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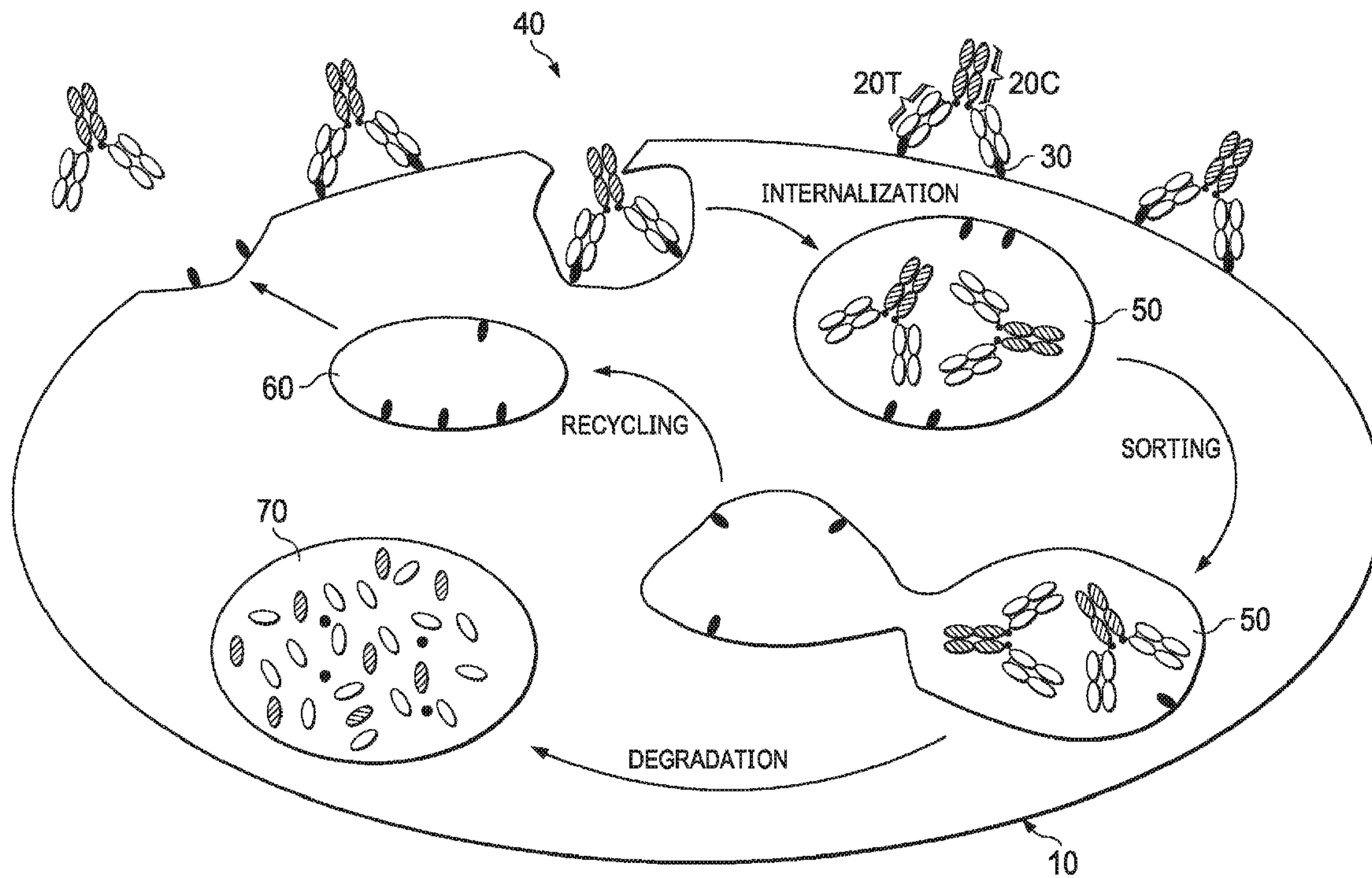


FIG. 1

(57) Abrégé/Abstract:

Endolysosomal targeting conjugates that are engineered to deliver cargo molecules such as cytotoxic drugs or imaging labels with improved efficiency to late endosomes and/or lysosomes in target cells such as tumor cells are described. The endolysosomal

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(57) **Abrégé(suite)/Abstract(continued):**

targeting conjugate includes a targeting component and a cargo component. The targeting component is configured to bind to a cell surface molecule of a target cell and the cargo component includes a cargo molecule. The targeting component and the cargo component may be fused by a covalent bond or associated by a non-covalent bond. The targeting component may bind to the cell surface molecule or the cargo component with higher affinity in the extracellular space than in an endolysosomal compartment of the target cell.

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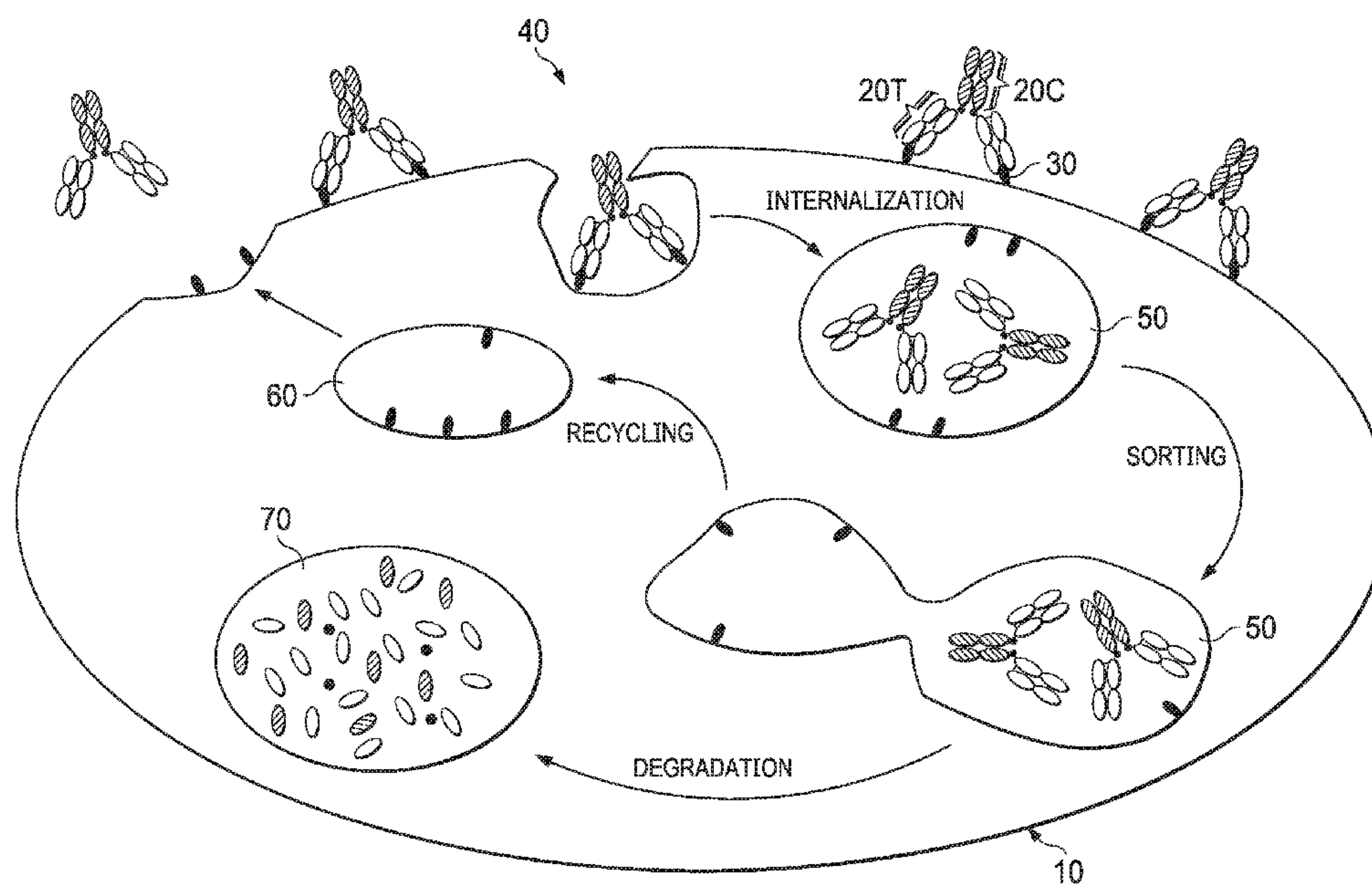
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ENDOLYSOSOMAL COMPARTMENT OF TARGET CELLS

FIG. 1

(57) Abstract: Endolysosomal targeting conjugates that are engineered to deliver cargo molecules such as cytotoxic drugs or imaging labels with improved efficiency to late endosomes and/or lysosomes in target cells such as tumor cells are described. The endolysosomal targeting conjugate includes a targeting component and a cargo component. The targeting component is configured to bind to a cell surface molecule of a target cell and the cargo component includes a cargo molecule. The targeting component and the cargo component may be fused by a covalent bond or associated by a non-covalent bond. The targeting component may bind to the cell surface molecule or the cargo component with higher affinity in the extracellular space than in an endolysosomal compartment of the target cell.

[Continued on next page]

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**ENDOLYSOSOMAL TARGETING CONJUGATES FOR IMPROVED
DELIVERY OF CARGO MOLECULES TO THE ENDOLYSOSOMAL
COMPARTMENT OF TARGET CELLS**

FIELD OF THE INVENTION

[0001] The invention disclosed herein generally relates to the generation of engineered proteins that can be used as platforms to deliver cytotoxic drugs, imaging labels or other cargo molecules to target cells such as tumor cells. These proteins are engineered to deliver the cargo molecules more efficiently to late endosomes and lysosomes in the targeted cells.

BACKGROUND

[0002] Antibody-drug conjugates (ADCs) or protein-drug conjugates (PDCs) represent a class of therapeutics that combine the high specificity of antibodies, antibody fragments or other proteins that bind to cancer cells or other unwanted cells such as inflammatory or virally infected cells with the delivery of highly toxic drugs. A problem with current ADCs or PDCs is that they have toxicities that can limit the dose. However, the development of ADCs and PDCs that allow more efficient delivery of cytotoxic drugs to tumor cells remains challenging.

[0003] In addition, antibodies, antibody fragments or other targeting proteins can be labeled with radiolabel, fluorescent label or near infrared label to be used as labeled conjugates (LCs) in diagnostic imaging. However, the development of LCs that allow more efficient labeling of tumors with higher contrast to background tissues also remains challenging.

SUMMARY

[0004] The present disclosure relates to engineered proteins, herein referred to as endolysosomal targeting conjugates, that are configured to allow improved delivery of cargo

molecules such as cytotoxic drugs (e.g., in ADCs, or PDCs) or imaging labels (e.g., in LCs) to the endolysosomal pathway in target cells such as cancer cells or other cell types.

[0005] According to a first aspect, an endolysosomal targeting conjugate is described. The endolysosomal targeting conjugate has a targeting component that includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain, wherein the targeting component is configured to bind to a cell surface molecule of a target cell with a lower dissociation constant in an extracellular space than in an endolysosomal compartment of the target cell. The endolysosomal targeting conjugate also has a cargo component that includes a cargo molecule conjugated to an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain. The targeting component is fused directly or indirectly to the cargo component. Upon entry to the endolysosomal compartment, the targeting component is configured to dissociate from the cell surface molecule. The endolysosomal targeting conjugate is configured to deliver the cargo molecule to the endolysosomal compartment of the target cell.

[0006] According to a second aspect, an endolysosomal targeting conjugate is described. The endolysosomal targeting conjugate has a targeting component that includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain, wherein the targeting component is configured to bind to a cell surface molecule of a target cell. The endolysosomal targeting conjugate also has a cargo component that includes a cargo molecule conjugated to an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain. The targeting component is configured to bind to the cargo component with a lower dissociation constant in an extracellular space than in an endolysosomal compartment of the target cell. Upon entry to the endolysosomal compartment, the targeting component is configured to dissociate from the cargo component. The endolysosomal targeting conjugate is configured to deliver the cargo molecule to the endolysosomal compartment of the target cell.

[0007] According to a third aspect, a composition is described. The composition includes an endolysosomal targeting conjugate and a pharmaceutically acceptable vehicle.

[0008] According to a fourth aspect, a method of treating cancer is described. The method includes administering a subject with an effective dose of an endolysosomal targeting conjugate composition to a subject, wherein the cargo molecule is a cytotoxic drug and the administering of the composition suppresses growth of a tumor in the subject.

[0009] According to a fifth aspect, a method of imaging a tumor in a subject is described. The method includes the steps of: (1) administering a subject with an effective dose of an endolysosomal targeting conjugate composition to a subject, wherein the cargo molecule is an imaging label; and (2) performing an imaging method suitable for detecting the imaging label in the subject. The administering of the composition provides a sufficient concentration of the imaging label detectable by the imaging method.

[0010] According to a sixth aspect, a method of providing an endolysosomal targeting conjugate for the treatment of cancer is described. The method includes the steps of: (1) selecting a targeting component, wherein the targeting component includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain configured to selectively bind to a cell surface molecule on a selected type of tumor target cell, wherein the targeting component is configured to bind to a cell surface molecule with a lower dissociation constant in an extracellular space than in an endolysosomal compartment; (2) selecting a cargo component, wherein the cargo component includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain conjugated to a cargo molecule, wherein the cargo molecule is a cytotoxic drug having efficacy for suppressing growth of the selected type of tumor target cell; and (3) providing the endolysosomal targeting conjugate including the targeting component fused directly or indirectly to the cargo component.

[0011] According to a seventh aspect, a method of providing an endolysosomal targeting conjugate for the treatment of cancer is described. The method includes the steps of (1) selecting a targeting component, wherein the targeting component includes an antibody, an antibody fragment, a nanobody, a protein, a protein fragment, or a protein domain configured to selectively bind to a cell surface molecule on a selected type of tumor target cell; (2) selecting a cargo component, wherein the cargo component includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain conjugated to a cargo molecule, wherein the cargo molecule is a cytotoxic drug having efficacy for suppressing growth of the selected type of tumor target cell. In the method, the targeting component is engineered to further include a first protein domain; the cargo component is engineered to further include a second protein domain; and the first protein domain is configured to bind to the second domain with a lower dissociation constant in an extracellular space than in an endolysosomal compartment.

[0012] The above endolysosomal targeting conjugates and methods may further include the following details, which may be combined with one another unless clearly

mutually exclusive: i) the targeting component may include an antibody, an antibody fragment or a nanobody that is configured to bind to the cell surface molecule in the extracellular space with a dissociation constant less than 500 nM.; ii) the targeting component may include an antibody, an antibody fragment or a nanobody that is configured to bind to the cell surface molecule with a lower dissociation constant at a near neutral pH than at an acidic endolysosomal pH; iii) near-neutral pH may be greater than about pH 6.8 and less than about pH 7.5 and the acidic endolysosomal pH may be greater than about pH 5.0 and less than about pH 6.5; iv) the targeting component may include an antibody, an antibody fragment or a nanobody that is configured to bind to a cell surface molecule with a lower dissociation constant at an extracellular Ca^{2+} concentration than at an endolysosomal Ca^{2+} concentration; v) the extracellular Ca^{2+} concentration may be about 2mM and the endolysosomal Ca^{2+} concentration may be about 2 μM ; vi) the targeting component may include a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule in the extracellular space with a dissociation constant less than 500 nM; vii) the targeting component may be a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at a near neutral pH than at an acidic endolysosomal pH; viii) the targeting component may include a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at an extracellular Ca^{2+} concentration than at an endosomal Ca^{2+} concentration; ix) the cargo component may include an antibody, an antibody fragment, which may be an antibody Fc region or a domain of the antibody Fc fragment; x) the antibody Fc region or the domain of the antibody Fc fragment may be derived from human IgG1; xi) the cargo component may include an albumin molecule or a domain of albumin; xii) the targeting component may include a Fab fragment or a scFv fragment of a HER2-specific antibody, wherein a heavy chain variable domain of the Fab fragment or the scFv fragment has mutations of Ser55 to histidine and Gly57 to glutamic acid; xiii) the targeting component may include a Fab fragment or a scFv fragment of a HER2-specific antibody, wherein in the Fab fragment or the scFv fragment a heavy chain variable domain has a mutation of Ser103 to histidine and a light chain variable domain has a mutation of Tyr55 to histidine; xiv) the endolysosomal targeting conjugate may include at least a first targeting component and a second targeting component, wherein the first targeting component is configured to bind to a different cell surface molecule than the second targeting component; xv) the at least first and second targeting components may be fused to a heterodimer of two immunoglobulin Fc fragments; xvi) the targeting component may include

a phosphatidylserine-binding protein and the target cell may be a cell having a phosphatidylserine on the cell surface; xvii) the phosphatidylserine-binding protein may be selected from the group consisting of a core domain of AnxA1, a C2A domain of Syt1, and a C2 domain of PKC α ; xviii) the phosphatidylserine-binding protein may be the C2A domain of Syt1; xix) the targeting component may include two of the C2A domains of Syt1; xx) the targeting component may include four of the C2A domains of Syt1; xxi) the targeting component may include more than four of the C2A domains of Syt1; xxii) the targeting component may include a phosphatidylserine-binding protein, the cargo component comprises an Fc portion of human IgG1, and the targeting component may be covalently fused to the cargo component by a linker protein; xxiii) the linker protein may be a Gly4Ser linker; xxiv) the cargo molecule may be a cytotoxic drug; xxv) the cytotoxic drug may be monomethylauristatin E (MMAE); xxvi) the cargo molecule may be an imaging label; xxvii) the targeting component may be configured to bind to the cargo compartment with a lower dissociation constant at near neutral pH than at acidic endosomal pH; xxviii) the targeting component may be configured to bind to the cargo component with a lower dissociation constant at an extracellular Ca²⁺ concentration than at an endosomal Ca²⁺ concentration; xxix) the targeting component may include a calbindin D9K domain 2 and the cargo component may include a calbindin D9K domain 1; xxx) the calbindin domain 1 D9K may be fused to the cargo component by a linker peptide and/or the calbindin D9K domain 2 may be fused to the targeting component by a linker peptide; xxxi) the composition may include at least one endolysosomal targeting conjugate configured to target tumors of one or more types of target cells; xxxii) the cargo molecule may be conjugated to the antibody fragment, the antibody domain, the nanobody, the protein, the protein fragment, or the protein domain by a polypeptide linker or through a chemical conjugation reaction; xxxiii) the imaging label may be a radiolabel, a fluorescent or a near infrared label; xxxiv) an N-terminus of the targeting component may be fused to a C-terminus of the cargo component; xxxv) a C-terminus of the targeting component may be fused to an N-terminus of the cargo component; xxxvi) the targeting component may be fused to the cargo component at a non-terminal location of the cargo component; xxxvii) the cargo component may include an immunoglobulin Fc fragment and the targeting component may be fused to the immunoglobulin Fc fragment of the cargo component at an N-terminus or a C-terminus of a hinge-CH2-CH3 domain of the immunoglobulin Fc fragment; xxxviii) the targeting component may include an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to human epidermal growth factor receptor 2; xxxix) the targeting component may include an antibody,

an antibody fragment, an antibody domain, or a nanobody that is configured to bind to prostate-specific membrane antigen; xl) the endolysosomal targeting conjugate may include one or more proteins having an amino acid sequence of at least one of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, or a homolog thereof; xli) the endolysosomal targeting conjugate may include a heterodimer of proteins having amino acid sequences of SEQ ID NO: 2 plus SEQ ID NO: 4, SEQ ID NO: 6 plus SEQ ID NO: 8, SEQ ID NO: 42 plus SEQ ID NO: 44, or SEQ ID NO: 46 plus SEQ ID NO: 48, or homologs thereof; xlii) the endolysosomal targeting conjugate may include a heterotrimer of proteins having amino acid sequence of SEQ ID NO: 18 plus SEQ ID NO: 20 plus SEQ ID NO: 22, SEQ ID NO: 22 plus SEQ ID NO: 24 plus SEQ ID NO: 26, or homologs thereof; xliii) the targeting component may include an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to the cell surface molecule at an acidic pH with a dissociation constant greater than 1.5 μ M; xliv) the targeting component may include a protein, a protein fragment or a protein domain that is configured to bind to the cell surface molecule at an acidic pH with a dissociation constant greater than 1.5 μ M; xlv) the acidic pH may be about 5.8; xlvi) the targeting component may include an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to the cell surface molecule with a lower dissociation constant at a pH from about 6.8 to about 7.5 than at a pH from about 5.0 to about 6.5; xlvii) the targeting component may include an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to a cell surface molecule with a lower dissociation constant at a Ca^{2+} concentration of about 2mM than at a Ca^{2+} concentration of about 2 μ M; xlviii) the targeting component may include a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at a pH from about 6.8 to about 7.5 than at a pH from about 5.0 to about 6.5; xlix) the targeting component may include a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at a Ca^{2+} concentration of about 2mM than at a Ca^{2+} concentration of about 2 μ M; xlx) the targeting component may be configured to bind to the cargo compartment with a lower dissociation constant at a pH from about 6.8 to about 7.5 than at a pH from about 5.0 to about 6.5; xlx) the targeting

component may be configured to bind to the cargo component with a lower dissociation constant at a Ca^{2+} concentration of about 2mM than at a Ca^{2+} concentration of about 2 μM ; xlxii) the cargo molecule may be a cytotoxic radiolabel; xlxiii) the cargo molecule may be a drug or other agent that modifies a behavior of the target cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] For a more complete understanding of the present invention and its features and advantages, reference is now made to the following description, taken in conjunction with the accompanying drawings, which are not to scale, in which like numerals refer to like features, and in which:

[0014] FIGURE 1 is an exemplary schematic diagram of selected cellular events that lead to the delivery of cargo molecules into late endosomes and lysosomes by the endolysosomal targeted conjugates;

[0015] FIGURE 2 is an exemplary schematic diagram of selected cellular events that lead to the dissociation of the cargo component and the targeting component of the endolysosomal targeting conjugates in endosomes followed by delivery of the cargo component into late endosomes and lysosomes;

[0016] FIGURE 3A is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0017] FIGURE 3B is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody scFv-Fc fusion protein that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0018] FIGURE 3C is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody variable domain/antibody variable domain fragment-Fc fusion protein that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0019] FIGURE 3D is a schematic diagram of an exemplary endolysosomal targeting conjugate including Fab fragments fused to the N-terminal locations of immunoglobulin hinge and CH3 domains that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0020] FIGURE 3E is a schematic diagram of an exemplary endolysosomal targeting conjugate including scFv fragments fused to the N-terminal locations of immunoglobulin hinge and CH3 domains that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0021] FIGURE 3F is a schematic diagram of an exemplary endolysosomal targeting conjugate including antibody variable domain/antibody variable domain fragments fused to the N-terminal locations of immunoglobulin hinge and CH3 domains that bind to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0022] FIGURE 3G is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody variable domain/antibody variable domain fragments fused to albumin that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0023] FIGURE 3H is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody Fab fragment fused to albumin that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0024] FIGURE 3I is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody scFv fragment fused to albumin that binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0025] FIGURE 3J is a schematic diagram of an exemplary endolysosomal targeting conjugate including a protein, protein domain or fragment fused to a N-terminal location of an Fc fragment. The protein, protein domain or protein fragment binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0026] FIGURE 3K is a schematic diagram of an exemplary endolysosomal targeting conjugate including a protein, protein domain or fragment fused to a C-terminal location of an Fc fragment. The protein, protein domain or protein fragment binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0027] FIGURE 3L is a schematic diagram of an exemplary endolysosomal targeting conjugate including a protein, protein domain or fragment fused to both the N-terminal and C-terminal locations of an Fc fragment. The protein, protein domain or protein fragment binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0028] FIGURE 3M is a schematic diagram of an exemplary endolysosomal targeting conjugate including a protein, protein domain or protein fragment fused to both the N-terminal and C-terminal locations of an Fc fragment to form a heterodimer with two protein domains or fragments molecule per Fc fragment. The protein, protein domain or protein fragment binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0029] FIGURE 3N is a schematic diagram of an exemplary endolysosomal targeting conjugate including a protein, protein domain or protein fragment fused to a C-terminal location of an Fc fragment to form a heterodimer with one protein, protein domain or fragment molecule per Fc fragment. The protein, protein domain or protein fragment binds to a cell surface protein or cell surface receptor with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0030] FIGURE 3O is a schematic diagram of an exemplary endolysosomal targeting conjugate including a Fab-protein (domain) and protein (domain)-Fc fusion that are

designed to associate with each other with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0031] FIGURE 3P is a schematic diagram of an exemplary endolysosomal targeting conjugate including an scFv fragment-protein (domain) and protein (domain)-Fc fusion that are designed to associate with each other with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0032] FIGURE 3Q is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody variable domain-protein (domain) and protein (domain)-Fc fusion that are designed to associate with each other with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0033] FIGURE 3R is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody that comprises two different Fab fragments that bind to two or more cell surface receptors or cell surface molecules with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0034] FIGURE 3S is a schematic diagram of an exemplary endolysosomal targeting conjugate including an antibody that comprises two different scFv fragments that bind to two or more cell surface receptors or cell surface molecules with higher affinity (lower dissociation constant) at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at lower, endosomal Ca^{2+} concentrations);

[0035] FIGURE 4A shows exemplary data for binding analyses of exemplary HER2-targeting endolysosomal targeting agents (without conjugated drug) to HER2 at different pH values;

[0036] FIGURE 4B shows exemplary data for dissociation constants of exemplary HER2-targeting endolysosomal targeting agents (without conjugated drug) to HER2 at different pH values;

[0037] FIGURE 5A shows graphs of exemplary data for flow cytometry analyses of expression levels of HER2 on different tumor cell lines; FIGURE 5B shows graphs of exemplary data for flow cytometry analyses of internalization and accumulation of exemplary HER2-targeting endolysosomal targeting conjugates in different tumor cell lines;

[0038] FIGURE 5C shows a series of exemplary microscopic images comparing the localization of exemplary HER2-targeting endolysosomal targeting conjugates in dextran-positive lysosomes;

[0039] FIGURE 6 shows graphs reporting exemplary data for the effects of the HER2-targeting endolysosomal targeting conjugates and control MMAE-conjugate on the viability of tumor cells;

[0040] FIGURE 7A shows graphs reporting exemplary whole body counts and blood counts against time for exemplary HER2-targeting endolysosomal targeting conjugates in mice;

[0041] FIGURE 7B shows graphs reporting exemplary data for the effects of exemplary HER2-targeting endolysosomal targeting conjugates and control proteins on tumor growth (MDA-MB-453 cells) in mice;

[0042] FIGURE 8A shows exemplary binding data for the interaction of exemplary HER2-targeting fusion proteins comprising calbindin domains 2 and 1 fused to antibody Fab (HER2-specific) and Fc fragments, respectively;

[0043] FIGURE 8B shows graphs of exemplary data for flow cytometry analyses of internalization and accumulation of exemplary prostate-specific membrane antigen (PSMA)-targeting antibodies comprising calbindin domains 2 and 1 fused to antibody Fab (026, PSMA-specific) and Fc fragments, respectively, in prostate cancer cells;

[0044] FIGURE 9 shows graphs reporting exemplary gel filtration chromatography analyses of exemplary PS-targeting Fc fusion proteins;

[0045] FIGURE 10A is a schematic representation and analysis of exemplary PS-targeting agents (Fc fusions comprising human IgG1-derived Fc linked to PS-targeting proteins, AnxA1, C2A domain of synaptotagmin 1 (Syt1) and PKC α). Filled circles and rectangles represent the PS-targeting domains and IgG1 hinge region, respectively. Right panel shows SDS-PAGE analyses of the endolysosomal targeting agents, with molecular weights (MW) shown in kDa on the left;

[0046] FIGURE 10B shows exemplary lipid binding profiles of exemplary PS-targeting agents using lipid-coated nitrocellulose membranes;

[0047] FIGURE 10C shows graphs reporting exemplary binding of exemplary PS-targeting agents to PS-positive 2H11 and MDA-MB-231 cells using flow cytometry analysis;

[0048] FIGURE 10D shows graphs reporting exemplary whole body counts vs. time of exemplary PS-targeting agents;

[0049] FIGURE 10E shows graphs reporting exemplary areas under curves for the data shown in FIGURE 10D for exemplary PS-targeting agents;

[0050] FIGURE 10F shows exemplary whole body images of tumor-bearing mice injected with exemplary PS-targeting agents labeled with a near infrared dye (IRDye800CW);

[0051] FIGURE 10G shows graphs reporting exemplary tumor-associated fluorescence for exemplary PS-targeting agents labeled with a near infrared dye (IRDye800CW) for the images shown in FIGURE 10F;

[0052] FIGURE 10H shows exemplary images of tumors and a graph reporting mean dye intensities for exemplary PS-targeting agents labeled with a near infrared dye (IRDye800CW) following 48 hours of injection into tumor-bearing mice;

[0053] FIGURE 11A is a schematic representation and analysis of exemplary bivalent and tetravalent PS-targeting agents with filled circles representing the Syt1 C2A domain (left panel). Right panel shows SDS-PAGE analyses of the Syt1-Fc fusions, with molecular weights (MW) shown in kDa on the right;

[0054] FIGURE 11B shows exemplary gel filtration chromatography analyses of an exemplary PS-targeting Fc-fusion proteins containing four Syt1 molecules per Fc fragment;

[0055] FIGURE 11C shows exemplary binding of exemplary PS-targeting agents to PS on PS-coated nitrocellulose membranes and to lipid-coated nitrocellulose membranes;

[0056] FIGURE 11D shows graphs reporting exemplary binding of exemplary PS-targeting agents and control protein to cells with exposed PS;

[0057] FIGURE 11E shows graphs reporting exemplary internalization of exemplary PS-targeting agents into cells with exposed PS;

[0058] FIGURE 11F shows a series of exemplary microscopic images of exemplary PS-targeting agents and control protein in endothelial cells (2H11) with the lysosomes in the cells labeled with LAMP-1-specific antibody;

[0059] FIGURE 11G shows another series of exemplary microscopic images of exemplary PS-targeting agents and control protein in tumor cells (MDA-MB-231) with the lysosomes in the cells labeled with LAMP-1-specific antibody;

[0060] FIGURE 12A shows a schematic representation and analysis of exemplary PS-targeting endolysosomal targeting conjugates (left panel), with the cargo component conjugated with MMAE (small filled circles). Right panel shows SDS-PAGE analyses of the unconjugated or MMAE-conjugated PS-targeting agents, with molecular weights (MW) shown in kDa on the left;

[0061] FIGURE 12B shows exemplary mass spectrometry analyses of exemplary MMAE-conjugated PS-targeting endolysosomal targeting conjugates;

[0062] FIGURE 12C shows exemplary gel filtration chromatography analyses of exemplary PS-targeting endolysosomal targeting conjugates;

[0063] FIGURE 12D shows an exemplary analysis of the binding of exemplary PS-targeting endolysosomal targeting conjugates to PS in the presence of the indicated Ca^{2+} concentrations (left panel) or pH levels (right panel). Bead-associated proteins were analyzed using immunoblotting;

[0064] FIGURE 13A shows an exemplary series of microscopic images of exemplary PS-targeting endolysosomal targeting conjugates and control MMAE-conjugate in tumor cells (MDA-MB-231) with the early endosomes in the cells labeled with EEA-1-specific antibody. Specific endosomes are cropped and enlarged (labeled a and b);

[0065] FIGURE 13B shows an exemplary series of microscopic images of exemplary PS-targeting endolysosomal targeting conjugates and control MMAE-conjugate in tumor cells (MDA-MB-231) with the lysosomes in the cells labeled with LAMP-1-specific antibody;

[0066] FIGURE 13C shows a series of exemplary microscopic images of the effects of exemplary PS-targeting endolysosomal targeting conjugates and control MMAE conjugate on tubulin in endothelial cells (2H11) and in tumor cells (MDA-MB-231);

[0067] FIGURE 14A shows graphs of exemplary flow cytometry data to indicate the levels of exposed PS on tumor cells;

[0068] FIGURE 14B shows graphs reporting exemplary data for the effects of the exemplary PS-targeting endolysosomal targeting conjugates and control MMAE-conjugate on the viability of tumor cells;

[0069] FIGURE 14C shows graphs of exemplary flow cytometry data to indicate the levels of internalized exemplary PS-targeting endolysosomal targeting conjugates into tumor cells following two hours of incubation;

[0070] FIGURE 14D shows graphs of exemplary data indicating that exemplary PS-targeting Fc fusions (without conjugated drug) do not affect cell viability;

[0071] FIGURE 15A shows graphs reporting exemplary whole body counts vs. time of exemplary PS-targeting endolysosomal targeting conjugates;

[0072] FIGURE 15B shows exemplary graphs reporting the areas under curves for the data shown in FIGURE 15A for exemplary PS-targeting endolysosomal targeting conjugates; FIGURE 15C show graphs reporting exemplary data for the effects of exemplary PS-targeting endolysosomal targeting conjugates and control proteins on tumor growth (MDA-MB-231 cells) in mice;

[0073] FIGURE 15D show graphs reporting exemplary data for the effects of exemplary PS-targeting endolysosomal targeting conjugates and control proteins on the body weight of tumor-bearing mice;

[0074] FIGURE 15E shows graphs reporting exemplary data for the effects of exemplary PS-targeting endolysosomal targeting conjugates and control proteins on tumor growth (LNCaP cells) in mice;

[0075] FIGURE 16A shows SDS-PAGE analyses of a control endolysosomal targeting conjugate that has been engineered to reduce PS-binding activity (using DN mutations) and control proteins with and without conjugation to MMAE;

[0076] FIGURE 16B shows exemplary lipid binding profiles of exemplary PS-targeting endolysosomal targeting conjugates that bind to PS or a control endolysosomal targeting conjugate that is engineered to bind with low affinity (DN) to PS to lipid-coated nitrocellulose membranes;

[0077] FIGURE 16C shows graphs reporting exemplary binding of exemplary PS-targeting endolysosomal targeting conjugates that bind to PS or are engineered to bind with low affinity (DN) to PS-positive 2H11 cells using flow cytometry analysis;

[0078] FIGURE 16D shows graphs reporting exemplary data for the effects of exemplary PS-targeting endolysosomal targeting conjugates and control proteins on tumor growth (LNCaP cells) in mice;

[0079] FIGURE 16E shows exemplary images of isolated tumors (LNCaP cells) from mice treated with exemplary PS-targeting endolysosomal targeting conjugates and control proteins;

[0080] FIGURE 17A shows a series of exemplary microscopic images showing the location of exemplary PS-targeting endolysosomal targeting conjugates and control (PBS vehicle) with respect to CD31-positive endothelial cells in tumor tissue; and

[0081] FIGURE 17B shows a series of exemplary microscopic images showing the location of exemplary PS-targeting endolysosomal targeting conjugates and control (PBS vehicle) with respect to F4/80-positive macrophages in tumor tissue.

DETAILED DESCRIPTION

[0082] This disclosure relates to engineered antibodies or fusion proteins that are configured to allow improved delivery of a cargo molecule, such as a cytotoxic drug or an imaging label to late endosomes and lysosomes in a target cell. The terms “endolysosomal” and “endolysosomal compartment” as used herein refer to the early endosomes, late endosomes, lysosomes and associated tubulovesicular transport carriers of cells. Accordingly, the term “endolysosomal targeting conjugate” as used herein refers to an engineered antibody or fusion protein that is configured for improved delivery of a cargo molecule to the endolysosomal compartment of a target cell.

[0083] The antibodies or fusion proteins described herein include a targeting component and a cargo component. The targeting component may include an antibody, an antibody fragment, antibody domain, nanobody, protein, protein fragment, or protein domain that is configured to bind to a cell surface molecule, such as a cell surface receptor or other cell surface molecule that may be present on the extracellular surface of a cell's plasma membrane, wherein the molecule is at least partially exposed to an extracellular space. The cargo component may include an antibody, an antibody fragment, antibody domain, nanobody, protein, protein fragment, or protein domain that is conjugated to a cargo molecule, such as a cytotoxic drug or imaging label.

[0084] The terms “antibody-drug conjugate” or “ADC” as used herein refer to antibody-based conjugates that are configured to deliver a drug to a cell. In particular, the terms “antibody-drug conjugate” or “ADC” as used herein refers to an antibody that includes a targeting component and a cargo component. The targeting component may include an antibody Fab fragment, an antibody variable domain, or a nanobody. The targeting component is linked to a cargo component that may include an antibody constant region (Fc fragment) or a domain of a constant region. One or more cytotoxic drug molecules may be conjugated to the antibody constant region (Fc fragment) or a domain of a constant region of the cargo component of the ADC.

[0085] The terms “protein-drug conjugate”, or “PDC” as used herein refer to protein-based conjugates that are configured to deliver a drug to a cell. In particular, the term “protein-drug conjugate” or “PDC” as used herein refers to a protein that includes a targeting component and a cargo component. The targeting component may include a protein that binds to a cell surface receptor or cell surface molecule, or a Fab fragment, an antibody variable domain, or a nanobody. The targeting component is linked to a cargo component that may include an antibody constant region (Fc fragment), a domain of a constant region, or a protein such as albumin. One or more cytotoxic drug molecules may be conjugated to the antibody constant region (Fc fragment), domain of a constant region, or a protein such as albumin of the cargo component of the PDC.

[0086] The term “labeled conjugate” or “LC” as used herein refers to an antibody or protein that includes a targeting component linked to a cargo component. Accordingly, the targeting component may include an antibody Fab fragment, an antibody variable domain, nanobody, or a protein that binds to a cell surface receptor or cell surface molecule. The cargo component may include an antibody constant region (Fc fragment) or domains of a constant region, or a protein such as albumin. The antibody constant region (Fc fragment), or domains of a constant region, or the protein of the cargo component may be conjugated to one or more imaging labels, such as radiolabels, fluorescent molecules or other labeled molecules.

[0087] In general, for ADCs or PDCs to effectively deliver a drug to a cell, they should selectively bind to target cells, be internalized into the cells, and enter degradative compartments called late endosomes and lysosomes. Prior to entering lysosomes, ADCs or PDCs enter early endosomes where sorting into the recycling or lysosomal pathway occurs. Improving delivery to late endosomes and lysosomes (the endolysosomal pathway) would result in more potent ADCs or PDCs and enable the use of lower dosages.

[0088] In addition, LCs that allow improved delivery of imaging labels to late endosomes and lysosomes in target cells would result in higher contrast for imaging target cells against a background of non-targeted cells or tissue.

[0089] Accordingly, the current invention generally relates to endolysosomal targeting conjugates that are engineered to more efficiently deliver cargo molecules to the endolysosomal compartment of target cells, whereby the engineered conjugates described herein are configured to respond to differences in chemical composition in endosomes or late endosomes and the extracellular environment, thereby achieving more efficient delivery of a cargo molecule such as a cytotoxic drug or an imaging label to target cells. Examples of the endolysosomal targeting conjugates described herein include improved ADCs, PDCs, and LCs that are configured for improved targeting of the conjugated cargo molecules such as drugs or imaging labels to the endolysosomal compartment of target cells, thereby resulting in more potent ADCs or PDCs, or higher contrast LCs.

[0090] Cargo molecules as described herein can include any molecule having a function useful to cause an effect in a target cell. For example, in addition to cytotoxic drugs or imaging labels, additional cargo molecules could be radiolabels that kill cells through radiation damage i.e. can be used therapeutically rather than for imaging. Examples of such radiolabels are Yttrium (Y)-90 and Iodine (I)-131. Additional types of cargo molecules are identifiable by skilled persons upon reading the present disclosure.

[0091] In addition to tumor cells, target cells in the sense of the disclosure may include other types of unwanted cells, such as inflammatory cells, or virally-infected cells, among others identifiable by skilled persons upon reading the present disclosure.

[0092] The term “cell surface molecule” as used herein refers to a protein or other biological molecule (e.g. phospholipid, carbohydrate) that is at least partially exposed on the extracellular surface of a plasma membrane of a cell.

[0093] FIGURES 1 and 2 show schematic diagrams of exemplary mechanisms of improved delivery of cargo molecules by the endolysosomal targeting conjugates of the disclosure. Upon binding of the targeting component to the cell surface molecule, the endolysosomal targeting conjugate is internalized into cells (FIGURE 1 and 2).

[0094] As shown in exemplary schematic form in FIGURE 1, the endolysosomal targeting conjugate may dissociate in early or late endosomes from the receptor or cell surface molecule due to lower (acidic) pH, lower Ca^{2+} concentrations, lower Cl^- or Na^+ concentrations, higher K^+ concentrations or other environmental conditions that distinguish endosomes from the extracellular space (for example, as described in Scott, C.C., Gruenberg,

J. (2010) Ion flux and the function of endosomes and lysosomes: pH is just the start. *Bioessays* 33, 103-110). Accordingly, in some examples of the endolysosomal targeting conjugates herein described, the affinity of the targeting component for the cell surface molecule is higher in the extracellular space than in the endolysosomal compartment. Thus, the targeting component and the cell surface molecule may bind with a lower dissociation constant in the extracellular space. The endolysosomal targeting conjugate is delivered to late endosomes or lysosomes, resulting in release of its drug or label.

[0095] In another example, as shown in exemplary schematic form in FIGURE 2, the targeting and cargo components of the endolysosomal targeting conjugate may be associated with each other by non-covalent interactions that are stable at near neutral pH or extracellular Ca^{2+} concentrations, but not at endosomal (acidic) pH or endosomal Ca^{2+} concentrations or other environmental conditions that distinguish endosomes from the extracellular space. The cargo component is released in early or late endosomes and enters lysosomes, whereas the targeting component can be recycled and reloaded with cargo component. Accordingly, in some examples of the endolysosomal targeting conjugates herein described, the affinity of the targeting component for cargo component is higher in the extracellular space than in the endolysosomal compartment. Thus, the targeting component may bind the cargo component with a lower dissociation constant in the extracellular space.

[0096] The targeting component and the cargo component may be linked by a covalent bond or may be non-covalently associated with each other.

[0097] Examples of endolysosomal targeting conjugates described herein include targeting components that are configured to bind to cell surface molecules such as HER2 or prostate-specific membrane antigen (PSMA). In particular, the exemplary endolysosomal targeting conjugates may bind to cell surface molecules such as HER2 or PSMA with affinities of less than 500 nM at near-neutral pH.

[0098] Examples of endolysosomal targeting conjugates described herein include targeting components that are configured to bind to cell surface molecules such as PS. In particular, the exemplary PDCs may bind to cell surface molecules such as PS with affinities of less than 500 nM at near-neutral pH.

[0099] HER2 and PSMA are cell surface receptors that are overexpressed on tumors and are therefore well-characterized tumor targets. This endolysosomal targeting conjugates of the present disclosure are not limited to targeting these receptors or cell surface molecules such as prostate stem cell associated antigen, EpCAM, c-MET, carcinoembryonic antigen (CEA), CD19, CD20, CD20, CD33, CD38, epidermal growth factor receptor

(EGFR), glypican-2, CD56, insulin-like growth factor receptor 1, tumor endothelial marker-8 (TEM-8), CD46 and many other targets are identifiable by persons with skill in the art upon reading the present disclosure.

[00100] PS resides in the inner leaflet of the plasma membrane of normal cells, but in response to oxidative stress and inflammatory factors in the tumor microenvironment, becomes exposed on the outer leaflet of the membrane of cancer endothelial cells. Based on studies in rodent models, exposure of PS is considered to be a ‘universal’ marker for tumor vasculature. Typically, less than half of tumor blood vessels are PS-positive and exposure can be increased by radiation and chemotherapy. In addition to tumor endothelium, PS has also been reported to be exposed on many non-apoptotic cancer cells, including melanoma, breast, prostate and renal carcinoma.

[00101] Accordingly, the targeting component can include any type of molecule that is configured to specifically bind to a cell surface receptor or other cell surface molecule. Such molecules can include proteins, protein fragments, polynucleotides such as ribonucleic acids or deoxyribonucleic acids, polypeptides, polysaccharides, lipids, amino acids, peptides, sugars and/or other small or large molecules and/or polymers identifiable by skilled persons upon reading the present disclosure.

[00102] As shown in FIGURE 1, an endolysosomal targeting conjugate 20 including a targeting component 20T and a cargo component 20C. The cargo component has attached drug or label that is indicated by filled black circles. may reversibly bind to cell surface receptor or other molecule 30 on the surface of a cell 10. This binding typically occurs at near-neutral pH, such as at a pH greater than about 6.8 and less than about 7.5, because that is the typical pH of extracellular space 40. Binding to the cell surface receptor or cell surface molecule can also occur at typical extracellular Ca^{2+} concentrations (approximately 2 mM). Cell surface receptor or molecule 30 with attached endolysosomal targeting conjugate 20 are internalized into the cell 10 through receptor-mediated uptake into endosome 50. The complex of endolysosomal targeting conjugate 20 and cell surface receptor or other molecule 30 dissociate from the receptor or other molecule 30 in the early or late endosome due to the acidic pH (from about pH 5.0 to about pH 6.5) or low Ca^{2+} concentration (approximately 2 μM) in these compartments. Accordingly, receptor or cell surface molecule 30 may recycle back in a recycling endosome 60 to the cell surface and be reloaded with endolysosomal targeting conjugate 20, whereas the endosomally dissociated endolysosomal targeting conjugate 20 enters the lysosomes and is degraded into fragments (70). The endolysosomal targeting conjugate is configured to bind to the cell surface receptor

or other molecule under the conditions in the extracellular space, with dissociation in the endosomes or late endosomes of at least 10% of the endolysosomal targeting conjugate that is internalized into the cell.

[00103] As shown in FIGURE 2, an endolysosomal targeting conjugate including a targeting component 80 and cargo component 90 (with attached drug or other label) indicated by filled circles) may reversibly bind to cell surface receptor or other molecule 30 on the surface of a cell 10. The association of the targeting component 80 and cargo component 90 typically occurs at near-neutral pH, such as at a pH greater than 6.8 and less than 7.5, because that is the typical pH of extracellular space 40. Association of the targeting component 80 and cargo component 90 can occur at extracellular Ca^{2+} concentrations (approximately 2 mM). Cell surface receptor or molecule 80 with attached endolysosomal targeting conjugate are internalized into the cell 10 through receptor-mediated uptake into endosome 50. The targeting component 80 and cargo component 90 dissociate in the early or late endosome due to the acidic pH (typically less than pH 6.8) or low Ca^{2+} concentrations (approximately 2 μM) in these compartments. Accordingly, the targeting component 80 bound to the receptor or cell surface molecule may recycle back to the cell surface in a recycling endosome 60 and be reloaded with cargo component 90, whereas the endosomally dissociated cargo component 90 enters the lysosomes and is degraded into fragments (70). The endolysosomal targeting conjugate is configured to bind to the cell surface receptor or other molecule under the conditions in the extracellular space, with dissociation in the endosomes or late endosomes of at least 10% of the cargo component that is internalized into the cell.

[00104] Endolysosomal targeting conjugates according to this disclosure are configured to specifically bind a cell surface receptor/molecule at near-neutral pH or extracellular Ca^{2+} concentrations via a targeting component. The term “specifically bind” as used herein refers to a detectable selective inter-molecular interaction between the targeting component and the cell surface receptor/molecule. For example, to specifically bind, the targeting component needs to show a detectable interaction with the cell surface receptor or cell surface molecule that is being targeted, whilst not showing a detectable interaction, or much lower affinity interaction, with other cell surface receptors or cell surface molecules. Techniques for detecting specific binding are known within the art, such as ELISA and other methods identifiable by skilled persons.

[00105] Accordingly, endolysosomal targeting conjugates allow the linked cargo component to be internalized into cells that express the targeted cell surface receptor or other targeted cell surface molecule and thereafter intracellularly degraded.

[00106] Endolysosomal targeting conjugates can include at least a first targeting component and a second targeting component, wherein the antibody Fab fragment, single chain Fv (scFv), nanobody, protein or protein fragment of the first targeting component is different to the antibody Fab fragment, single chain Fv (scFv), nanobody, protein or protein fragment of the second targeting component. Accordingly, endolysosomal targeting conjugates comprising at least a first targeting component and a second targeting component can bind to two different cell surface receptors or cell surface molecules.

[00107] In addition, endolysosomal targeting conjugates may contain human or humanized proteins or protein fragments to avoid or decrease the possibility of an immune reaction to the endolysosomal targeting conjugates when administered to a human. The targeting component and cargo component are preferably a human protein or protein fragment for administration of the endolysosomal targeting conjugate to a human. The targeting component and cargo component are preferably a human protein or protein fragment, such as a human antibody, antibody fragment or human albumin or albumin fragment, or a humanized antibody or humanized antibody fragment for administration of the endolysosomal targeting conjugate to a human. If an endolysosomal targeting conjugate is developed for use in a non-human animal, then proteins or protein fragments derived from or engineered to be immunologically compatible with that animal may be used instead.

[00108] FIGURE 3A is a schematic representation of an exemplary endolysosomal targeting conjugate (20a) including Fab fragments (100) of an antibody that is configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The Fc fragment (110) is homodimeric. As understood by persons skilled in the art, the Fc fragment of an IgG is all of the lower base of the antibody's Y-shape, which includes a sulfhydryl-bridged hinge region and CH2 and CH3 domains. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00109] FIGURE 3B is a schematic representation of an exemplary endolysosomal targeting conjugate (20b) including scFv fragments (260) of an antibody that is configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+}

concentrations). The Fc fragment (110) is homodimeric. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00110] FIGURE 3C is a schematic representation of an exemplary endolysosomal targeting conjugate (20c) including antibody variable domains or fragments (130) that are configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The Fc fragment (110) is homodimeric. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00111] Antibody variable region 130 may include portions of a non-variable region of an antibody that is configured to bind to a cell surface receptor or cell surface molecule. For example, antibody variable region 130 may be a single-domain antibody (sdAb) or camelid-derived VHH domain (also commonly referred to as a nanobody). Such variable regions have the overall fold of an immunoglobulin domain, comprising two anti-parallel β -sheets, and can also include domains from other members of the immunoglobulin superfamily such as T cell receptor variable domains, constant region domains of antibodies or domains of the coreceptor, CD4, among others identifiable by persons skilled-in-the-art. Antibody variable regions can also include heterodimers of heavy chain variable (VH) domains linked by peptide linkers to light chain variable (VL) domains to form scFv fragments (260). The linker sequences that are used to link VH and VL domains are well known to those with skill in the art and include the GGGGSGGGGSGGGGS $[(\text{G}_4\text{S})_3]$ sequence that connect the C-terminus of the VH domain to the N-terminus of the VL domain. The C-terminus of the VL domain can be connected to the N-terminus of the VH domain with similar linker sequences. ScFvs that bind to a cell surface receptor or other cell surface molecule with pH-dependence, Ca^{2+} -dependence etc. can be isolated from libraries of scFvs using phage display, yeast display or other antibody display approaches. The targeting component of an endolysosomal targeting conjugate may include Fab fragments of an antibody that can be isolated from libraries of Fab fragments using phage display or yeast display among other techniques known to skilled persons. For nanobodies, scFvs and Fab fragments, the desired binding properties (pH-dependence, Ca^{2+} -dependence) for the targeted cell surface receptor or cell surface molecule can be further improved by randomly mutating residues in the complementarity determining regions (CDRs), or by using error-prone polymerase chain reaction, to generate libraries of mutated nanobodies or variable domains, followed by selection. Exemplary CDR residues that would be targeted are those in CDR3 of

the light chain variable domain (residues 89-97; Kabat numbering) and CDR3 of the heavy chain variable domain (residues 95-102; Kabat numbering). These libraries can be displayed on phage or yeast and variants with the required binding behavior selected using approaches known to those with skill in the art.

[00112] FIGURE 3D is a schematic representation of an exemplary endolysosomal targeting conjugate (20d) including Fab fragments (100) of an antibody that is configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The Fab fragments are linked to immunoglobulin CH3 domains (140) to form a homodimer. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00113] FIGURE 3E is a schematic representation of an exemplary endolysosomal targeting conjugate (20e) including scFv fragments (260) of an antibody that is configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The scFv fragments are linked to immunoglobulin CH3 domains (140) to form a homodimer. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00114] FIGURE 3F is a schematic representation of an exemplary endolysosomal targeting conjugate (20f) including antibody variable domains or fragments (130) that are configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The protein fragments or domains are linked to immunoglobulin CH3 domains (140) to form a homodimer. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00115] FIGURE 3G is a schematic representation of an exemplary endolysosomal targeting conjugate (20g) including an antibody variable domain or fragment (130) that is configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The antibody variable domain or fragment is linked to albumin or an albumin fragment (150), which may be mutated or modified so that it binds with increased affinity to a neonatal Fc receptor (FcRn). For example, mutations can be inserted into the FcRn binding domain (DIII) of (human serum) albumin using error prone PCR followed by display of libraries of mutated albumin variants on yeast or phage, and

selection of higher affinity variants. Alternatively, higher affinity variants can be generated by mutating residues at or near the albumin:FcRn interface and either selecting or screening for albumin variants with increased binding affinity. Although FIGURE 3G illustrates antibody variable domain or fragment (130) at a terminal location of albumin or albumin fragment 150, it may instead be located at a non-terminal location. The antibody variable domain or fragment may be fused to albumin or albumin fragment 150 in any suitable manner, including attachment via a chemical reaction or attachment through a peptide linker. In this example, the cargo molecule (120) is attached via chemical conjugation to an exposed amino acid such as cysteine or lysine.

[00116] In the exemplary schematic representations shown in FIGURE 3H and FIGURE 3I, the antibody variable domain or fragment 130 as shown in FIGURE 3G is replaced by a Fab fragment 100 (FIGURE 3H) or scFv fragment 260 (FIGURE 3I) that are configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). In this example, the cargo molecule (120) is attached via chemical conjugation to an exposed amino acid such as cysteine or lysine.

[00117] FIGURE 3J is a schematic representation of an exemplary endolysosomal targeting conjugate (20j) including protein fragments or domains (160) that are configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The protein fragments or domains are linked to the N-termini of an Fc fragment (110) to form homodimers. In this example, the cargo molecule (120) is attached to the hinge region via chemical conjugation to cysteine residues.

[00118] In the exemplary schematic representations shown in FIGURE 3K and FIGURE 3L, the protein fragments or domains (160) are linked to the C-termini of an Fc fragment (110) to form homodimers (FIGURE 3K) or to both the N- and C-termini of an Fc fragment (110) to form homodimers (FIGURE 3L). In these examples, the cargo molecules (120) are attached to the hinge regions via chemical conjugation to cysteine residues.

[00119] FIGURE 3M is a schematic representation of an exemplary endolysosomal targeting conjugate (20m) including protein fragments or domains (160) that are configured to bind to a cell surface protein or cell surface molecule with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The protein fragments or domains are linked to the N- and C-termini of an Fc fragment (170) that is configured to heterodimerize with another Fc fragment 180

lacking a protein fragment or domain, to produce a heterodimeric endolysosomal targeting conjugate 20m as shown in FIGURE 3M. FIGURE 3N shows a schematic representation of an exemplary PDC or LC 20n wherein the protein fragment or domain (160) is fused to the C-terminus of the Fc fragment (170). In the examples shown in FIGURES 3M and 3N, the cargo molecules (120) are attached to the hinge regions via chemical conjugation to cysteine residues. In order to avoid Fc fragment homodimers in which both Fc fragments have a fused protein fragment or domain (160), or no fused protein or protein domain, the endolysosomal targeting conjugate can be designed with knobs-into-holes mutations (for example, as described in Moore, G.L., Bautista, C., Pong, E., Nguyen, D.H., Jacinto, J., Eivazi, A., Muchhal, U.S., Karki, S., Chu, S.Y., Lazar, G.A. (2011) A novel bispecific antibody format enables simultaneous bivalent and monovalent co-engagement of distinct target antigens. *MAbs* 3, 546-557) and/or electrostatic steering mutations (for example, as described in Gunasekaran, K., Pentony, M., Shen, M., Garrett, L., Forte, C., Woodward, A., Ng, S.B., Born, T., Retter, M., Manchulenko, K., Sweet, H., Foltz, I.N., Wittekind, M., Yan, W. (2010) Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. *J. Biol. Chem.* 285, 19637-19646) to promote heterodimer formation. Other approaches can also be used to generate heterodimers, such as the insertion of a (G₄S)₁₃ linker peptide between the C-terminus of the antigen-Fc fusion and N-terminus of a second Fc fragment (for example, as described in Zhou, L., Wang, H-Y., Tong, S., Okamoto, C.T., Shen, W-C., Zaro, J.L. (2016) Single chain Fc-dimer-human growth hormone fusion protein for improved drug delivery. *Biomaterials*, 117, 24-31). DNA and protein sequences of examples of endolysosomal targeting conjugate that include knobs-into-holes mutations, electrostatic steering mutations and/or other mutations are described in Example 14.

[00120] Examples of knobs-into-holes mutations include Y349T/T394F: S364H/F405A and Y349T/F405F: S364H/T394F (for example, as described in Moore, G.L., Bautista, C., Pong, E., Nguyen, D.H., Jacinto, J., Eivazi, A., Muchhal, U.S., Karki, S., Chu, S.Y., Lazar, G.A. (2011) A novel bispecific antibody format enables simultaneous bivalent and monovalent co-engagement of distinct target antigens. *MAbs* 3, 546-557) and T366W:T366S:L368A/Y407V (for example, as described in Atwell, S., Ridgway, J.B.B., Wells, J.A., Carter, P. (1997) Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. *J. Mol. Biol.*, 270, 26-35) among others identifiable by persons skilled in the art. The residue numbering of these exemplary knobs-into-holes

mutations refers to the EU antibody numbering system, as would be understood by persons skilled in the art.

[00121] Examples of electrostatic steering mutations include E356K/D399K:K392D/K409D and K409D/K370D:D357K/D399K (for example as described in Gunasekaran, K., Pentony, M., Shen, M., Garrett, L., Forte, C., Woodward, A., Ng, S.B., Born, T., Retter, M., Manchulenko, K., Sweet, H., Foltz, I.N., Wittekind, M., Yan, W. (2010) Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. *J. Biol. Chem.* 285, 19637-19646) among others identifiable by persons skilled in the art. The residue numbering of these exemplary electrostatic steering mutations refers to the EU antibody numbering system, as would be understood by persons skilled in the art.

[00122] FIGURE 3O is a schematic representation of an exemplary endolysosomal targeting conjugate (20o) including Fab fragments (210) of an antibody that are fused to domain 2 of calbindin (CalD2; 190). Fc fragments (110) are fused to domain 1 of calbindin (CalD1; 200). CalD1 (200) and CalD2 (190) associate with each other with higher affinity at extracellular Ca^{2+} concentrations than at lower, endosomal Ca^{2+} concentrations. Other examples may include protein or protein fragments (190, 200) that are fused to the Fab fragment (210) or Fc (110) and associate with each other in a Ca^{2+} - or pH-dependent way. Other examples of proteins that interact in a Ca^{2+} -dependent way are : calmodulin and the calmodulin binding peptide M13 (for example, as described in Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., Tsien, R.Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature*, 388, 882-887.); S100C and the N-terminal 13 residues of annexin I (for example, as described in Seeman, J., Weber, K., Gerke, V. (1996) Structural requirements for annexin I-S100C complex-formation. *Biochem. J.*, 319, 123-129; Malliard, W.S., Haigler, H.T., Schlaepfer, D.D. (1995) Calcium-dependent binding of S100C to the N-terminal domain of Annexin I. *J. Biol. Chem.*, 270, 719-725); osteonectin and collagen IV (for example, as described in Maurer, P., Hohenadl, C., Hohenester, E., Gohring, W., Timpl, R., Engel, J. (1995) The C-terminal protein of BM-40 (SPARC/Osteonectin) is an autonomously folding and crystallizable domain that binds calcium and collagen IV. *J. Mol. Biol.*, 253, 347-357). In other examples of endolysosomal targeting conjugates, the Fab fragment as shown in FIGURE 3O is replaced by a scFv fragment (220) as shown in FIGURE 3P, or an antibody variable domain, fragment or nanobody (230) as shown in FIGURE 3Q. In the examples shown in FIGURES 3O, 3P and

3Q, the cargo molecules (120) are attached to the hinge regions via chemical conjugation to cysteine residues

[00123] FIGURE 3R is a schematic representation of an exemplary endolysosomal targeting conjugate (20a) including Fab fragments (100, 240) of two antibodies that are configured to bind to two different cell surface proteins or cell surface molecules with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). The Fc fragments (170, 180) are each fused to the different Fab fragments (100, 240) to form heterodimers. Heterodimer formation may be driven by knobs-into-holes mutations (for example, as described in Moore, G.L., Bautista, C., Pong, E., Nguyen, D.H., Jacinto, J., Eivazi, A., Muchhal, U.S., Karki, S., Chu, S.Y., Lazar, G.A. (2011) A novel bispecific antibody format enables simultaneous bivalent and monovalent co-engagement of distinct target antigens. *MAbs* 3, 546-557) and/or electrostatic steering mutations (for example, as described in Gunasekaran, K., Pentony, M., Shen, M., Garrett, L., Forte, C., Woodward, A., Ng, S.B., Born, T., Retter, M., Manchulenko, K., Sweet, H., Foltz, I.N., Wittekind, M., Yan, W. (2010) Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. *J Biol Chem* 285, 19637-19646). In another example shown in FIGURE 3S, the Fab fragments as shown in FIGURE 3R are replaced by two scFv fragments (250, 260) that are configured to bind to two different cell surface proteins or cell surface molecules with higher affinity at near neutral pH (or at extracellular Ca^{2+} concentrations) than at acidic, endosomal pH (or at endosomal Ca^{2+} concentrations). In the examples shown in FIGURES 3R and 3S, the cargo molecules (120) are attached to the hinge regions via chemical conjugation to cysteine residues

[00124] In the examples shown in FIGURE 3A – FIGURE 3S, the endolysosomal targeting conjugate has a targeting component that includes Fab fragments, scFvs or nanobodies, and the cargo component includes an Fc fragment, a sub-fragment of an Fc (e.g. CH_2 domains) or albumin linked to a cargo molecule such as a cytotoxic drug or imaging label ~~molecule~~—such as radiolabel or fluorescent label. The cargo molecules shown in FIGURES 3A, 3B, 3C, 3D, 3E, 3F, 3J, 3K, 3L, 3M, 3N, 3O, 3P, 3Q, 3R and 3S are attached to the hinge regions via one or more cysteine residues. In other examples, one or more cargo molecules can be attached to the cargo component through different chemistries known to those with skill in the art, such as: amine-to-amine (NHS esters), sulfhydryl-to-sulfhydryl (maleimide), amine-to-sulfhydryl (NHS ester/maleimide), sulfhydryl-to-carbohydrate (maleimide/hydrazide), or attachment to via an unnatural amino acid with the desired

chemical reactivity, among other approaches identifiable by skilled persons. The unnatural amino acid may be inserted during recombinant production of the targeting component. In other examples, such as those shown in FIGURES 3R and FIGURE 3S, the targeting components may bind to two or more different targets. Each of the targeting components may be fused to an Fc fragment with knobs-into-holes mutations and/or electrostatic steering mutations to drive heterodimer formation.

[00125] In the examples shown in FIGURES 3J, 3K, 3L, 3M and 3N, the endolysosomal targeting conjugate may include, for example, PS-targeting agents produced by fusing PS-binding domains to the Fc fragment of human IgG1 or other Fc fragment. For example, the targeting component may be conjugated to a cargo component that includes a cytotoxic drug to produce endolysosomal targeting conjugate PDCs or the targeting component may be conjugated to a cargo component that includes an imaging label such as a radiolabel, fluorescent label or near infrared label, to produce endolysosomal targeting conjugate LCs. Accordingly, endolysosomal targeting conjugates described herein may include proteins or protein fragments or domains that are configured to dissociate from a target cell surface molecule upon exposure to the significant decrease in Ca^{2+} levels in endosomes, for example by using PS-binding domains that interact with PS in a Ca^{2+} -dependent manner. For example, an endolysosomal targeting conjugate PDC or LC can include a targeting component such as Fc-Syt1. An endolysosomal targeting conjugate PDC that includes Fc-Syt1 may be bivalent, for example, as shown in FIGURES 3J, 3K, or tetravalent as shown in FIGURE 3L.

[00126] The endolysosomal targeting conjugate may include one targeting domain fused to an Fc fragment that forms a heterodimer with an Fc fragment that does not have a fused protein. To promote heterodimer formation, the Fc fragments may be engineered with knobs-into-holes mutations and/or electrostatic steering mutations as shown in the examples in FIGURES 3M and 3N.

[00127] In some examples described herein, the targeting component and cargo component are not covalently linked, but instead are configured to associate with each other through a linkage to engineered domains that associate with each other non-covalently. In particular, the non-covalent association may have stronger affinity when in contact with the chemical environment in extracellular space than in the endolysosomal compartment. For example, the engineered domains may include those such as calbindin domain 1 (CalD1) and calbindin domain 2 (CalD2). CalD1 and CalD2, which bind to each other at extracellular Ca^{2+} concentrations, but not at endosomal Ca^{2+} concentrations (see Example 6). Other examples of

proteins that interact in a Ca^{2+} -dependent way are : calmodulin and the calmodulin binding peptide M13 (for example, as described in Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., Tsien, R.Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature*, 388, 882-887.); S100C and the N-terminal 13 residues of annexin I (for example, as described in Seeman, J., Weber, K., Gerke, V. (1996) Structural requirements for annexin I-S100C complex-formation. *Biochem. J.*, 319, 123-129; Malliard, W.S., Haigler, H.T., Schlaepfer, D.D. (1995) Calcium-dependent binding of S100C to the N-terminal domain of Annexin I. *J. Biol. Chem.*, 270, 719-725); osteonectin and collagen IV (for example, as described in Maurer, P., Hohenadl, C., Hohenester, E., Gohring, W., Timpl, R., Engel, J. (1995) The C-terminal protein of BM-40 (SPARC/Osteonectin) is an autonomously folding and crystallizable domain that binds calcium and collagen IV. *J. Mol. Biol.*, 253, 347-357). Examples of several configurations containing such domains are shown in FIGURES 3O, 3P and 3Q, although other configurations are possible.

[00128] Endolysosomal targeting conjugates may include different numbers of targeting components and cargo components. The configurations shown in FIGURES 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, 3I, 3J, 3K, 3L, 3M, 3N, 3O, 3P, 3Q, 3R and 3S are examples and are not limiting, since multiple other configurations are identifiable by those with skill in the art upon reading the present disclosure.

[00129] In some examples herein described, endolysosomal targeting conjugates may include a toxin, such as monomethyl auristatin E (MMAE), conjugated via a valine-citrulline-PAB linker. The endolysosomal targeting conjugate may include, for example, an antibody that binds a cell-surface molecule such as HER2 with higher affinity at near neutral pH than at acidic, endosomal pH (HER2-ADCs; see Examples 2-5). The engineered antibodies and proteins described herein can be expressed by mammalian cells at high yields and conjugated with the drug at high efficiency. Upon binding to HER2-positive cells, the exemplary HER2-ADCs are internalized into early endosomes, where the pH drops from near neutral to around pH 5.5-6.5. This decrease in pH causes HER2-ADCs to dissociate from the early/sorting endosomal membrane, leading to effective lysosomal delivery of the MMAE drug. In particular, for example (e.g., see FIGURE 4B) the pertuzumab variants SG and YS have dissociation constants greater than 1.5 μM at pH 5.8, whereas the WT pertuzumab has a much lower dissociation constant at the same pH. Accordingly, the HER2-ADCs can effectively eradicate HER2-positive cells, and treatment with HER2-ADCs can suppress the

tumor growth effectively without any sign of adverse effects in mouse models of HER2-expressing breast cancer.

[00130] In another example described herein, the endolysosomal targeting conjugate has a targeting component including a PS-binding domain such as the C2A domain of Synaptotagmin 1 (Syt1) fused to a cargo component such as the Fc region of an antibody, conjugated to MMAE via a maleimidocaproyl valine-citrulline-PAB linker (PS-PDCs; see Examples 7-13). The PS-targeting proteins can also be expressed by mammalian cells at high yields and conjugated with the drug at high efficiency. The exemplary PS-PDCs are configured to bind PS specifically in a calcium-dependent manner. Upon binding to PS-positive cells, PS-PDCs are internalized into early endosomes efficiently, where the calcium level drops from 2 mM to less than 2 μ M. This drop of calcium concentration causes PS-PDCs to dissociate from the early/sorting endosomal membrane, leading to effective lysosomal delivery of the MMAE drug. The PS-PDCs can effectively eradicate PS-positive cells which include but are not limited to tumor endothelial cells, breast and prostate cancer cells. Treatment with PS-PDCs can suppress the tumor growth effectively without any sign of adverse effects in mouse models of human triple negative breast cancer as well as prostate cancer, while the unconjugated proteins have no efficacy. Since PS is a universal marker for cancer endothelial cells as well as stressed tumor cells, the PDCs can be applied to treatment of most solid tumors.

[00131] The targeting components of the endolysosomal targeting conjugates described herein that bind to a target cell surface molecule in a pH-dependent or Ca^{2+} -dependent way can be isolated from libraries of immunoglobulin variable domains, scFvs (VH:VL heterodimers in which VH and VL domains are connected to each other by linker peptides such as GGGGSGGGGSGGGGS) or Fab fragments using phage display, yeast display or other methods identifiable by those with skill in the art. These libraries can either be derived from naturally occurring antibody variable genes, can be generated using approaches that result in 'semi-synthetic' libraries wherein complementarity determining regions (CDRs) are produced using randomized oligonucleotide sequences, or can be derived from VH and VL domain genes of existing antibodies by insertion of random mutations into the CDRs. Random mutations in the CDRs can be inserted using error-prone PCR or with biases towards histidine residues (for pH-dependence) followed by selection using phage display or yeast display. Exemplary CDR residues that would be targeted are those in CDR3 of the light chain variable domain (residues 89-97; Kabat numbering) and CDR3 of the heavy chain variable domain (residues 95-102; Kabat numbering). Selection of scFvs or Fab

fragments with desired pH-dependence or Ca^{2+} -dependence can be carried out using methods known to those with skill in the art. In addition, to isolate pH-dependent binders, CDR residues can be systematically mutated to histidine, the resulting Fab or scFv fragments expressed and analyzed for binding to target using, for example, surface plasmon resonance or ELISAs.

[00132] The endolysosomal targeting conjugates described herein may have variations in numbers of targeting components (e.g. Fab fragments or scFv fragments) that range from 1-4 targeting components (FIGURE 3). These targeting components may be linked to immunoglobulin Fc fragments or other proteins such as albumin, and include linker sequences that vary in length and composition between the fusion proteins, domains or fragments e.g. GGGGS or 2-3 repeats of this linker, among other linker sequences identifiable by skilled persons. Domains such as CalD1 or CalD2 can also be linked to targeting components and cargo components using similar linkers. The Fc fragments of an endolysosomal targeting conjugate may also have mutations such as knobs-into-holes and/or electrostatic steering mutations so that heterodimers of Fc fragments with and without linked targeting components are formed.

[00133] The targeting component may be fused to an Fc region of an antibody, which retains the therapeutic functions and *in vivo* persistence elicited by the Fc region while reducing the size of the protein. In other examples, the Fc region may be replaced by albumin, or domain III of albumin, which have prolonged *in vivo* persistence due to the interaction of albumin (or DIII) with the recycling receptor, FcRn.

[00134] The exemplary endolysosomal targeting conjugate ADCs and PDCs described herein show improved efficacy in killing cancer cells through improved intracellular release of the toxin (see Examples 3-5, 9-13). In addition to the exemplary cytotoxic drug, MMAE, other cytotoxic drugs may be used such as a maytansinoid, tubulysin, benzodiazepine, duocarmycin, among drugs identifiable by those with skill in the art. The drug may be conjugated to the antibody fragment, antibody domain, nanobody, protein, protein fragment or protein domain of the cargo component through chemical conjugation. Examples of chemical coupling that may be used are: amine-to-amine (NHS esters), sulfhydryl-to-sulfhydryl (maleimide), amine-to-sulfhydryl (NHS ester/maleimide), sulfhydryl-to-carbohydrate (maleimide/hydrazide), or attachment via an unnatural amino acid with the desired chemical reactivity, among other approaches identifiable by skilled persons. The unnatural amino acid may be inserted during recombinant production of the targeting component. Polyethyleneglycol (PEG) spacers may be inserted between the chemically

conjugated proteins, protein fragments or other molecules. Linkers may be cleavable, such as valine-citrulline to enable release of the cytotoxic drug in the late endosomes or lysosomes by resident proteases such as cathepsins. In cases where the linkage is not cleavable, such as for trastuzumab-DM1, the antibody may be proteolysed to release the drug. Linkage chemistry, sites of linkage and choice of peptide can be guided by molecular modeling, and can be designed to minimize loss of binding activity of the ADC or PDC for cell surface receptor or other cell surface molecule, as would be understood by skilled persons.

[00135] The cargo component of endolysosomal targeting conjugate LCs can be generated by conjugation of an imaging label identifiable by persons skilled in the art to the antibody fragment, antibody domain, protein, protein fragment or protein domain of the cargo component. Non-limiting examples of imaging labels include near infrared dyes such as IRDye800CW, or radiolabels such as I-124, Cu-64 or Zr-89. Conjugation to Cu-64 or Zr-89 can be achieved through chelation to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) which chelates these radiolabels, among other methods identifiable by persons skilled in the art.

[00136] In further examples, the cargo component of the endolysosomal targeting conjugate can be a cytotoxic radiolabel (e.g. Yttrium-90, Y-90, or iodine-131, I-131) or drug or other agent that modifies the behavior of the targeted cell. For example, the drug could be an antagonistic ligand for the androgen receptor (AR) and could be used to downregulate AR activity.

[00137] The term “behavior” in relation to a target cell or other cell may refer to an activity, a function, an output, or any other attribute or action regarding the phenotype or genotype of the target cell or other cell. In general, a drug or other agent can be used to produce an effect, such as a particular therapeutic effect, a cytotoxic effect, and so on, with regard to a target cell, as would be understood by skilled persons upon reading the present disclosure.

[00138] In several examples described herein, the endolysosomal targeting conjugate may include the amino acid sequences of SEQ ID NO: 2 plus SEQ ID NO: 4, SEQ ID NO: 6 plus SEQ ID NO: 8, SEQ ID NO: 10; SEQ ID NO:12; SEQ ID NO:14; SEQ ID NO:16; SEQ ID NO: 18 plus SEQ ID NO:20 plus SEQ ID NO: 22, SEQ ID NO: 22 plus SEQ ID NO: 24 plus SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42 plus SEQ ID NO: 44, SEQ ID NO: 46 plus SEQ ID NO: 48, or homologs thereof.

[00139] The endolysosomal targeting conjugate may include an amino acid sequence having at least 50% identity with SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48.

[00140] As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the nucleotide bases or residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity or similarity is used in reference to proteins, it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted with a functionally equivalent residue of the amino acid residues with similar physiochemical properties and therefore do not change the functional properties of the molecule.

[00141] A functionally equivalent residue of an amino acid used herein typically refers to other amino acid residues having physiochemical and stereochemical characteristics substantially similar to the original amino acid. The physiochemical properties include water solubility (hydrophobicity or hydrophilicity), dielectric and electrochemical properties, physiological pH, partial charge of side chains (positive, negative or neutral) and other properties identifiable to a person skilled in the art. The stereochemical characteristics include spatial and conformational arrangement of the amino acids and their chirality. For example, glutamic acid is considered to be a functionally equivalent residue to aspartic acid in the sense of the current disclosure. Tyrosine and tryptophan are considered as functionally equivalent residues to phenylalanine. Arginine is considered to be a functionally equivalent residue to lysine.

[00142] A person skilled in the art would understand that similarity between sequences is typically measured by a process that includes the steps of aligning the two polypeptide or polynucleotide sequences to form aligned sequences, then detecting the number of matched characters, i.e. characters similar or identical between the two aligned sequences, and calculating the total number of matched characters divided by the total number of aligned characters in each polypeptide or polynucleotide sequence, including gaps. The similarity result is expressed as a percentage of identity.

[00143] As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window,

wherein the portion of the polynucleotide sequence in the comparison window may include additions or deletions (gaps) as compared to the reference sequence (which does not include additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

[00144] As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length protein or protein fragment. A reference sequence can be, for example, a sequence identifiable in a database such as GenBank and UniProt and others identifiable to those skilled in the art.

[00145] As understood by those skilled in the art, determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Computer implementations of suitable mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL, ALIGN, GAP, BESTFIT, BLAST, FASTA, among others identifiable by skilled persons.

[00146] For example, endolysosomal targeting conjugates according to the present disclosure may include an amino acid sequence having at least 50% sequence identity, preferably at least 80%, more preferably at least 90%, most preferably at least 95% sequence identity compared to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, or SEQ ID NO: 8, or SEQ ID NO: 10, or SEQ ID NO: 12, or SEQ ID NO: 14, or SEQ ID NO: 16, or SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 34, or SEQ ID NO: 36, or SEQ ID NO: 38, or SEQ ID NO: 40, or SEQ ID NO: 42, or SEQ ID NO: 44, or SEQ ID NO: 46, or SEQ ID NO: 48.

[00147] The endolysosomal targeting conjugates herein described may be provided in a composition that includes an endolysosomal targeting conjugate and a pharmaceutically acceptable vehicle.

[00148] The endolysosomal targeting conjugates or compositions thereof described herein may be administered using any suitable method to deliver them to a subject, such as a cell, a plurality of cells, or a multicellular organism, in particular an animal or a human, and in particular an animal or a human that may have one or more tumors, such as via injection,

particularly intravenous, subcutaneous or intramuscular injection, among other methods identifiable by skilled persons.

[00149] The endolysosomal targeting conjugates or compositions thereof herein described may be used in a method of treating cancer. The method includes administering a subject with an effective dose of an endolysosomal targeting conjugate or a composition thereof to a subject, wherein the cargo molecule is a cytotoxic drug and the administering of the composition suppresses growth of a tumor in the subject.

[00150] The endolysosomal targeting conjugates or compositions thereof herein described may be used in a method of imaging a tumor in a subject. The method includes the steps of: administering a subject with an effective dose of an endolysosomal targeting conjugate composition to a subject, wherein the cargo molecule is an imaging label; and performing an imaging method suitable for detecting the imaging label in the subject. In the method, the administering of the composition is performed at an effective dose to provide a sufficient concentration of the imaging label that is detectable by the imaging method, as would be identifiable by skilled persons.

[00151] The endolysosomal targeting conjugates or compositions thereof herein described may be administered at suitable time intervals, for example weekly, monthly or, for example, whenever 50% of subjects are expected to have shown tumor regression.

[00152] In diagnostic/theranostic imaging, it is expected that the administration to a subject of an endolysosomal targeting conjugate LC that includes an imaging label such as a radiolabel, near infrared label, or fluorescent label may be followed by a period of 1-7 days to allow localization of the target cell in the subject, for example, tumor localization in the subject. Following this period, the subject may be imaged using positron emission tomography or other suitable imaging modality, such as localized or whole-body imaging, to allow detection of the location of the target cell such as the tumor.

[00153] The endolysosomal targeting conjugates described herein may be designed to selectively target a particular cell type, and thereby deliver a cargo molecule to a selected target cell. In particular, the endolysosomal targeting conjugates described herein may be designed to target a particular type of tumor cell in a subject. Accordingly, a method of providing the endolysosomal targeting conjugates described herein may include the steps of: (1) selecting a targeting component, wherein the targeting component includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain configured to selectively bind to a cell surface molecule on a selected type of cell, such as a tumor target cell, wherein the targeting component is configured to bind to a

cell surface molecule that is expressed on the target cell with higher affinity in an extracellular space than in an endolysosomal compartment; (2) selecting a cargo component, wherein the cargo component includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain conjugated to a cargo molecule, wherein the cargo molecule may be, for example, a cytotoxic drug having efficacy for suppressing growth of the selected type of tumor target cell or an imaging label suitable for imaging of the selected tumor cell; and (3) providing the endolysosomal targeting conjugate including the targeting component fused directly or indirectly to the cargo component.

[00154] Alternatively, the method of providing an endolysosomal targeting conjugate for the treatment of cancer and/or imaging of tumors in a subject may include the steps of: (1) selecting a targeting component, wherein the targeting component includes an antibody, an antibody fragment, a nanobody, a protein, a protein fragment, or a protein domain configured to selectively bind to a cell surface molecule on a selected type of tumor target cell; (2) selecting a cargo component, wherein the cargo component includes an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain conjugated to a cargo molecule, wherein the cargo molecule is a cytotoxic drug having efficacy for suppressing growth of the selected type of tumor target cell or an imaging label suitable for imaging of the selected tumor cell. In particular, in the method, the targeting component is engineered to further include a first protein domain, the cargo component is engineered to further include a second protein domain, and the first protein domain is configured to bind to the second domain with higher affinity in an extracellular space than in an endolysosomal compartment, as described herein.

EXAMPLES

[00155] The following non-limiting examples are provided to further illustrate the endolysosomal targeting conjugates and methods disclosed herein. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches that have been found to function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in

the specific examples that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1. Materials and Methods

[00156] Cell lines and culture conditions. The mouse endothelial cell line 2H11 (ATCC, CRL-2163) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). The human breast cancer cell line MDA-MB-231 (ATCC, HTB-26) was cultured in DMEM with supplemented 10% FBS. The human breast cancer cell lines T-47D (ATCC, HTB-133), MDA-MB-453 (ATCC, HTB-131), and MDA-MB-468 (ATCC, HTB-132) were cultured in RPMI 1640 medium supplemented with 10% FBS. The human breast cancer cell line SK-BR-3 (ATCC, HTB-30) and human ovarian cancer cell line SK-OV-3 (ATCC, HTB-77) were cultured in McCoy's 5A medium supplemented with 10% FBS. The human prostate cancer cell lines LNCaP and 22Rv1 (ATCC, CRL-1740 and CRL-2505, respectively) were cultured in RPMI 1640 medium supplemented with 10% FBS. The human breast cancer cell line HCC1954 (Gazdar, A.F., Kurvari, V., Virmani, A., Gollahon, L., Sakaguchi, M., Westerfield, M., Kodagoda, D., Stasny, V., Cunningham, H.T., Wistuba, I.I., Tomlinson, G., Tonk., V, Ashfaq., R., Leitch, A.M., Minna, J.D., Shay, J.W. (1998) Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. *Int. J. Cancer.* 78, 766-774) was cultured in RPMI 1640 medium supplemented with 10% FBS. All cancer cell lines were authenticated with DNA fingerprinting by the University of Arizona Genetics Core (UAGC). Cells were cultured at 37°C with 5% CO₂. Expi293F cells (Life Technologies, catalog number A14635) used for protein expression were cultured in Expi293 expression medium at 37°C with 8% CO₂ and 80% humidity.

[00157] Antibodies, antibody-drug conjugates and dextrans. The following antibodies were used in this study: rat anti-mouse LAMP1, mouse anti-human LAMP1 and mouse anti-beta tubulin antibodies (Developmental Studies Hybridoma Bank, clone # 1D4B, H4A3 and E7); mouse anti-human EEA1 and rat anti-mouse CD31 antibodies (BD Biosciences, catalog # 610456 and 557355); goat anti-human IgG (H+L) antibody conjugated with HRP, donkey anti-rat (H+L) antibody conjugated with Alexa Fluor 488 and donkey anti-human IgG (H+L) antibody conjugated with Cy3 (Jackson ImmunoResearch, catalog # 109-035-003, 712-545-153 and 709-165-149); goat anti-human IgG (H+L) antibody conjugated

with Alexa Fluor 555, goat anti-mouse IgG (H+L) antibody conjugated with Alexa Fluor 488 and goat anti-human IgG (H+L) antibody conjugated with Alexa Fluor 647 (Life Technologies, catalog # A21433, A11029 and A21445); rabbit anti-human Ki-67 antibody (Abcam, catalog # 92742). Trastuzumab-DM1 (T-DM1, Kadcyla®) was obtained from the UT Southwestern Medical Center Pharmacy (Dallas). Alexa Fluor 647-labeled dextran, 10 kDa molecular weight, was purchased from Life Technologies (catalog # D22914).

[00158] Generation of expression constructs for production of protein-drug conjugates. For use as controls, the Fc region including the hinge region (residues 215-447 of the heavy chain; EU numbering) of the hen egg lysozyme-specific human IgG1, HuLys10 (Foote, J., Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J. Mol. Biol. 224, 487-499), was cloned into the pcDNA3.4 vector with the N-terminal leader peptide derived from a mouse IgG heavy chain (Foote, J., Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J. Mol. Biol. 224, 487-499; Neuberger, M.S. (1983) Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. EMBO J. 2:1373-1378). Similarly, the genes encoding the heavy and light chain genes (cDNA) of the HuLys10 antibody were cloned into pcDNA3.4. For the control IgG heavy and light chain constructs, Cys214 (EU numbering) in the light chain, which forms a sulfhydryl bridge with Cys220 (EU number) in the heavy chain of HuLys10 were both mutated to serine residues using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, catalog # 200523).

[00159] cDNA clones for human Annexin A1 (AnxA1), human synaptotagmin 1 (Syt1) and human PKC α were purchased from Open Biosystems (clone ID: 3459615, clone ID: 6187902 and clone ID: 40028305, respectively). Genes encoding the AnxA1 PS-binding core domain (amino acids 41-346), the Syt1 PS-binding C2A domain (amino acids 141-266) and PKC α PS-binding C2 domain (amino acids 157-288) were fused via a Gly4Ser linker sequence to the CH3 domain of the human IgG1 Fc region (residues 215-447; EU numbering) with a leader peptide derived from the mouse IgG heavy chain (Foote, J., Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J. Mol. Biol. 224, 487-499; Neuberger, M.S. (1983) Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. EMBO J. 2:1373-1378). Cys220 (EU numbering) in the hinge region was mutated in all Fc fusion constructs so that there are two cysteine residues per hinge. The genes encoding the Fc fusions were cloned into the pcDNA3.4 vector (Invitrogen, catalog # 14308).

[00160] To generate Syt1-Fc-Syt1, the Syt1 PS-binding C2A domain (amino acids 141-266) was linked to the N-terminus of the hinge region of the Fc-Syt1 construct via a Gly₄Ser linker sequence. The leader peptide derived from a mouse IgG heavy chain (Foote, J., Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224, 487-499; Neuberger, M.S. (1983) Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. *EMBO J.* 2:1373-1378) was appended to the N-terminus of the hinge-linked Syt1 PS-binding C2A domain, and the resulting Fc fusion was cloned into the pcDNA3.4 vector. Mutations to reduce PS-binding of the Syt1 C2A domain (D173N, D179N, D231N, D233N and D239N) (Striegel, A.R., Biela, L.M., Evans, C.S., Wang, Z., Delehoy, J.B., Sutton, R.B., Chapman, E.R. and Reist, N.E. 2012. Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission. *J. Neurosci.* 32, 1253-1260) were inserted in the Syt1-Fc construct to generate Fc-Syt1(DN) and cloned into the pcDNA3.4 vector. All constructs were generated using standard methods of molecular biology and designed oligonucleotides.

[00161] Generation of expression constructs for antibody-drug conjugates. Synthetic genes encoding the heavy chain variable domain and pertuzumab light chain variable domain of the HER2-specific antibody, pertuzumab (Franklin, M.C., Carey, K.D., Vajdos, F.F., Leahy, D.J., de Vos, A.M., Sliwkowski, M.X. (2004) Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*, 4, 317-328), were purchased from Genescript and cloned into an expression vector for Fab fragment production, containing the human heavy chain constant region domain 1 (CH1) and human light chain constant domain (kappa chain, C κ). To identify residues in the pertuzumab heavy and light chain variable domains to target for histidine scanning, the crystal structure of pertuzumab in complex with antigen, HER2, (Protein Data Bank accession code 1N8Z) was analyzed in PyMOL. Residues located in the CDRs of pertuzumab (VH domain: Asp31, Tyr32, Asn54, Tyr60, Leu100, Gly101, Pro102, Ser103, Tyr105, Asp107; VL domain: Tyr55; amino acid numbers referred to are those in the protein sequence of the pertuzumab heavy and light chain variable domains, and do not refer to other numbering conventions) that interact with residues in domain II of HER2 (His245, Val286, Ser288, Leu295, His296, and Lys311 as described in Franklin, M.C., Carey, K.D., Vajdos, F.F., Leahy, D.J., de Vos, A.M., Sliwkowski, M.X. (2004) Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*, 4, 317-328) were systematically replaced by histidine using splicing by overlap extension (Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease,

L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77, 61-68). The resulting genes were cloned and expressed as periplasmically secreted Fab fragments in *E. coli*.

[00162] To generate the pertuzumab scFv gene in the phage display vector, pHEN1 (Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P., Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucl. Acids Res.* 19, 4133-4137), the Fab fragment expression vector for pertuzumab was modified using standard methods of molecular biology to insert a linker peptide [(Gly₄Ser)₃ linker] between the heavy and light chain variable domain gene, followed by recloning of the scFv gene into pHEN1. To generate libraries of mutated pertuzumab scFvs with randomly mutated residues in the CDRs, the following oligonucleotides were used. For each oligonucleotide, DNA sequence is shown in 5' to 3' direction: CDRH1Back, GCTTCTGGATTCACATTCACANNBNNBNNBATGGATTGGGTGAGACAGGCT (SEQ ID NO:49); CDRH1For, TGTGAATGTGAATCCAGAAGC (SEQ ID NO:50); CDRH2Back, TGGGTGGCTGATGTGAATCCTNNBNNBNNBNNBTCTATCTACAATCAGAGATTC (SEQ ID NO:51); CDRH2For, AGGATTCACATCAGCCACCCA (SEQ ID NO: 52); CDRH3Back, TACTACTGTGCTAGAAATCTGNNBCCTNNBTTCNNBTTCGATNNBTGGGGACAGGGAACACTG (SEQ ID NO:53); CDRH3For, CAGATTTCTAGCACAGTAGTA (SEQ ID NO:54); CDRL2-1Back, CCTAAGCTGCTGATCTACTCTNNBTCTNNBAGANNBACAGGAGTGCCTTCTAGA (SEQ ID NO: 55); CDRL2-1For, AGAGTAGATCAGCAGCTTAGG (SEQ ID NO: 56); CDRL2-2Back, GGAAAGGCTCCTAAGCTGCTGNNBNNBNNBGCTTCTTACAGATACACAGGA (SEQ ID NO: 57); and CDRL2-2For5, CAGCAGCTTAGGAGCCTTTCC (SEQ ID NO: 58). Molecular biology methods known to those with skill in the art were used to generate the libraries of scFvs genes, and electroporation of *E. coli* TG1 (Lucigen, catalog # 60502) was used to generate libraries of around 5×10^7 mutants for each targeted CDR.

[00163] Colonies from libraries were pooled and used to inoculate cultures supplemented with M13KO7 helper phage (NEB, catalog # N0315S), 100 µg/mL ampicillin, and 50 µg/mL kanamycin overnight at 30°C. Extruded phage were harvested from the supernatant by precipitation with 4% polyethylene glycol 8000, 3% NaCl. Phage (100 µL of

2×10^{12} pfu/mL) were pre-panned using 4% skimmed milk/phosphate-buffered saline (PBS) coated Maxisorp 96 well microtiter plates (Thermofisher, catalog # 44-2404-21) prior to panning using Maxisorp 96 cell plates coated with 2 μ g/mL recombinant human HER2 (extracellular domain)-Fc fusion protein (HER2-ECD-Fc; R&D Research, catalog # 1129-ER-050). Phage were incubated with 4% skimmed milk pH 7.4 for 2 hours in the 96 well plate. Plates were extensively washed with PBS pH 7.4 supplemented with 0.1% Tween-20 (PBST) followed by PBS pH 7.4. Phage with pH-dependent binding were selectively eluted using 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.8 for 10 minutes at room temperature. Eluted phage were used to infect exponentially growing *E.coli* TG1. Four rounds of panning were carried out, and isolated phage screened for higher affinity binding to HER2 at pH 7.0 relative to pH 5.8. Recombinant scFvs encoded by the selected phage were analyzed for binding to HER2 by ELISA and/or surface plasmon resonance.

[00164] The heavy and light chain variable domain genes for pertuzumab scFv or Fab fragments with higher affinity at pH 7.0 relative to pH 5.8 were cloned into cassette vectors for the expression of human IgG1 heavy chains and light chain (C κ) sequences, respectively, using pcDNA3.4 as backbone vector. The hinge disulfide bonds that link the C κ domain to hinge region, and one hinge disulfide that links the two heavy chains to each other, were removed from the heavy and light chains by mutating the light chain cysteine (Cys214; EU numbering) and two heavy chain cysteines (Cys220, Cys229; EU numbering) to serine.

[00165] To generate an expression construct encoding the HER2-specific Fab fragment of trastuzumab fused to the Calbindin D9K domain 2 (CalD2), the genes encoding trastuzumab heavy and light chain variable domains (Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., Shepard, H.M. (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA*, 89, 4285-4289) were synthesized by Genescript. The Calbindin D9K gene (Berggard, T., Julenius, K., Ogard, A., Drakenberg, T., Linse, S. (2001) Fragment complementation studies of protein stabilization by hydrophobic core residues. *Biochemistry*, 5, 1257-1264) was synthesized by Genescript and the trastuzumab Fab heavy chain (VH-CH1-linker including part of hinge), and the CalD2 domain genes were fused together with a Ser-Gly-Gly linker using splicing by overlap extension (Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77, 61-68). The VH-CH1-CalD2 fusion

protein gene and gene encoding the trastuzumab light chain (with a C-terminal polyhistidine tag) were cloned separately into the pcDNA3.4 vector.

[00166] Similar methods were used to generate expression constructs encoding the PSMA-specific (026) VH-CH1-linker peptide fused to the CalD2 domain. Genes encoding the 026 heavy and light chain variable domains (US patent no. 7850971B2) were synthesized by Genescript. The CalD2 domain gene and 026 VH-CH1-linker were fused together by splicing by overlap extension and the resulting fusion protein gene cloned into pcDNA3.4. The gene encoding the 026 light chain with a C-terminal polyhistidine tag was cloned into a separate pcDNA3.4 vector.

[00167] To generate an expression construct encoding Calbindin D9K domain 1 (CalD1) fused to the human IgG1-derived Fc fragment, the Fc domain (hinge-CH2-CH3, human IgG1-derived) gene was fused via a Gly-Ser-Ser linker to the Calbindin D9K domain 1 gene through splicing by overlap extension (Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77, 61-68) and cloned into the pcDNA3.4 vector.

[00168] Protein expression and purification. Recombinant antibodies and Fc fusion proteins were produced using the Expi293 expression system from Life Technologies following the manufacturer's instructions. Briefly, cells were transfected with expression constructs (above) for 6-7 days and recombinant PDCs or ADCs purified from culture supernatants using protein G-Sepharose. Bound proteins were eluted using 50 mM diethylamine with 150 mM NaCl. The eluted protein was neutralized by 2 M Tris pH 7.0, dialyzed against PBS, concentrated and loaded onto a Hiload 16/600 Superdex 200 gel filtration column (GE Healthcare). The monomeric form of the protein was separated, concentrated and analyzed using a Superdex 200 5/150 gel filtration column (GE Healthcare) or Yarra 3 μ m SEC-3000 column (Phenomenex).

[00169] Trastuzumab Fab-CalD2, 026 Fab-CalD2 and CalD1-Fc fusion proteins were produced using the Expi293 expression system described above and recombinant proteins purified from culture supernatants using Ni^{2+} -NTA-agarose.

[00170] Pertuzumab scFv and Fab fragments were expressed as recombinant proteins using *E. coli* as a host. scFv and Fab fragments were secreted into the periplasm and purified from osmotically shocked *E. coli* cells using Ni^{2+} -NTA-agarose.

[00171] Protein conjugation with maleimidocaproyl-val-cit-PAB-MMAE. Fc fusion or control antibody in PBS were reduced by addition of 16 molar equivalents (8 molar equivalents multiplied by the number of hinge disulfides) of TCEP at room temperature for 3 hours to reduce the hinge disulfide bonds. Maleimidocaproyl-val-cit-PAB-MMAE (MC-VC-PAB-MMAE; Levena Biopharma, catalog # SET0201) was then added to the reduced Fc fusion at 8 molar equivalents (4 molar equivalents multiplied by the number of free cysteines) and incubated at room temperature for 3 hours. Following the conjugation reaction, free MMAE was removed by extensively dialyzing the protein against PBS. The conjugated Fc fusion or control antibody was stored at 4°C.

[00172] To generate ADCs, analogous methods were used except 8 molar equivalents of TCEP and 4 molar equivalents of MC-VC-PAB-MMAE were used due to the presence of only one hinge disulfide. Additionally, the antibody was reduced at 37°C for 2 hours.

[00173] Surface plasmon resonance analyses. Binding analyses were carried out using a BIAcore T200 (GE Healthcare). Flow cells of CM5 sensor chips were coupled using amine coupling chemistry with recombinant HER2-ECD-Fc (fusion of HER2 extracellular domain to immunoglobulin Fc fragment), wild type (WT) pertuzumab, mutated pertuzumab variants or coupling buffer (10 mM sodium acetate pH 4.8) as a reference. Antibodies were injected over immobilized HER2-ECD-Fc, or HER2-ECD-Fc over the immobilized antibodies, at a flow rate of 5 or 10 μ L/minute at 25°C in phosphate buffered saline (PBS) plus 0.01% (v/v) Tween-20 and 0.05% (v/v) NaN_3 (pH 7.4, 7.0, 6.5, and 5.8). Flow cells were regenerated following each injection using 0.15M NaCl/0.1M glycine (pH 2.8) buffer. To determine equilibrium dissociation constants, antibodies were injected over immobilized HER2-ECD-Fc and the interactions were modeled as a 1:1 interaction using custom written software (Ober, R.J., Ward, E.S. (2002) Compensation for loss of ligand activity in surface plasmon resonance experiments. Anal. Biochem., 306, 228-236) to yield apparent dissociation constants (due to bivalent binding of the antibodies to immobilized HER2-ECD-Fc).

[00174] To investigate the calcium-dependence of the interaction of the Fab-CalD2 fusion protein and CalD1-Fc fusion protein, Fab-CalD2 was injected followed by co-injection of CalD1-Fc in buffer containing different calcium concentrations. Specifically, 100 nM trastuzumab Fab-CalD2 fusion was injected over immobilized HER2-ECD-Fc at a flow rate of 10 μ L/min at 25°C in PBS, 0.01% (v/v) Tween-20, 0.05% (v/v) NaN_3 pH 7.4 (PBS^+) plus

2 mM CaCl_2 , followed by 100 nM CalD1-Fc fusion at a flow rate of 10 $\mu\text{L}/\text{min}$ at 25°C in PBS^+ plus 2 mM CaCl_2 . PBS^+ with different concentrations of CaCl_2 or EDTA_2Na were injected during the dissociation phase. Data were processed using BIAevaluation and custom-written software.

[00175] Membrane lipid strip binding assay for PS-targeting proteins. Lipid-coated membrane strips (Echelon, catalog # P-6002) were first hydrated with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and then incubated with blocking solution (4% fatty acid free BSA dissolved in TBST) at room temperature for 1 hour. Proteins were diluted at 2 $\mu\text{g}/\text{ml}$ in blocking buffer and incubated with membranes at room temperature for 2 hours. The lipid strip was then washed with TBST and bound proteins detected using horse-radish peroxidase (HRP)-conjugated goat anti-human IgG (H+L) antibody.

[00176] Flow cytometry analyses of internalization of antibody-drug conjugates. Cancer cells were plated in 48 well plates and incubated at 37°C overnight. Cells were treated with 10 nM Alexa 488-labeled ADCs for 0.5, 4, and 20 hours. Treated cells were cooled on ice and surface signal of Alexa 488 was quenched with 5 $\mu\text{g}/\text{mL}$ rabbit anti-Alexa 488 antibody for 30 minutes at 4°C. Samples were washed, harvested by trypsinization, resuspended in PBS, analyzed using FACS-Accuri and data processed with FlowJo (FLOWJO, LLC).

[00177] To analyze the accumulation of CalD1-Fc within cells, LNCaP cells were plated in a 48 well plate and allowed to adhere. LNCaP cells were then pulsed with 100 nM Alexa 647-labeled CalD1-Fc or with a mixture of 100 nM Alexa 647-labeled CalD1-Fc plus 100 nM 026 Fab-CalD2 for 1 or 2 hours. Cells were washed, trypsinized using trypsin-EDTA (Gibco catalog # 25200056) to detach the cells and dissociate Alexa 647-labeled CalD1-Fc from cell surface bound 026 Fab-CalD2. Cells were harvested, washed and analyzed using a BD Accuri C6 flow cytometer.

[00178] Fluorescence microscopy analyses of PS- and HER2-targeting agents. To study the subcellular localization of PS-targeting agents, 2H11 or MDA-MB-231 cells were grown on coverslips (Zeiss, ref # 0109030091) and incubated with either 50 nM control IgG (HuLys10) or 50 nM PS-targeting agents diluted in growth medium for 3 hours. Cells were then washed with PBS and fixed with ice-cold 4% paraformaldehyde (PFA) at room

temperature for 20 minutes. Following fixation, cells were permeabilized with 0.1% Triton X-100 and incubated with blocking buffer (PBS, 5% serum and 0.1% Tween 20) at room temperature for 30 minutes. Primary antibodies specific for mouse LAMP-1 (clone 1D4B), human LAMP-1 (clone H4A3) or mouse EEA1 were diluted in blocking buffer and incubated with cells at room temperature for 2 hours. Cells were then washed with PBST (PBS with 0.1% Tween 20) and incubated with fluorescently-labeled secondary antibodies diluted in the blocking buffer for 1 hour at room temperature. To detect internalized PS-targeting agents, fluorescently-labeled goat or donkey anti-human IgG (H+L) antibody was used. Following the incubation, cells were washed with PBST and mounted with ProLong Gold antifade mountant (Life Technologies, catalog # P36930). Fluorescent images were acquired using a Zeiss Axiovert 200M inverted fluorescence microscope with a 63X, 1.4 NA plan apochromat objective (Carl Zeiss) and a 1.6X internal optovar. The acquired data were processed using the microscopy image analysis tool (MIATool) software (www4.utsouthwestern.edu/wardlab/miatool.asp).

[00179] To study the subcellular fate of internalized HER2-targeting ADCs, MDA-MB-453 cells were plated on Mattek dishes. Cells were pre-treated with 5 μ M Alexa 647-labeled dextran (pulsed for 2 hours, chased for 3 hours), and subsequently treated with 10 nM Alexa 488-labeled mutated variants of pertuzumab conjugated to MMAE (SG-MMAE, YS-MMAE) or T-DM1 for 20 hours. Wild type (WT) pertuzumab conjugated to MMAE was used as a control. Samples were treated with 33.3 nM rabbit anti-Alexa 488 antibody (ThermoFisher, catalog # A11094) for 30 minutes on ice to quench surface fluorescence. Cells were fixed with 1.7% (w/v) paraformaldehyde supplemented with 0.025% glutaraldehyde for 10 minutes at room temperature. Samples were imaged and data processed as above.

[00180] PS pull-down assay for PS-targeting agents. To study the Ca^{2+} -dependence of binding of the PS-targeting Fc fusions to PS, proteins were diluted at 100 nM in binding buffer (10 mM HEPES pH 7.4 and 150 mM NaCl with 2 mM or 2 μ M Ca^{2+}). 50 μ l PS-coated beads (Echelon, catalog # P-B0PS) were added and incubated at room temperature for 2 hours. The beads were then washed with the binding buffer and bound proteins detected by immunoblotting with HRP-conjugated goat anti-human IgG (H+L) antibody. To examine the pH-dependent binding to PS, proteins were diluted at 100 nM in PBS pH 7.4 or 6.0. 50 μ l (bed volume) PS-coated beads were added and incubated at room temperature for 2 hours.

The beads were then washed with PBS and bound proteins detected by immunoblotting with HRP-conjugated goat anti-human IgG (H+L) antibody.

[00181] Annexin V binding assay to analyze levels of exposed PS on cells. One million cells were suspended in Annexin V binding solution (10 mM HEPES pH 7.4 with 150 mM NaCl and 2.5 mM CaCl_2). Annexin V conjugated with Alexa 488 Fluor (Life Technologies, catalog # A13201) was added to the cell suspension at a 1:100 dilution and incubated with the cells for 10 minutes at room temperature. Cells were then washed once with the Annexin V binding solution and analyzed by flow cytometry (BD FACSCalibur). Flow cytometry data were processed using FlowJo (FLOWJO, LLC).

[00182] Flow cytometry analyses of PS-targeting agents. Cells were trypsinized and resuspended with flow cytometry buffer (PBS w/ Ca^{2+} / Mg^{2+} and 1% BSA). 50 nM PS-targeting Fc fusions were incubated with the cells for 30 minutes at either room temperature or on ice depending on the assay. Cells were washed with flow cytometry buffer and incubated with fluorophore-conjugated secondary antibodies on ice for 30 minutes. Cells were then washed and analyzed by flow cytometry (BD FACSCalibur). Flow cytometry data were processed using FlowJo (FLOWJO, LLC).

[00183] Cell growth and survival assays. Cancer cell lines (2H11, MCF-7, SK-BR-3, SK-OV-3, LNCaP, 22Rv1, MDA-MB-231, MDA-MB-453, MDA-MB-468, and HCC1954) were plated in 96 well plates. Cells were grown overnight followed by the addition of PS-targeting PDCs or HER2-targeting ADCs. Cell growth and survival were measured after 3-5 days incubation with the Cell Proliferation AQ One Solution Cell Proliferation Assay kit (Promega, catalog # G3581). Dose-response curves were plotted using GraphPad Prism software.

[00184] Whole body imaging, pharmacokinetic and therapy studies in mice. Animal procedures used in all mouse studies were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center and Texas A&M University. BALB/c SCID mice were purchased from the Jackson Laboratory (stock # 001803) and bred in-house. Pharmacokinetic studies were performed as described previously (19). Briefly, Lugol solution was added to drinking water 96 hours before the experiments. SCID BALB/c female mice (8 weeks old; 18-22 g weight) were anesthetized using 2%

isoflurane in oxygen and injected (i.v.) with ^{125}I -labeled proteins (100-120 μCi , 10-12 $\mu\text{g}/\text{mouse}$). Whole body counting was performed using a dose calibrator (Capintec Inc.) at different time points.

[00185] For whole body, near infrared imaging (NIR) with PS-targeting agents, female nude mice (6-7 weeks old; purchased from Envigo, catalog # 6903F) or BALB/c SCID mice (6-8 weeks old) were used. For implantation of MDA-MB-231 tumors, mice were anesthetized with 2% isoflurane in oxygen and a small surgical cut was performed to expose the mammary fat pad. MDA-MB-231 cells were trypsinized and dispersed into single cell suspensions in PBS. 5×10^6 cells/mouse were injected in 100 μl into the mammary fat pad, using a 25G needle, and the wound was then sealed with a wound clip. Buprenorphine was administered (s.c.) at 50 $\mu\text{g}/\text{kg}$ immediately following the surgery and 12 hours later. Mice were monitored daily and the wound clip was removed one week post-surgery. For imaging of nude mice, mice were divided into 3 groups ($n = 3$ mice per group) when tumors reached sizes of approximately 150 mm^3 and anesthetized with 2% isoflurane in oxygen. Anesthetized mice were injected (i.v.) with 1 nmol IRDye800CW-labeled PS-specific agents in PBS. Fluorescence imaging (FLI) was performed using a Caliper Xenogen IVIS Spectrum (Perkin Elmer) in vivo imaging system at 0 (before injection) and 3, 24 and 48 hours post-injection. FLI was performed using 745 nm excitation, 800 nm emission, binning 8, FOV 12.9 cm, f-stop 2 and auto-exposure. Data were quantitated with the Living Imaging software using absolute Radiant Efficiency (photons/s) in an ROI, manually drawn to outline the FLI signal of the tumor and normalized to the tumor volumes. For imaging in BALB/c SCID mice, mice were divided into 3 groups ($n = 3$ mice per group) when tumors reached sizes of approximately 300 mm^3 and injected (i.v.) with 1 nmol of IRDye800CW-labeled PS-specific agents in PBS. 48 hours following injection, tumors were dissected out and imaged as above. Fluorescence in an ROI, manually drawn to outline the FLI signal of the tumor was quantitated and normalized to the tumor weight.

[00186] For tumor treatment studies with PS-targeting PDCs, implantation of MDA-MBA-231 tumor xenografts in BALB/c SCID mice was carried out as described for the whole body imaging experiments. For implantation of LNCaP tumors, 7-8 week old male BALB/c SCID mice (18-22 g weight) were anesthetized with 2% isoflurane in oxygen and 5×10^6 LNCaP cells suspended in 50% RPMI and 50% Matrigel (BD Biosciences) were injected (s.c.). When MDA-MBA-231 or LNCaP tumors reached a size of $\sim 100 \text{ mm}^3$, mice were injected (i.p.) with 5 mg/kg docetaxel 72 and 48 hours before the treatment. Mice were then injected (i.v.) with 1 nmole unconjugated proteins, PDCs or PBS vehicle twice per week.

Tumors and body weights were measured twice a week. For the treatment experiment using Fc-Syt1(DN)_MMAE (FIGURE 16D), mice were treated for 4 weeks and monitored for another 2.5 weeks. Experiments were terminated when the tumor size reached 2 cm in any dimension.

[00187] For therapy with HER2-specific ADCs, 6-8 week old female BALB/c SCID mice were implanted with $4-5 \times 10^6$ MDA-MB-453 cells using methods described for the whole body imaging experiments. When the tumors reached a size of $\sim 60-100 \text{ mm}^3$, mice were injected (i.v.) with 2 mg/kg ADC, T-DM1, unconjugated protein, or with PBS vehicle once every 3 weeks (two doses total).

[00188] Immunohistochemical analyses. Female BALB/c SCID mice bearing MDA-MB-231 tumors were treated (i.p.) with 5 mg/kg docetaxel 72 and 48 hours before delivery (i.v.) of either PBS or 1 nmole Fc-Syt1 conjugated with MMAE. At different time points, mice were perfused with PBS followed by 4% PFA. Tumors were then dissected out, embedded in OCT (Fisher Scientific, catalog # 23-730-571) and stored at -80°C . $10 \mu\text{m}$ tissue sections were cut and hydrated with PBS at room temperature before fixation with 4% PFA for 30 minutes. Tumor sections were then washed with PBS and incubated with the permeabilization / blocking solution (PBS + 0.5% Triton X-100 and 3% BSA) at room temperature for 1 hour. Primary antibodies for human Ki-67 and mouse CD31 were diluted in the blocking buffer (PBS + 0.1% Tween 20 + 5% serum) and incubated with the tissue sections at 4°C overnight. The next day, tissue sections were washed with PBST (PBS + 0.1% Tween 20) and incubated with the fluorophore-conjugated secondary antibodies diluted in the blocking buffer at room temperature for 2 hours. After washing with PBST, tissue sections were mounted with ProLong Gold antifade mounting medium. Confocal images were acquired using a Nikon A1R confocal microscope with a 40X, 1.3 NA plan fluor objective and processed with the NIS-Elements software (Nikon).

Example 2. Generation of HER2-targeting agents with pH-dependent binding to HER2

[00189] FIGURE 4A and 4B show analyses of the binding of two mutated variants of pertuzumab: SG mutant, Ser55 mutated to histidine and Gly57 mutated to glutamic acid in the heavy chain variable domain (SEQ ID NO: 4); YS mutant, Tyr55 mutated to histidine in the light chain variable domain and Ser103 mutated to histidine in the heavy chain variable domain (SEQ ID NOS: 6, 8) that target HER2. The data were obtained using surface plasmon resonance. FIGURE 4A shows representative sensorgrams at pH 7.0 and 5.8 for the

interactions of 1 μ M HER-extracellular domain (ECD)-Fc fusion with mutated variants and wild type (WT) pertuzumab. For the analyses shown in FIGURE 4A, the antibodies were immobilized on the flow cells. FIGURE 4B shows the equilibrium dissociation constants (nM) for WT pertuzumab, SG and YS obtained by injecting the antibodies over immobilized HER2-ECD-Fc. The data demonstrate that both the SG and YS variants of pertuzumab have greater pH dependence than WT pertuzumab for binding to HER2.

Example 3. Internalization and accumulation of HER2-targeting agents in HER2-expressing cells

[00190] Pertuzumab (WT) and mutated variants SG and YS were conjugated through two hinge cysteine residues to maleimidocaproyl-val-cit-PAB-MMAE (MC-VC-PAB-MMAE) at a drug to antibody ratio (DAR) of 2 drugs per antibody, followed by analyses of binding and accumulation of the HER2-targeting ADCs (HER2-ADCs) within a panel of different HER2-expressing cell lines. FIGURE 5A shows the expression levels of HER2 on the different cancer cell lines, detected using Alexa 647-labeled pertuzumab (solid lines) or Alexa 647-labeled control antibody (dotted lines) and flow cytometry. FIGURE 5B shows the levels of internalized Alexa 488-labeled WT pertuzumab-MMAE (WT-MMAE), SG-MMAE, YS-MMAE, control antibody-MMAE (C-MMAE) or trastuzumab-DM1 (T-DM1) following 0.5, 4 and 20 hours incubation. Cell surface bound ADC was quenched using an Alexa 488-specific antibody. Error bars indicate standard deviation, with * indicating statistically significant differences (Student's t-test, $p < 0.05$). The data show that for all cancer cell lines tested, SG-MMAE and YS-MMAE accumulate to higher levels relative to WT-MMAE and T-DM1. The delivery of the ADCs to lysosomes in MDA-MB-453 cancer cells, that express intermediate levels of HER2 (FIGURE 5A) was also investigated using fluorescence microscopy (FIGURE 5C). Lysosomes in MDA-MB-453 cells were labeled by pulse chasing with Alexa 647-labeled dextran. Cells were subsequently incubated with 10 nM Alexa 488-labeled ADCs for 20 hours, washed and surface signal quenched using an Alexa-488 specific antibody. The microscopy images (size bar = 3 μ m) show substantially higher levels of accumulation of SG-MMAE and YS-MMAE in lysosomes compared with WT-MMAE or T-DM1.

Example 4. Inhibition of growth and survival of HER2-positive cells by HER2-ADCs

[00191] Analyses of the effects of the HER2-ADCs on the viability of HER2+ breast cancer cells show that SG-MMAE and YS-MMAE are more effective in reducing the viability of MDA-MBA-MB-453, SK-OV-3 and JIMT-1 cells than WT pertuzumab conjugated to MMAE (WT-MMAE) or T-DM1 (FIGURE 6). Error bars indicate standard deviation, with * indicating statistically significant differences (Student's *t*-test, $p < 0.05$).

Example 5. Inhibition of tumor growth by HER2-ADCs in mouse xenograft models

[00192] Analyses of the pharmacokinetics of HER2-ADCs (WT-MMAE, SG-MMAE and YS-MMAE) demonstrates that the in vivo persistence of SG-MMAE and YS-MMAE in BALB/c SCID mice is similar to that of WT-MMAE (FIGURE 7A; $n = 5$ mice/group). ADCs were radiolabeled with ^{125}I , injected into mice (5 mice/group) and remaining radioactivity in blood and at the whole body level determined at the indicated time points. Therapy studies with the ADCs in BALB/c SCID mice bearing MDA-MB-453 xenografts (intermediate HER2 expression levels) show that SG-MMAE and YS-MMAE are more effective in treatment than WT-MMAE or T-DM1 (FIGURE 7B). Mice were treated with two doses of 2 mg/kg ADC at days 17 and 38 (experiment 1; indicated by arrows) or days 24 and 45 (experiment 2; indicated by arrows). Error bars indicate standard errors, with statistically significant difference SG-MMAE vs. WT-MMAE or T-DM1; YS-MMAE vs. WT-MMAE or T-DM1) at treatment endpoints are indicated by * (Student's *t*-test; $p < 0.05$; $n = 5-8$ mice/group). Collectively, the data indicate that SG-MMAE and YS-MMAE have favorable pharmacokinetics and are also more effective than their parent, WT pertuzumab and clinically approved HER2-specific ADC, T-DM1, in reducing tumor growth.

Example 6. Generation and characterization of ADCs with calcium dependent association

[00193] The interaction of a HER2-specific Fab fragment (derived from trastuzumab) fused at the C-terminus of the CH1 domain via a hinge-SGG linker to domain 2 of calbindin D9K (CalD2) (trastuzumab Fab-CalD2; SEQ ID NO: 18, associated with trastuzumab light chain, SEQ ID NO: 20) and a human-IgG1-derived Fc fragment fused with domain 1 of calbindin D9K (CalD1-Fc; SEQ ID NO: 22) using surface plasmon resonance demonstrates Ca^{2+} -dependent association (FIGURE 8A). The sensorgrams show binding of

100 nM trastuzumab Fab-CalD2 to immobilized HER2-ECD, followed by injection of 100 nM CalD1-Fc and then buffer with different concentrations of Ca^{2+} ranging from 0-2 mM (boxed region, shown enlarged in right panel). The data show representative sensorgrams, demonstrating that as the Ca^{2+} -concentration decreases, the dissociation of the Fab-CalD2 and CalD1-Fc increases. In additional experiments, a fusion protein including the PSMA-specific VH-CH1 domains (PRGX1-XG1-029; abbreviated to 026; Schülke, N., Varlamova, O.A., Donovan, G.P., Ma, D., Gardner, J.P., Morrissey, D.M., Arrigale, R.R., Zhan, C., Chodera, A.J., Surowitz, K.G., Maddon, P.J., Heston, W.D.W., Olson, W.C. (2003) The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy. *Proc. Natl. Acad. Sci., USA*, 100, 1259-12595) fused at the C-terminus via a hinge-SGG linker to CalD2 (026-CalD2; SEQ ID NO: 24) was also generated. In association with the 026 light chain (SEQ ID NO: 26), this CalD2 fusion forms Fab-CalD2. Flow cytometry analyses demonstrated that the accumulation of fluorescently labeled CalD1-Fc (SEQ ID NO: 22) within PSMA-expressing LNCaP cells was enhanced when cells were treated with a mixture (100 nM each) of 026-CalD2 and Alexa 647-labeled CalD1-Fc compared with treatment with 100 nM Alexa 647-labeled CalD1-Fc without added 026-CalD2 ('no Fab') (FIGURE 8B).

Example 7. Generation and characterization of PS-targeting agents

[00194] A panel of PS-targeting agents was generated by fusing the Fc region of human IgG1 to the following PS-binding domains: core domain of Annexin A1 (AnxA1), C2A domain of Synaptotagmin 1 (Syt1) and C2 domain of PKC α . The resulting fusion proteins were designated Fc-AnxA1, Fc-Syt1 and Fc-PKC α , respectively. The PS-targeting agents were purified as homodimers (FIGURE 9, FIGURE 10A; assessed using SDS-PAGE and HPLC), and bound to PS in a lipid binding assay using lipid strips (FIGURE 10B). They also bound to cardiolipin, which is located on the inner mitochondrial membrane of eukaryotic cells and therefore not relevant to targeting. Fc-AnxA1 exhibited a broad lipid binding profile and bound both neutral and negatively-charged lipids (FIGURE 10B). Importantly, none of the PS-binding agents bound phosphatidylcholine (PC) and sphingomyelin, lipids that are present in the plasma membrane outer leaflet.

[00195] The tumor endothelial cell line 2H11 was used to investigate the ability of the PS-binding agents to interact with lipids on the cell surface. Binding of fluorescent Annexin V showed that these cells exposed PS and that PS exposure increased following

docetaxel treatment. Flow cytometry analyses showed that all PS-binding agents interacted with PS-positive cells, with Fc-Syt1 showing lower levels of binding (FIGURE 10C; 2nd only indicates secondary antibody control, and Fc indicates Fc fragment without PS-targeting protein or domain).

[00196] The pharmacokinetic behavior and tumor localization of the three PS-binding agents in mice were evaluated to determine which recombinant proteins were suitable for further development as protein-drug conjugates (PDCs). Pharmacokinetic studies of the PS-binding agents revealed that Fc-Syt1 had a significantly longer half-life in mice (FIGURE 10D, E). Whole body counts following injection of radioiodinated PS-targeting fusion proteins into mice ($n = 5$ mice/group) are shown in the graph in FIGURE 10D, and 10E shows the corresponding areas under the curve for each radiolabeled protein. In addition, proteins were labeled with the residualizing dye, IRDye800CW, and injected (i.v.) into female nude mice bearing MDA-MB-231 xenografts ($n = 3$ mice/group) and imaged at the indicated time points (FIGURE 10F), with tumor fluorescence quantitated in extracted tumors at 48 hours post-injection (FIGURE 10G). Similar experiments were carried out in tumor-bearing BALB/c SCID mice ($n = 3$ mice/group) and 48 hours later, tumors were excised and dye levels determined (FIGURE 10H). Amongst the three PS-specific agents, Fc-Syt1 exhibited the highest level of tumor localization. Statistically significant differences for FIGURES 10E, 10G and 10H were analyzed using one-way ANOVA followed by Tukey post-hoc test (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$), and error bars represent SEM.

Example 8. Tetravalency of the PS-targeting agent increases binding and internalization into target cells

[00197] In several receptor systems it has been shown that multivalent ligands, or mixtures of cross-linking ligands such as antibodies, promote receptor internalization and degradation. To study the role of avidity in the behavior of PS-targeting PDCs, the tetravalent Syt1-Fc-Syt1 that contains four Syt1 C2A domains was generated (shown schematically in FIGURE 11A). The tetravalent protein was purified as homodimers (FIGURE 11A, B; assessed using SDS-PAGE and HPLC). Binding analyses using lipids immobilized on nitrocellulose demonstrated that Syt1-Fc-Syt1 had higher affinity/avidity for PS and the same lipid selectivity as its bivalent parent, Fc-Syt1 (FIGURE 11C). Consistent with the binding

data shown in FIGURE 11C, the tetravalent Syt1-Fc-Syt1 bound to 2H11 cells at significantly higher levels when analyzed using flow cytometry (FIGURE 11D).

[00198] The internalization of Fc-Syt1 and Syt1-Fc-Syt1 using Alexa 647-labeled proteins was also studied. 2H11 cells were incubated with labeled Fc-Syt1 and Syt1-Fc-Syt1 on ice at different concentrations to achieve similar surface binding followed by incubation at 37°C to allow internalization for different times. Surface-bound proteins were stripped off with EDTA (due to the Ca^{2+} -dependence of binding) and internalized levels (resistant to stripping) were determined by flow cytometry. These studies indicated that although both proteins efficiently accumulated within cells, the tetravalent Syt1-Fc-Syt1 was internalized more quickly (FIGURE 11E). For FIGURES 11D and 11E, statistically significant differences were analyzed using two-way ANOVA followed by Tukey post hoc test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars in FIGURES 11D and 11E represent SEM.

[00199] Fluorescent microscopy was also used to study the subcellular trafficking behavior of the Syt1-Fc fusion proteins. 2H11 and MDA-MB-231 cells were incubated with 50 nM PS-targeting agent or control IgG for 4 hours, followed by washing, fixation and staining with Cy3/Alexa 555-labeled anti-human IgG (H+L). The lysosomal marker, LAMP-1, was detected using a LAMP-1 specific antibody followed by Alexa 488-labeled secondary conjugate. Fc-Syt1 and Syt1-Fc-Syt1 were internalized and delivered into LAMP-1 positive lysosomes in 2H11 (FIGURE 11F) and MDA-MB-231 (FIGURE 11G) cells. Scale bars: 10 μm (F) and 5 μm (G).

Example 9. Calcium sensing and endosomal release of PS-PDCs

[00200] The lysosomal trafficking and internalization behavior of Fc-Syt1 and Syt1-Fc-Syt1 indicated that they could be effective as delivery vehicles for conjugated drugs. Maleimidocaproyl-val-cit-PAB-MMAE was conjugated to hinge cysteines (FIGURE 12A, left panel). For use as a negative control, maleimidocaproyl-val-cit-PAB-MMAE was conjugated to a hen egg lysozyme-specific human IgG1 in which the light/heavy chain-interacting cysteine residues were mutated to serine. SDS-PAGE analyses indicated that the conjugations had gone to completion and resulted in a drug to antibody ratio (DAR) of four (FIGURE 12A, right panel). This was confirmed by MALDI-TOF mass spectrometry for Fc-Syt1_MMAE. Since complete conjugation disrupted the two disulfide bonds in the hinge region, a molecular weight of 43.6 kDa was obtained using mass spectrometry (FIGURE

12B). This contrasted with the unconjugated or partially conjugated protein that retained two or one disulfide bonds respectively, resulting in an apparent molecular weight of approximately 82 kDa (FIGURE 12B). Importantly, HPLC analyses demonstrated that the conjugation process did not lead to protein aggregation (FIGURE 12C).

[00201] The Syt1 C2A domain requires Ca^{2+} for PS binding. The lower Ca^{2+} concentration in early/sorting endosomes ($\sim 2 \mu\text{M}$) compared with the extracellular Ca^{2+} levels (1 – 2 mM) suggests that following internalization, PS-targeting PDCs (PS-PDCs) will dissociate from the limiting membrane of these endosomes. This dissociation is expected to lead to improved lysosomal delivery. Both PS-PDCs containing Syt1 domains bound to PS-beads in buffer containing 2 mM Ca^{2+} , but no detectable interaction was observed when the Ca^{2+} concentration was decreased to 2 μM (FIGURE 12D). In addition, because the pH within sorting endosomes is acidic (pH 6.0-6.5), the effect of pH on PDC:PS interactions was analyzed. Both PDCs bound to PS at similar levels in the pH range 6.0-7.4 (FIGURE 12D). For FIGURE 12D, bead-associated proteins were analyzed using immunoblotting and detection with goat anti-human IgG (H+L) conjugated with horse-radish peroxidase.

[00202] Consistent with the in vitro binding analyses demonstrating Ca^{2+} -dependent binding, fluorescence microscopy analyses following incubation of MDA-MB-453 cells with 100 nM PS-PDC or MMAE-conjugated control for 30 minutes, followed by washing and staining early endosomes with early endosomal antigen 1 (EEA1)-specific antibody, showed the presence of the PS-PDCs in the lumen, rather than limiting membrane of sorting endosomes following internalization into the cells (FIGURE 13A). The PS-PDCs were detected using an Alexa 555-labeled anti-human IgG (H+L) antibody in these experiments, and intensity analyses of early endosomes (labeled a and b) that have been cropped and expanded are shown on the right side of the figure panel. In addition, Fc-Syt1_MMAE and Syt1-Fc-Syt1_MMAE could be detected in lysosomes (detected using a LAMP-1-specific antibody) within four hours of delivery (FIGURE 13B). Both PS-PDCs disrupted the microtubular network in 2H11 and MDA-MB-231 cells following incubation of 2H11 or MDA-MB-453 cells with 100 nM or 50 nM PS-PDCs for 10 or 20 hours, respectively (FIGURE 13C). Scale bars = 5 μm (FIGURE 13A), 10 μm (FIGURE 13B), 15 μm (FIGURE 13C, upper panels) and 10 μm (FIGURE 13C, lower panels).

Example 10. Inhibition of growth and survival of PS-positive cells by PS-PDCs

[00203] The effects of PS-specific PDCs on the growth of multiple cell lines were examined, including tumor endothelium (2H11), ER positive breast cancer (T-47D), HER2-positive breast cancer (SK-BR-3), triple negative breast cancer (MDA-MB-231), androgen sensitive prostate cancer (LNCaP) and androgen insensitive prostate cancer (22Rv1). Staining of the cells with fluorescently labeled Annexin V followed by flow cytometry analyses showed that all of these cell lines were PS-positive (FIGURE 14A). Incubation of the cells with PS-PDCs effectively inhibited the growth and survival of the cells in a dose-dependent manner (FIGURE 14B). Cell viability following 72 hours (2H11), 96 hours (SK-BR-3, MDA-MB-231 and 22Rv1) or 120 hours (T-47D) incubation is shown. Although the tetravalent Syt1-Fc-Syt1_MMAE was more potent in inhibiting T-47D cell growth than the divalent Fc-Syt1_MMAE, the two PDCs exhibited similar effects on the other cell lines. By contrast, relatively high concentrations of the control IgG (hen egg lysozyme-specific human IgG1) conjugated with MMAE resulted in inhibition of cell growth (FIGURE 14B), possibly due to nonspecific fluid phase uptake of the drug. Consistent with the growth inhibitory effects of the PS-PDCs, flow cytometry analyses of the internalization of the two PDCs at concentrations close to their corresponding IC50s showed similar behavior for all other cell lines following 2 hours incubation, except that Syt1-Fc-Syt1_MMAE was internalized at a higher level in T-47D cells (FIGURE 14C). For FIGURE 14C, statistically significant differences were analyzed using two-way ANOVA followed by Bonferroni *post hoc* test (n.s., no significant difference; *, $p < 0.05$; ***, $p < 0.001$, and error bars represent SEM). In addition, unconjugated PS-targeting proteins showed no effect on cell growth in cell viability assays when added to cells at a concentration of 1 μ M for 96 hours (FIGURE 14D). Thus, the data indicate that Syt1-based PDCs are potent inhibitors of tumor endothelial and cancer cell growth *in vitro*.

Example 11. Inhibition of tumor growth by PS-PDCs in mouse xenograft models

[00204] The therapeutic effects of the PS-PDCs against tumor xenografts in BALB/c SCID mice were investigated. Prior to therapy, pharmacokinetic studies of the PS-PDCs demonstrated that tetravalent Syt1-Fc-Syt1_MMAE had a shorter half-life than bivalent Fc-Syt1_MMAE (FIGURE 15A, B), possibly due to increased target-mediated uptake. FIGURE 15A shows whole body counts in BALB/c SCID mice (n = 5 mice/group) following injection of radioiodinated PS-PDC. FIGURE 15B shows the areas under the curve for the clearance curves shown in FIGURE 15A, with statistically significant differences

analyzed using unpaired Student's t-test (****, $p < 0.0001$). To investigate their therapeutic effects, the PDCs were delivered into female BALB/c SCID mice ($n = 5-6$ mice/group) bearing orthotopic MDA-MB-231 breast tumors. Tumor-bearing mice were pretreated with docetaxol, and the following doses (equivalent to 1 nmole protein) of PDCs or control unconjugated proteins were delivered twice per week: 4.1 mg/kg for Fc-Syt1 or Fc-Syt1_MMAE, 5.6 mg/kg for Syt1-Fc-Syt1 or Syt1-Fc-Syt1_MMAE. In these experiments, bivalent Fc-Syt1_MMAE potently blocked breast tumor growth (FIGURE 15C). Tetravalent Syt1-Fc-Syt1_MMAE also inhibited tumor growth, but less effectively than bivalent Fc-Syt1_MMAE. Treatment of unconjugated Fc-Syt1 and Syt1-Fc-Syt1 had no effect on tumor growth (FIGURE 15C). Importantly, no weakness or loss of body weight in any of the treatment groups was observed (FIGURE 15D), indicating that PS-specific PDCs are well-tolerated *in vivo*.

[00205] Similar to the efficacy in the breast tumor model, bivalent Fc-Syt1_MMAE completely blocked tumor growth in male BALB/c SCID mice bearing prostate cancer LNCaP xenografts that had been pretreated with docetaxel (FIGURE 15E). Tumor-bearing mice were dosed as for the MDA-MB-231 xenograft experiments (above). Consistent with the *in vitro* data (FIGURE 14D), the unconjugated PS-targeting fusion proteins had no effect. However, tetravalent Syt1-Fc-Syt1_MMAE did not significantly inhibit tumor growth in the LNCaP model. The most likely explanation for this difference is the shorter *in vivo* persistence of the tetravalent PDC. For both FIGURES 15C and 15E, statistical analyses at treatment end points were analyzed using one-way ANOVA followed by Bonferroni *post hoc* test (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars in FIGURES 15A, 15C and 15E represent SEM.

Example 12. Inhibition of tumor growth by Fc-Syt1 MMAE is dependent on PS-binding

[00206] To exclude the possibility that the drug accumulated in the tumor through non-specific mechanisms such as the enhanced permeability and retention (EPR) effect and to show that the *in vivo* efficacy was dependent on PS binding, a mutated variant of the synaptotagmin 1 C2A domain was generated with decreased affinity for PS. The C2A domain of synaptotagmin 1 interacts with PS through three Ca^{2+} ions chelated by five aspartic acids (D) in domain loops I and III (Striegel, A.R., Biela, L.M., Evans, C.S., Wang, Z., Delehoy, J.B., Sutton, R.B., Chapman, E.R. and Reist, N.E. 2012. Calcium binding by synaptotagmin's

C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission. J. Neurosci. 32, 1253-1260). To ablate the Ca^{2+} binding, all five aspartic acid residues (D173N, D179N, D231N, D233N and D239N) were mutated to asparagines (N) to produce Fc-Syt1(DN). Fc-Syt1(DN) was purified and conjugated to MMAE with a DAR of four (FIGURE 16A), and interacted with PS at essentially background levels in the protein-lipid overlay assay using lipid-coated nitrocellulose strips (FIGURE 16B). Moreover, flow cytometry analyses demonstrated that Fc-Syt1(DN) had significantly reduced binding to PS-positive cells compared with its wild-type counterpart (FIGURE 16C). In FIGURE 16C, statistically significant differences were analyzed using two-way ANOVA followed by Tukey *post hoc* test (***, $p < 0.001$; ****, $p < 0.0001$), and error bars indicate SEM.

[00207] BALB/c SCID mice bearing orthotopic MDA-MB-231 tumors (n = 6 mice/group) were pretreated with docetaxel prior to treatment with PS-PDCs or controls for four weeks, until mice in the control (PBS) group were euthanized due to their large tumor sizes. Mice were treated twice per week for four weeks (days 28-56) with the following doses (equivalent to 1 nmole protein): 4.1 mg/kg for Fc-Syt1_MMAE or Fc-Syt1(DN)_MMAE, 2.6 mg/kg for Fc_MMAE. Treatment of MDA-MB-231 tumors with Fc-Syt1_MMAE led to potent growth inhibition (FIGURE 16D). More importantly, tumor growth remained inhibited after the delivery of Fc-Syt1_MMAE was stopped at four weeks. Statistically significant differences between Fc-Syt1_MMAE and Fc-Syt1(DN)_MMAE treatment groups at the treatment end point were analyzed using one-way ANOVA followed by Bonferroni *post hoc* test (***, $p < 0.001$), and error bars indicate SEM. Tumors from mice within each group were isolated at the end of the experiment, and no tumors could be isolated from three of six mice in the Fc-Syt1_MMAE treatment group (FIGURE 16E). Although the delivery of Fc_MMAE or Fc-Syt1(DN)_MMAE slowed tumor growth initially, rapid proliferation was observed following the end of treatment (FIGURE 16D). Collectively, the data indicate that PS-binding is essential for the activity of Fc-Syt1_MMAE.

EXAMPLE 13. Fc-Syt1 MMAE targets multiple cell types including both tumor endothelium and cancer cells in tumor tissues

[00208] To further validate that Fc-Syt1_MMAE binds PS-positive cells in tumor tissue following docetaxel treatment, immunohistochemistry was performed at 1 hour

(FIGURE 17A) or 24 hours (FIGURE 17B) following delivery of this PDC into tumor-bearing mice. PBS was injected as a vehicle control, and Fc-Syt1_MMAE was detected using Alexa 555-labeled anti-human IgG (H+L). Fc-Syt1_MMAE was localized to CD31-positive blood vessels (FIGURE 17A), tumor cells and tumor-infiltrating F4/80-positive macrophages (FIGURE 17B) which can expose PS. The data indicate that cancer cells not only expose PS *in vitro* (FIGURE 14A), but retain this loss of PS asymmetry *in vivo*.

Example 14. DNA and protein sequences of exemplary antibody-drug conjugates, protein-drug conjugates

[00209] Table 1 shows DNA sequences of polynucleotides encoding exemplary proteins described herein, and Table 2 shows amino acid sequences of exemplary proteins encoded by the polynucleotides shown in Table 1, wherein the DNA sequence of SEQ ID NO: 1 encodes the protein of SEQ ID NO: 2, the DNA sequence of SEQ ID NO: 3 encodes the protein of SEQ ID NO: 4, the DNA sequence of SEQ ID NO: 5 encodes the protein of SEQ ID NO: 6, the DNA sequence of SEQ ID NO: 7 encodes the protein of SEQ ID NO: 8, the DNA sequence of SEQ ID NO: 9 encodes the protein of SEQ ID NO: 10, the DNA sequence of SEQ ID NO: 11 encodes the protein of SEQ ID NO: 12, the DNA sequence of SEQ ID NO: 13 encodes the protein of SEQ ID NO: 14, the DNA sequence of SEQ ID NO: 15 encodes the protein of SEQ ID NO: 16, the DNA sequence of SEQ ID NO: 17 encodes the protein of SEQ ID NO: 18, the DNA sequence of SEQ ID NO: 19 encodes the protein of SEQ ID NO: 20, the DNA sequence of SEQ ID NO: 21 encodes the protein of SEQ ID NO: 22, the DNA sequence of SEQ ID NO: 23 encodes the protein of SEQ ID NO: 24, the DNA sequence of SEQ ID NO: 25 encodes the protein of SEQ ID NO: 26, the DNA sequence of SEQ ID NO: 27 encodes the protein of SEQ ID NO: 28, the DNA sequence of SEQ ID NO: 29 encodes the protein of SEQ ID NO: 30, the DNA sequence of SEQ ID NO: 31 encodes the protein of SEQ ID NO: 32, the DNA sequence of SEQ ID NO: 33 encodes the protein of SEQ ID NO: 34, the DNA sequence of SEQ ID NO: 35 encodes the protein of SEQ ID NO: 36, the DNA sequence of SEQ ID NO: 37 encodes the protein of SEQ ID NO: 38, the DNA sequence of SEQ ID NO: 39 encodes the protein of SEQ ID NO: 40, the DNA sequence of SEQ ID NO: 41 encodes the protein of SEQ ID NO: 42, the DNA sequence of SEQ ID NO: 43 encodes the protein of SEQ ID NO: 44, the DNA sequence of SEQ ID NO: 45 encodes the protein of SEQ ID NO: 46 and the DNA sequence of SEQ ID NO: 47 encodes the protein of SEQ ID NO: 48.

Table 1. DNA sequences of polynucleotides encoding exemplary proteins.

Polynucleotide encoding protein	DNA sequence	SEQ ID NO:
Pertuzumab light chain with Cys mutation	GACATCGAGCTCACACAGTCTCCTTCTTCTCTGTCTGCTTC TGTGGGAGATAGAGTGACAATCACATGTAAGGCTTCTCA GGATGTGTCTATCGGAGTGGCTTGGTACCAGCAGAAGCC TGGAAAGGCTCCTAAGCTGCTGATCTACTCTGCTTCTTAC AGATACACAGGAGTGCCTTCTAGATTCTCTGGATCTGGAT CTGGAACAGATTTACACTGACAATCTCTTCTCTACAGCC TGAGGATTTTCGCTACATACTACTGTCAGCAGTACTACATC TACCCTTACACATTCGGACAGGGAACAAAGCTCGAGATC AAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGC CATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGT GTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGT ACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTC CCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCA CCTACAGCCTCAGCAGCACCTTGACGCTGAGCAAAGCAG ACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCC ATCAGGGCCTGAGTTCGCCCCGTCACAAAGAGCTTCAACA GGGGAGAGTCT	1
SG heavy chain with Cys mutations	CAGGTCCAAGTGCAGGAGTCTGGAGGAGGACTGGTGCAG CCTGGAGGATCTCTGAGACTGTCTTGTGCTGCTTCTGGAT TCACATTCACAGATTACACAATGGATTGGGTGAGACAGG CTCCTGGAAAGGGACTGGAGTGGGTGGCTGATGTGAATC CTAATCACGGAGAATCTATCTACAATCAGAGATTCAAGG GAAGATTCACACTGTCTGTGGATAGATCTAAGAATACAC TGTACCTACAGATGAACTCTCTGAGAGCTGAGGATACAG CTGTGTACTACTGTGCTAGAAATCTGGGACCTTCTTTCTA CTTCGATTACTGGGGACAGGGAACACTGGTCAACCGTCTCC TCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCAC CCTCCTCCAAGAGCACCTCTGGGGGGCACAGCGGCCCTGG GCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGG TGTCGTGGAAGTCAAGGCGCCCTGACCAGCGGCGTGCACA CCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCT CAGCAGCGTGGTGACTGTGCCCTCCAGCAGCTTGGGCAC CCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAA CACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTCTGA CAAACTCACACATGCCACCGTCCCCAGCACCTGAACT CCTGGGGGGGACCGTCAGTCTTCTCTTCCCCCAGAAACCC AAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACA TGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTC AAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAAT GCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCAC GTACCGTGTGGTCAGCGTCCTACCGTCCTGCACCAGGAC TGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGCCCTCCAGCCCCCATCGAGAAAACCATCTCCAAA	3

	GCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTG CCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGC CTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCG CCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAAC TACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCT TCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGT GGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATG AGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCC TGTCTCCGGGTAAA	
YS light chain with Cys mutation	GACATCGAGCTCACACAGTCTCCTTCTTCTGTCTGCTTC TGTGGGAGATAGAGTGACAATCACATGTAAGGCTTCTCA GGATGTGTCTATCGGAGTGGCTTGGTACCAGCAGAAGCC TGGAAAGGCTCCTAAGCTGCTGATCTACTCTGCTTCTTAC AGACACACAGGAGTGCCTTCTAGATTCTCTGGATCTGGAT CTGGAACAGATTTCACACTGACAATCTCTTCTCTACAGCC TGAGGATTTTCGCTACATACTACTGTCAGCAGTACTACATC TACCCTTACACATTCGGACAGGGAACAAAGCTCGAGATC AAACGAACTGTGGCTGCACCATCTGTCTTTCATCTTCCCGC CATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGT GTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGT ACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTC CCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCA CCTACAGCCTCAGCAGCACCTTGACGCTGAGCAAAGCAG ACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCC ATCAGGGCCTGAGTTCGCCCCGTCACAAAGAGCTTCAACA GGGGAGAGTCT	5
YS heavy chain with Cys mutations	CAGGTCCAACCTGCAGGAGTCTGGAGGAGGACTGGTGCAG CCTGGAGGATCTCTGAGACTGTCTTGTGCTGCTTCTGGAT TCACATTCACAGATTACACAATGGATTGGGTGAGACAGG CTCCTGGAAAGGGACTGGAGTGGGTGGCTGATGTGAATC CTAATTCTGGAGGATCTATCTACAATCAGAGATTCAAGG GAAGATTCACACTGTCTGTGGATAGATCTAAGAATACAC TGTACCTACAGATGAACTCTCTGAGAGCTGAGGATACAG CTGTGTACTACTGTGCTAGAAATCTGGGACCTCACTTCTA CTTCGATTACTGGGGACAGGGAACACTGGTCAACCGTCTCC TCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCAC CCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGG GCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGG TGTCGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGACA CCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCT CAGCAGCGTGGTGAAGTGTGCCCTCCAGCAGCTTGGGCAC CCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAA CACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTCTGA CAAACTCACACATGCCACCGTCCCCAGCACCTGAACT CCTGGGGGGGACCGTCAGTCTTCTCTTCCCCCAAACCC AAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACA TGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTC AAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAAT GCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCAC	7

	GTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGAC TGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAAC AAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAA GCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTG CCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGC CTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCG CCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAAC TACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCT TCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGT GGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATG AGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCC TGTCTCCGGGTAAA	
SG scFv linked to N-terminus of heavy chain with Cys mutations	CAGGTGCAGCTGCAGGAGTCTGGAGGAGGACTGGTGCAG CCTGGAGGATCTCTGAGACTGTCTTGTGCTGCTTCTGGAT TCACATTCACAGATTACACAATGGATTGGGTGAGACAGG CTCCTGGAAAGGGACTGGAGTGGGTGGCTGATGTGAATC CTAATCACGGAGAATCTATCTACAATCAGAGATTCAAGG GAAGATTCACACTGTCTGTGGATAGATCTAAGAATACAC TGTACCTACAGATGAACTCTCTGAGAGCTGAGGATACAG CTGTGTACTACTGTGCTAGAAATCTGGGACCTTCTTTCTA CTTCGATTACTGGGGACAGGGAACACTGGTCACGGTCTC CTCGGGAGGTGGCGGATCTGGTGGAGGTGGCAGTGGTGG AGGTGGCTCAGACATCGAGCTCACACAGTCTCCTTCTTCT CTGTCTGCTTCTGTGGGAGATAGAGTGACAATCACATGTA AGGCTTCTCAGGATGTGTCTATCGGAGTGGCTTGGTACCA GCAGAAGCCTGGAAAGGCTCCTAAGCTGCTGATCTACTC TGCTTCTTACAGATACACAGGAGTGCCTTCTAGATTCTCT GGATCTGGATCTGGAACAGATTTACACTGACAATCTCTT CTCTACAGCCTGAGGATTTTCGCTACATACTACTGTCAGCA GTACTACATCTACCCTTACACATTCGGACAGGGAACAAA GCTCGAGATCAAACGGGGTGGCAGCGTTGAGCCCAAATC TTCTGACAAAACCTCACACATGCCACCGTCCCCAGCACCT GAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCAA AACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGG TCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAAC AGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACC AGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCT CCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCT CCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACA CCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGG TCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGA CATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGA ACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGACG GCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAG CAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGAT GCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCT CTCCCTGTCTCCGGGTAAA	9

YS scFv linked to N-terminus of heavy chain with Cys mutations	CAGGTGCAGCTGCAGGAGTCTGGAGGAGGACTGGTGCAG CCTGGAGGATCTCTGAGACTGTCTTGTGCTGCTTCTGGAT TCACATTACAGATTACACAATGGATTGGGTGAGACAGG CTCCTGGAAAGGGACTGGAGTGGGTGGCTGATGTGAATC CTAATTCTGGAGGATCTATCTACAATCAGAGATTCAAGG GAAGATTCACACTGTCTGTGGATAGATCTAAGAATACAC TGTACCTACAGATGAACTCTCTGAGAGCTGAGGATACAG CTGTGTACTACTGTGCTAGAAATCTGGGACCTCACTTCTA CTTCGATTACTGGGGACAGGGAACACTGGTCACGGTCTC CTCGGGAGGTGGCGGATCTGGTGGAGGTGGCAGTGGTGG AGGTGGCTCAGACATCGAGCTCACACAGTCTCCTTCTTCT CTGTCTGCTTCTGTGGGAGATAGAGTGACAATCACATGTA AGGCTTCTCAGGATGTGTCTATCGGAGTGGCTTGGTACCA GCAGAAGCCTGGAAAGGCTCCTAAGCTGCTGATCTACTC TGCTTCTTACAGACACACAGGAGTGCCTTCTAGATTCTCT GGATCTGGATCTGGAACAGATTTACACTGACAATCTCTT CTCTACAGCCTGAGGATTTTCGCTACATACTACTGTCAGCA GTACTACATCTACCCTTACACATTCGGACAGGGAACAAA GCTCGAGATCAAACGGGGTGGCAGCGTTGAGCCCAAATC TTCTGACAAAACCTCACACATGCCACCGTCCCCAGCACCT GAACTCCTGGGGGGGACCGTCAGTCTTCCTCTTCCCCCAA AACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGG TCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTG AGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAAC AGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACC AGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCT CCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCT CCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACA CCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGG TCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGA CATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGA ACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGACG GCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAG CAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGAT GCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCT CTCCCTGTCTCCGGGTAAA	11
Heavy chain with Cys mutations and SG scFv linked to C-terminus	GTTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCAC CGTCCCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTT CCTCTTCCCCCCTAAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA	13

	TGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGTGGCA GCCAGGTGCAGCTGCAGGAGTCTGGAGGAGGACTGGTGC AGCCTGGAGGATCTCTGAGACTGTCTTGTGCTGCTTCTGG ATTCACATTCACAGATTACACAATGGATTGGGTGAGACA GGCTCCTGGAAAGGGACTGGAGTGGGTGGCTGATGTGAA TCCTAATCACGGAGAATCTATCTACAATCAGAGATTCAA GGGAAGATTCACACTGTCTGTGGATAGATCTAAGAATAC ACTGTACCTACAGATGAACTCTCTGAGAGCTGAGGATAC AGCTGTGTACTACTGTGCTAGAAATCTGGGACCTTCTTTC TACTTCGATTACTGGGGACAGGGAACACTGGTCACGGTC TCCTCGGGAGGTGGCGGATCTGGTGGAGGTGGCAGTGGT GGAGGTGGCTCAGACATCGAGCTCACACAGTCTCCTTCTT CTCTGTCTGCTTCTGTGGGAGATAGAGTGACAATCACATG TAAGGCTTCTCAGGATGTGTCTATCGGAGTGGCTTGGTAC CAGCAGAAGCCTGGAAAGGCTCCTAAGCTGCTGATCTAC TCTGCTTCTTACAGATACACAGGAGTGCCTTCTAGATTCT CTGGATCTGGATCTGGAACAGATTTCACACTGACAATCTC TTCTCTACAGCCTGAGGATTTCGCTACATACTACTGTCAG CAGTACTACATCTACCCTTACACATTCGGACAGGGAACA AAGCTCGAGATCAAACGG	
Heavy chain with Cys mutations and YS scFv linked to C-terminus	GTTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCAC CGTCCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTT CCTCTTCCCCC AAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGTGGCA GCCAGGTGCAGCTGCAGGAGTCTGGAGGAGGACTGGTGC AGCCTGGAGGATCTCTGAGACTGTCTTGTGCTGCTTCTGG ATTCACATTCACAGATTACACAATGGATTGGGTGAGACA GGCTCCTGGAAAGGGACTGGAGTGGGTGGCTGATGTGAA TCCTAATTCTGGAGGATCTATCTACAATCAGAGATTCAAG GGAAGATTCACACTGTCTGTGGATAGATCTAAGAATACA CTGTACCTACAGATGAACTCTCTGAGAGCTGAGGATACA GCTGTGTACTACTGTGCTAGAAATCTGGGACCTCACTTCT	15

	ACTTCGATTACTGGGGACAGGGAACACTGGGTCACGGTCT CCTCGGGAGGTGGCGGATCTGGTGGAGGTGGCAGTGGTG GAGGTGGCTCAGACATCGAGCTCACACAGTCTCCTTCTTC TCTGTCTGCTTCTGTGGGAGATAGAGTGACAATCACATGT AAGGCTTCTCAGGATGTGTCTATCGGAGTGGCTTGGTACC AGCAGAAGCCTGGAAAGGCTCCTAAGCTGCTGATCTACT CTGCTTCTTACAGACACACAGGAGTGCCTTCTAGATTCTC TGGATCTGGATCTGGAACAGATTTCACACTGACAATCTCT TCTCTACAGCCTGAGGATTTTCGCTACATACTACTGTCAGC AGTACTACATCTACCCTTACACATTTCGGACAGGGAACAA AGCTCGAGATCAAACGG	
Trastuzumab VH-CH1-hinge- CalD2	CAGGTCCAACCTGCAGGAGTCTGGCGGTGGCCTGGTGCAG CCAGGGGGGCTCACTCCGTTTGTCTGTGCAGCTTCTGGCT TCAACATTAAAGACACCTATATACTGGGTGCGTCAGG CCCCGGGTAAAGGGCCTGGAATGGGTTGCAAGGATTTATC CTACGAATGGTTATACTAGATATGCCGATAGCGTCAAGG GCCGTTTCACTATAAGCGCAGACACATCCAAAAACACAG CCTACCTACAGATGAACAGCCTGCGTGCTGAGGACACTG CCGTCTATTATTGTTCTAGATGGGGAGGGGACGGCTTCTA TGCTATGGACTACTGGGGTCAAGGAACCCTGGTCACCGT CTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTG GCACCCTCCTCCAAGAGCACCTCTGGGGGGCACAGCGGCC CTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTG ACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCGGCGTG CACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACT CCCTCAGCAGCGTGGTGACTGTGCCCTCCAGCAGCTTGGG CACCCAGACCTACATCTGCAACGTGAATCACAACCCCAG CAACACCAAGGTCGACAAGAAAGTTGAGCCCAAATCTTC AACTAAGACGCACACATCAGGAGGTAACACCCTAGATGA TCTCTTTCAAGAACTGGACAAGAATGGAGATGGAGAAGT TAGTTTTGAAGAATTCCAAGTATTAGTAAAAAAGATATCC CAG	17
Trastuzumab light chain	GACATCGAGCTCACCCAGTCCCCAAGCTCCCTGTCCGCCT CTGTGGGCGATAGAGTCACCATCACCTGCCGTGCCAGTC AGGATGTGAATACTGCTGTAGCCTGGTATCAACAGAAAC CAGGAAAAGCTCCGAAACTACTGATTTACTCGGCATCCTT CCTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGATCCAGAT CTGGGACGGATTTCACTCTAACCATCAGCAGTCTACAGCC GGAAGACTTCGCAACTTATTACTGTCAGCAACATTATACT ACTCCTCCCACGTTCGGACAGGGTACCAAGCTCGAGATC AAACGAACGTGTGGCTGCACCATCTGTCTTCATCTTCCCGC CATCTGATGAGCAGTTGAAATCTGGAACCTGCCTCTGTTGT GTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGT ACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTC CCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCA CCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAG ACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCC ATCAGGGCCTGAGTTCGCCCCGTCACAAAGAGCTTCAACC GCGGAGAGTCACACCATCACCATCACCAT	19

CalD1-hinge-Fc	AAGTCTCCTGAGGAACTGAAGAGGATTTTTGAAAAATAT GCAGCCAAAGAAGGTGATCCAGACCAGTTGTCAAAGGAT GAACTGAAGCTATTGATTCAGGCTGAATTCCCCAGTTTAC TCAAAGGTCCAGGCTCGAGCGTTGAGCCCAAATCTTCTG ACAAAACCTCACACATGCCCCACCGTGCCCAGCACCTGAAC TCCTGGGGGGGACCGTCAGTCTTCCTCTTCCCCCCTGAGGTCAC CAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAC ATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGT CAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAA TGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCA CGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGA CTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAA CAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAA AGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCT GCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAG CCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATC GCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAA CTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTC CTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAG GTGGCAGCAGGGGAACGTCTTCTCATGCTCTGTGATGCAT GAGGCTCTGCATAACCACTACACGCAGAAGAGCCTCTCC CTGTCTCCGGGTAAA	21
026-VH-CH1-hinge-CalD2	CAGGTCCAACCTGCAGGAGTCTGGGGGAGGCGTGGTCCAG CCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGAT TCACCTTCAGTAACTATGTCATGCACTGGGTCCGCCAGGC TCCAGGCAAGGGGCTGGAGTGGGTGGCAATTATATGGTA TGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGG CCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCT GTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGC TGTGTATTACTGTGCGGGTGGATATAACTGGAACCTACGA GTACCACTACTACGGTATGGACGTCTGGGGCCAAGGGAC CACGGTCACCGTCTCCTCAGCCTCCACCAAGGGCCCATCG GTCTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGG GCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCC CCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGA CCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTC AGGACTCTACTCCCTCAGCAGCGTGGTGACTGTGCCCTCC AGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAAT CACAACCCAGCAACACCAAGGTCGACAAGAAAGTTGAG CCCAAATCTTCAACTAAGACGCACACATCAGGAGGTAAC ACCCTAGATGATCTCTTTCAAGAACTGGACAAGAATGGA GATGGAGAAGTTAGTTTTGAAGAATTCCAAGTATTAGTA AAAAAGATATCCCAG	23
026 light chain	GACATCGAGCTCACCCAGTCTCCATCCTCACTGTCTGCAT CTGTAGGAGACAGAGTCACCATCACTTGTCGGGCGAGTC AGGGCATTACCAATTATTTAGCCTGGTTTCAGCAGAAACC AGGGAAAGCCCCTAAGTCCCTTATCTATGCTGCATCCAGT TTGCAAAGTGGGGTCCCATCAAAGTTCAGCGGCAGTGGA TCTGGGACAGATTTTCAGTCTCACCATCAGCAGCCTCCAGC	25

	CTGAAGATTTTGCAACTTATTACTGCCAACAGTATAATAG TTACCCGATCACCTTCGGCCAAGGGACACGACTCGAGAT CAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCG CCATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTG TGTGCCTGCTGAATAACTTCTATCCCAGAGAGGGCCAAAGT ACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTC CCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCA CCTACAGCCTCAGCAGCACCTTGACGCTGAGCAAAGCAG ACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCC ATCAGGGCCTGAGTTCGCCCCGTACAAAGAGCTTCAACC GCGGAGAGTCACACCATCACCATCACCAT	
Fc-Syt1	GTTGAGCCCCAAATCTTCTGACAAAACCTCACACATGCCAC CGTGCCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTT CCTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGAGGCG GTGGATCAGAGAAACTGGGAAAACCTTCAGTATTCACTGG ATTATGATTTCCAAAATAACCAGCTGCTGGTAGGGATCAT TCAGGCTGCCGAACCTGCCCGCCTTGGACATGGGGGGGCAC ATCTGATCCTTACGTGAAAGTGTTTCTGCTACCTGATAAG AAGAAGAAATTTGAGACAAAAGTCCACCGAAAAACCCTT AATCCTGTCTTCAATGAGCAATTTACTTTCAAGGTACCAT ACTCGGAATTGGGTGGCAAAACCCTAGTGATGGCTGTAT ATGATTTTGATCGTTTCTCTAAGCATGACATCATTGGAGA ATTTAAAGTCCCTATGAACACAGTGGATTTTGGCCATGTA ACTGAGGAATGGCGTGACCTGCAAAGTGCT	27
Syt1-Fc-Syt1	GAGAAACTGGGAAAACCTTCAGTATTCACTGGATTATGAT TTCCAAAATAACCAGCTGCTGGTAGGGATCATTCAAGGCT GCCGAACCTGCCCGCCTTGGACATGGGGGGGCACATCTGAT CCTTACGTGAAAGTGTTTCTGCTACCTGATAAGAAGAAG AAATTTGAGACAAAAGTCCACCGAAAAACCCTTAATCCT GTCTTCAATGAGCAATTTACTTTCAAGGTACCATACTCGG AATTGGGTGGCAAAACCCTAGTGATGGCTGTATATGATTT TGATCGTTTCTCTAAGCATGACATCATTGGAGAATTTAA GTCCCTATGAACACAGTGGATTTTGGCCATGTA AATGGCGTGACCTGCAAAGTGCTGGAGGCGGTGGATCAG	29

	TTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCCACC GTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTC CTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCCC GGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACG GCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG GAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTC ACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC GAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGCGGAG GTGGCAGCGAGAACTGGGAAAACCTTCAGTATTCAGTGG ATTATGATTTCCAAATAACCAGCTGCTGGTAGGGATCAT TCAGGCTGCCGAACCTGCCCGCCTTGGACATGGGGGGGCAC ATCTGATCCTTACGTGAAAGTGTTTCTGCTACCTGATAAG AAGAAGAAATTTGAGACAAAAGTCCACCGAAAAACCTT AATCCTGTCTTCAATGAGCAATTTACTTTCAAGGTACCAT ACTCGGAATTGGGTGGCAAAACCCTAGTGATGGCTGTAT ATGATTTTGATCGTTTCTCTAAGCATGACATCATTGGAGA ATTTAAAGTCCCTATGAACACAGTGGATTTTGGCCATGTA ACTGAGGAATGGCGTGACCTGCAAAGTGCT	
Fc-PKCα	GTTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCCAC CGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTT CCTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGAGGCG GTGGATCAGAGAAGAGGGGGCGGATTTACCTAAAGGCTG AGGTTGCTGATGAAAAGCTCCATGTCACAGTACGAGATG CAAAAAATCTAATCCCTATGGATCCAAACGGGCTTTCAG ATCCTTATGTGAAGCTGAACTTATTCCTGATCCCAAGAA	31

	TGAAAGCAAGCAAAAAACCAAAACCATCCGCTCCACACT AAATCCGCAGTGGAATGAGTCCTTTACATTCAAATTGAA ACCTTCAGACAAAGACCGACGACTGTCTGTAGAAATCTG GGACTGGGATCGAACAACAAGGAATGACTTCATGGGATC CCTTTCCTTTGGAGTTTCGGAGCTGATGAAGATGCCGGCC AGTGGATGGTACAAGTTGCTTAACCAAGAAGAAGGTGAG TACTACAACGTA	
Fc-AnxA1	GTTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCCAC CGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTT CCTCTTCCCCC AAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACA ACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGAGGCG GTGGATCAACCTTCAATCCATCCTCGGATGTCGCTGCCTT GCATAAGGCCATAATGGTTAAAGGTGTGGATGAAGCAAC CATCATTGACATTCTAACTAAGCGAAACAATGCACAGCG TCAACAGATCAAAGCAGCATATCTCCAGGAAACAGGAAA GCCCCTGGATGAAACACTGAAGAAAGCCCTTACAGGTCA CCTTGAGGAGGTTGTTTTAGCTCTGCTAAAACTCCAGCG CAATTTGATGCTGATGAACTTCGTGCTGCCATGAAGGGCC TTGGA ACTGATGAAGATACTCTAATTGAGATTTTGGCATC AAGAACTAACAAAGAAATCAGAGACATTAACAGGGTCTA CAGAGAGGAACTGAAGAGAGATCTGGCCAAAGACATAA CCTCAGACACATCTGGAGATTTTCGGAACGCTTTGCTTTC TCTTGCTAAGGGTGACCGATCTGAGGACTTTGGTGTGAAT GAAGACTTGGCTGATTCAGATGCCAGGGCCTTGTATGAA GCAGGAGAAAGGAGAAAGGGGACAGACGTAAACGTGTT CAATACCATCCTTACCACCAGAAGCTATCCACA ACTTCGC AGAGTGTTTCAGAAATACACCAAGTACAGTAAGCATGAC ATGAACAAAGTTCTAGACCTGGAGTTGAAAGGTGACATT GAGAAATGCCTCACAGCTATCGTGAAGTGCGCCACAAGC AAACCAGCTTTCTTTGCAGAGAAGCTTCATCAAGCCATGA AAGGTGTTGGA ACTCGCCATAAGGCATTGATCAGGATTA TGGTTTCCCGTTCTGAAATTGACATGAATGATATCAAAGC ATTCTATCAGAAGATGTATGGTATCTCCCTTTGCCAAGCC ATCCTGGATGAAACCAAGGAGATTATGAGAAAATCCTG GTGGCTCTTTGTGGAGGAAAC	33

Syt1-Fc	GAGAAACTGGGAAAACCTTCAGTATTCAGTGGATTATGAT TTCCAAAATAACCAGCTGCTGGTAGGGATCATTAGGCT GCCGAACTGCCCCGCCTTGGACATGGGGGGGCACATCTGAT CCTTACGTGAAAGTGTTTCTGCTACCTGATAAGAAGAAG AAATTTGAGACAAAAGTCCACCGAAAAACCCTTAATCCT GTCTTCAATGAGCAATTTACTTTCAAGGTACCATACTCGG AATTGGGTGGCAAAAACCCTAGTGATGGCTGTATATGATTT TGATCGTTTCTCTAAGCATGACATCATTGGAGAATTTAAA GTCCCTATGAACACAGTGGATTTTGGCCATGTAAGTGAAG AATGGCGTGACCTGCAAAGTGCTGGAGGCGGTGGATCAG TTGAGCCCCAAATCTTCTGACAAAACCTCACACATGCCACC GTGCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTTC CTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCCC GGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACG GCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG GAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTC ACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC GAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA	35
PKC α -Fc	GAGAAGAGGGGGCGGATTTACCTAAAGGCTGAGGTTGCT GATGAAAAGCTCCATGTCACAGTACGAGATGCAAAAAT CTAATCCCTATGGATCCAAACGGGCTTTCAGATCCTTATG TGAAGCTGAAACTTATTCCTGATCCCAAGAATGAAAGCA AGCAAAAACCAAAAACCATCCGCTCCACACTAAATCCGC AGTGGAATGAGTCCTTTACATTCAAATTGAAACCTTCAGA CAAAGACCGACGACTGTCTGTAGAAATCTGGGACTGGGA TCGAACAACAAGGAATGACTTCATGGGATCCCTTTCCTTT GGAGTTTCGGAGCTGATGAAGATGCCGGCCAGTGGATGG TACAAGTTGCTTAACCAAGAAGAAGGTGAGTACTACAAC GTAGGAGGCGGTGGATCAGTTGAGCCCAAATCTTCTGAC AAAACCTCACACATGCCACCGTGCCAGCACCTGAACTC CTGGGGGGGACCGTCAGTCTTCTCTTCCCCCCCCAAAACCCA AGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACAT GCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCA AGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACG TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACT GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACA AAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAG CCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGC CCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCC	37

	TGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGC CGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACA ACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTT CTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTG GCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGA GGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCT GTCTCCGGGTAAA	
AnxA1-Fc	ACCTTCAATCCATCCTCGGATGTCGCTGCCTTGCATAAGG CCATAATGGTTAAAGGTGTGGATGAAGCAACCATCATTG ACATTCTAACTAAGCGAAACAATGCACAGCGTCAACAGA TCAAAGCAGCATATCTCCAGGAAACAGGAAAGCCCCTGG ATGAAACACTGAAGAAAGCCCTTACAGGTCACCTTGAGG AGGTTGTTTTAGCTCTGCTAAAAACTCCAGCGCAATTTGA TGCTGATGAAC TTCGTGCTGCCATGAAGGGCCTTGGA ACTGATGAAGATACTCTAATTGAGATTTTGGCATCAAGAACT AACAAAGAAATCAGAGACATTAACAGGGTCTACAGAGA GGA ACTGAAGAGAGATCTGGCCAAAGACATAACCTCAGAC ACATCTGGAGATTTTTCGGAACGCTTTGCTTTCTCTTGCT AAGGGTGACCGATCTGAGGACTTTGGTGTGAATGAAGAC TTGGCTGATTCAGATGCCAGGGCCTTGTATGAAGCAGGA GAAAGGAGAAAGGGGACAGACGTAAACGTGTTCAATAC CATCCTTACCACCAGAAGCTATCCACA ACTTCGCAGAGTGTTTCAGAAATACACCAAGTACAGTAAGCATGACATGAAC AAAGTTCTAGACCTGGAGTTGAAAGGTGACATTGAGAAA TGCCTCACAGCTATCGTGAAGTGCGCCACAAGCAAACCA GCTTTCTTTGCAGAGAAGCTTCATCAAGCCATGAAAGGTG TTGGA ACTCGCCATAAGGCATTGATCAGGATTATGGTTTC CCGTTCTGAAATTGACATGAATGATATCAAAGCATTCTAT CAGAAGATGTATGGTATCTCCCTTTGCCAAGCCATCCTGG ATGAAACCAAAGGAGATTATGAGAAAATCCTGGTGGCTC TTTGTGGAGGAAACGGAGGCGGTGGATCAGTTGAGCCCA AATCTTCTGACAAA ACTCACACATGCCCACCGTGCCCAGCACCTGA ACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCC CCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCT GAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGAC CCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAG GTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTAC AACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGC ACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGG TCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCA TCTCCA AAGCCAAAGGGCAGCCCCGAGAACCACAGGTGT ACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACC AGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAG CGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGA GAACA ACTACAAGACCACGCCTCCCGTGCTGGACTCCGA CGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAG AGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTG ATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGC CTCTCCCTGTCTCCGGGTAAA	39

Syt1-Fc-Syt1 with knobs into holes mutations (TF)	GAGAAACTGGGAAAACCTTCAGTATTCAGTGGATTATGAT TTCCAAAATAACCAGCTGCTGGTAGGGATCATTAGGCT GCCGAACTGCCCCGCCTTGGACATGGGGGGGCACATCTGAT CCTTACGTGAAAGTGTTTCTGCTACCTGATAAGAAGAAG AAATTTGAGACAAAAGTCCACCGAAAAACCCTTAATCCT GTCTTCAATGAGCAATTTACTTTCAAGGTACCATACTCGG AATTGGGTGGCAAAAACCCTAGTGATGGCTGTATATGATTT TGATCGTTTCTCTAAGCATGACATCATTGGAGAATTTAAA GTCCCTATGAACACAGTGGATTTTGGCCATGTAAGTGGAGG AATGGCGTGACCTGCAAAGTGCTGGAGGGCGGTGGATCAG TTGAGCCCCAAATCTTCTGACAAAACCTCACACATGCCCACC GTGCCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTTC CTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCCC GGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACG GCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG GAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTC ACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC GAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGACCACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACAAGACCTTCCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGCGGAG GTGGCAGCGAGAACTGGGAAAACCTTCAGTATTCAGTGG ATTATGATTTCCAAAATAACCAGCTGCTGGTAGGGATCAT TCAGGCTGCCGAACTGCCCCGCCTTGGACATGGGGGGGCAC ATCTGATCCTTACGTGAAAGTGTTTCTGCTACCTGATAAG AAGAAGAAATTTGAGACAAAAGTCCACCGAAAAACCCTT AATCCTGTCTTCAATGAGCAATTTACTTTCAAGGTACCAT ACTCGGAATTGGGTGGCAAAAACCCTAGTGATGGCTGTAT ATGATTTTGATCGTTTCTCTAAGCATGACATCATTGGAGA ATTTAAAGTCCCTATGAACACAGTGGATTTTGGCCATGTA ACTGAGGAATGGCGTGACCTGCAAAGTGCT	41
Fc with knobs into holes mutations(HA)	GTTGAGCCCCAAATCTTCTGACAAAACCTCACACATGCCCAC CGTGCCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTT CCTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCCACCTGACCTGCCTGGTCAAAGG	43

	CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCGCCCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA	
Syt1-Fc-Syt1 with knobs into holes mutations (TF) and electrostatic steering mutations	GAGAAACTGGGAAAACCTTCAGTATTCAGTGGATTATGAT TTCCAAAATAACCAGCTGCTGGTAGGGATCATTAGGCT GCCGAAGTGGCCGCCTTGGACATGGGGGGGCACATCTGAT CCTTACGTGAAAGTGTTTCTGCTACCTGATAAGAAGAAG AAATTTGAGACAAAAGTCCACCGAAAAACCCTTAATCCT GTCTTCAATGAGCAATTTACTTTCAAGGTACCATACTCGG AATTGGGTGGCAAAAACCCTAGTGATGGCTGTATATGATTT TGATCGTTTCTCTAAGCATGACATCATTGGAGAATTTAAA GTCCCTATGAACACAGTGGATTTTGGCCATGTAAGTGGAGG AATGGCGTGACCTGCAAAGTGCTGGAGGCGGTGGATCAG TTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCCACC GTGCCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTTC CTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCCC GGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACG GCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG GAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTC ACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC GAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGACCACCCTGCCCCCATCCCGGGATGAGCT GACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACGACACCTTCCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCTCTACAGCGACCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGCGGAG GTGGCAGCGAGAACTGGGAAAACCTTCAGTATTCAGTGG ATTATGATTTCCAAAATAACCAGCTGCTGGTAGGGATCAT TCAGGCTGCCGAAGTGGCCGCCTTGGACATGGGGGGGCAC ATCTGATCCTTACGTGAAAGTGTTTCTGCTACCTGATAAG AAGAAGAAATTTGAGACAAAAGTCCACCGAAAAACCCTT AATCCTGTCTTCAATGAGCAATTTACTTTCAAGGTACCAT ACTCGGAATTGGGTGGCAAAAACCCTAGTGATGGCTGTAT ATGATTTTGATCGTTTCTCTAAGCATGACATCATTGGAGA ATTTAAAGTCCCTATGAACACAGTGGATTTTGGCCATGTA ACTGAGGAATGGCGTGACCTGCAAAGTGCT	45
Fc with knobs into holes mutations (HA) and electrostatic steering	GTTGAGCCCAAATCTTCTGACAAAACCTCACACATGCCCAC CGTGCCCAGCACCTGAACTCCTGGGGGGGACCGTCAGTCTT CCTCTTCCCCCCCCAAAACCCAAGGACACCCTCATGATCTCC CGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGC CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC	47

mutations	GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGA GGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCT CACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTA CAAGTGCAAGGTCTCCAACAAAGCCCCTCCCAGCCCCCAT CGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGA ACCACAGGTGTACACCCTGCCCCCATCCCGGGGATAAGCT GACCAAGAACCAGGTCCACCTGACCTGCCTGGTCAAAGG CTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAA TGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGT GCTGAAGTCCGACGGCTCCTTCGCCCTCTACAGCAAGCTC ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACA CGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA	
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Table 2. Amino acid sequences of exemplary proteins.

Protein	Amino acid sequence	SEQ ID NO:
Pertuzumab light chain with Cys mutation	DIELTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKA PKLLIYSASYRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYYIYPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTAS VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST YSLSSLTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES	2
SG heavy chain with Cys mutations	QVQLQESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAP GKGLEWVADVNPNHGESIYNQRFKGRFTLSVDRSKNTLYLQ MNSLRAEDTAVYYCARNLGPSFYFDYWGGQGLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSSDKTHTCPPSPAPELLGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK	4
YS light chain with Cys mutation	DIELTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKA PKLLIYSASYRHTGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYYIYPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTAS VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST YSLSSLTLTLKADYEKHKVYACEVTHQGLSSPVTKSFNRGES	6
YS heavy chain with Cys mutations	QVQLQESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAP GKGLEWVADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQ MNSLRAEDTAVYYCARNLGPHFYFDYWGGQGLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSSDKTHTCPPSPAPELLGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP	8

	REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK	
SG scFv linked to N-terminus of heavy chain with Cys mutations	QVQLQESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAP GKGLEWVADVNPNGESIYNQRFKGRFTLSVDRSKNTLYLQ MNSLRAEDTAVYYCARNLGPSFYFDYWGQGTLVTVSSGGGG SGGGGSGGGGSDIELTQSPSSLSASVGDRVTITCKASQDVSIG VAWYQQKPGKAPKLLIYSASYRYTGVPSTRFSGSGSGTDFTLTI SSLQPEDFATYYCQQYYIYPYTFGQGGTKLEIKRGGGSVEPKSSD KTHTCPPSPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPGK	10
YS scFv linked to N-terminus of heavy chain with Cys mutations	QVQLQESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAP GKGLEWVADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQ MNSLRAEDTAVYYCARNLGPHFYFDYWGQGTLVTVSSGGGG GSGGGGSGGGGSDIELTQSPSSLSASVGDRVTITCKASQDVSI GVAWYQQKPGKAPKLLIYSASYRHTGVPSRFSGSGSGTDFTL TISSLQPEDFATYYCQQYYIYPYTFGQGGTKLEIKRGGGSVEPKSS DKTHTCPPSPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGK	12
Heavy chain with Cys mutations and SG scFv linked to C-terminus	VEPKSSDKTHTCPPSPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGKGGSQVQLQESGGGLVQPGG SLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNPNG ESIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCAR NLGPSFYFDYWGQGTLVTVSSGGGGGSGGGGSGGGGSDIELTQ SPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIY SASYRYTGVPSTRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYI YPYTFGQGGTKLEIKR	14
Heavy chain with Cys mutations and YS scFv linked to C-terminus	VEPKSSDKTHTCPPSPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGKGGSQVQLQESGGGLVQPGG SLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNPNSGG SIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN	16

	LGPHFYFDYWGQGTLVTVSSGGGGSGGGGGSGGGGSDIELTQS PSSLSASVGDRVITITCKASQDVSIGVAWYQQKPGKAPKLLIYS ASYRHTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIY PYTFGQGGTKLEIKR	
Trastuzumab VH-CH1- hinge-CalD2	QVQLQESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPG KGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQM NSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLLVTVSSAST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHN PSNTKVDKKVEPKSSTKTHTSGGNTLDDLFQELDKNGDGEVS FEEFQVLVKKISQ	18
Trastuzumab light chain	DIELTQSPSSLSASVGDRVITITCRASQDVNTAVAWYQQKPGK APKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYY CQQHYTTPPTFGQGGTKLEIKRTVAAPS VFIFPPSDEQLKSGTAS VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKST YLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES HHHHHH	20
CalD1-hinge- Fc	KSPEELKRIFEKYAAKEGDPDQLSKDELKLLIQAEFPSLLKGP GSSVEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK	22
026-VH-CH1- hinge-CalD2	QVQLQESGGGVVQPGRSLRLSCAASGFTFSNYVMHWVRQAP GKGLEWVAIIWYDGSNKYYADSVKGRFTISRDN SKNTLYLQ MNSLRAEDTAVYYCAGGYNWNYEYHYYGMDVWGQGTTVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHNPSNTKVDKKVEPKSSTKTHTSGGNTLDDLFQELDKN GDGEVSFEEFQVLVKKISQ	24
026 light chain	DIELTQSPSSLSASVGDRVITITCRASQGITNYLAWFQQKPGKA PKSLIYAASSLQSGVPSKFSGSGSGTDFSLTISSLQPEDFATYYC QQYNSYPITFGQGRLEIKRTVAAPS VFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTY SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES HHHHHH	26
Fc-Syt1	VEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGKGGGGSEKLGKLQYSLDYDF QNNQLLVGIIQAAELPALDMGGTSDPYVKVFLLPDKKKKFFET KVHRKTLNPVFNEQFTFKVPYSELGGKTLVMAVYDFDRFSK HDIIGFEKVP MNTVDFGHVTEEWRDLQSA	28

Syt1-Fc-Syt1	EKLGKLQYSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYV KVFLLPDKKKKFETKVHRKTLNPFNEQFTFKVPYSELGGKT LVMAVYDFDRFSKHDIIGFEKVPMNTVDFGHVTEEWRDLQS AGGGGSVEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGKGGGGSEKLGKLQ YSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYVKVFLLPD KKKKKFETKVHRKTLNPFNEQFTFKVPYSELGGKTLVMAVY DFDRFSKHDIIGFEKVPMNTVDFGHVTEEWRDLQSA	30
Fc-PKCa	VEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGKGGGGSEKRGRIYLKAEVAD EKLHVTVRDAKNLIPMDPNGLSDPYVKLKLIPDPKNESKQKT KTIRSTLNPQWNESFTFKLKPSDKDRRLSVEIWDWDRTRND FMGSLSFGVSELMKMPASGWYKLLNQEEGEYYNV	32
Fc-AnxA1	VEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGKGGGGSTFNPSSDVAALHKAI MVKGVDEATIIDILTKRNNAQRQQIKAAYLQETGKPLDETLK KALTGHLEEVVLALLKTPAQFDADELRAAMKGLGTDEDTLIE ILASRTNKEIRDINRVYREELKRD LAKDITSDTSGDFRNALLSL AKGDRSEDFGVNEDLADSDARALYEAGERRKGTDVNVFNTI LTTRSYPQLRRVFQKYTKYSKHD MNK VLDLELKGDI EKCLTA IVKCATSKPAFFAEKLHQAMKGVGTRHKALIRIMVSRSEIDM NDIKAFYQKMYGISLCQAILDETKGDYEKILVALCGGN	34
Syt1-Fc	EKLGKLQYSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYV KVFLLPDKKKKFETKVHRKTLNPFNEQFTFKVPYSELGGKT LVMAVYDFDRFSKHDIIGFEKVPMNTVDFGHVTEEWRDLQS AGGGGSVEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK	36
PKC α -Fc	EKRGRIYLKAEVADEKLHVTVRDAKNLIPMDPNGLSDPYVKL KLIPDPKNESKQKTKTIRSTLNPQWNESFTFKLKPSDKDRRLS VEIWDWDRTRNDFMGSLSFGVSELMKMPASGWYKLLNQE EGEYYNVGGGGGSVEPKSSDKTHTCPPCPAPELLGGPSVFLFPP KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN	38

	AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK	
AnxA1-Fc	TFNPSSDVAALHKAIMVKGVDEATHIDILTKRNNAQRQQIKAA YLQETGKPLDETLKKALTGHLEEVVLALLKTPAQFDADELRA AMKGLGTDEDTLIEILASRTNKEIRDINRVYREELKRD LAKDI TSDTSGDFRNALLSLAKGDRSEDFGVNEDLADSDARALYEAG ERRKGTDVNVFNTILTTRSYPQLRRVFQKYTKYSKHDMNKV LDLELKGDIEKCLTAIVKCATSKPAFFAEKLHQAMKGVGTRH KALIRIMVSRSEIDMNDIKAFYQKMYGISLCQAILDETKGDYE KILVALCGGNGGGGSVEPKSSDKTHTCPPCPAPELLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK	40
Syt1-Fc-Syt1 with knobs into holes mutations (TF)	EKLGKLQYSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYV KVFLLPDKKKKFETKVHRKTLNPVFNEQFTFKVPYSELGGKT LVMAVYDFDRFSKHDIIGFEK VPMNTVDFGHVTEEWRDLQS AGGGGSVEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVTTLPSPRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFS CSVMHEALHNHYTQKSLSLSPGKGGGGSEKLGKLQ YSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYVKVFLLPD KKKKKFETKVHRKTLNPVFNEQFTFKVPYSELGGKTLVMAVY DFDRFSKHDIIGFEK VPMNTVDFGHVTEEWRDLQSA	42
Fc with knobs into holes mutations(HA)	VEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVHLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFALYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK	44
Syt1-Fc-Syt1 with knobs into holes mutations (TF) and electrostatic steering mutations	EKLGKLQYSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYV KVFLLPDKKKKFETKVHRKTLNPVFNEQFTFKVPYSELGGKT LVMAVYDFDRFSKHDIIGFEK VPMNTVDFGHVTEEWRDLQS AGGGGSVEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVTTLPSPRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYDTFPPVLDSDGSFFLYSDLTVDKSRWQ QGNVFS CSVMHEALHNHYTQKSLSLSPGKGGGGSEKLGKLQ YSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPYVKVFLLPD KKKKKFETKVHRKTLNPVFNEQFTFKVPYSELGGKTLVMAVY DFDRFSKHDIIGFEK VPMNTVDFGHVTEEWRDLQSA	46

Fc with knobs into holes mutations (HA) and electrostatic steering mutations	VEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDKLTKNQVHLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLKSDGSFALYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK	48
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[00210] The DNA sequence of SEQ ID NO:1 is of a polynucleotide encoding an exemplary light chain of a HER2-specific antibody, pertuzumab, (SEQ ID NO:2) with mutation of Cys214 to serine, and is configured, for example, for heterodimer formation with the SG heavy chain variant of pertuzuamb (SEQ ID NO: 4).

[00211] In particular, the amino acid sequence of the exemplary light chain of the HER2-specific antibody, pertuzumab, (SEQ ID NO: 2) has, in order from N- to C-terminus a HER2-specific VL domain at residues 1-108, and an immunoglobulin CL domain (human Cκ) at residues 109-214. The cysteine residue (214) that usually pairs with an immunoglobulin heavy chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO:2 are those of the protein sequence, and do not refer to the EU numbering convention.

[00212] The DNA sequence of SEQ ID NO: 3 is of a polynucleotide encoding an exemplary heavy chain of the HER2-specific antibody, pertuzumab, (SEQ ID NO: 4) with mutation of Cys222 and Cys231 to serines, and the SG mutations. The encoded heavy chain (SEQ ID NO: 4) is configured, for example, to form heterodimers with the light chain variant of pertuzumab (SEQ ID NO: 2) to generate the SG variant of pertuzumab.

[00213] In particular, the amino acid sequence of the exemplary antibody heavy chain of SEQ ID NO: 4 has, in order from N- to C-terminus, a HER2-specific VH domain at residues 1-119, an immunoglobulin CH1 domain (human IgG1-derived) at residues 120-216, an immunoglobulin hinge (human IgG1-derived) at residues 217-232, an immunoglobulin CH2 domain (human IgG1-derived) at residues 233-342, an immunoglobulin CH3 domain (human IgG1-derived) at residues 343-449. The exemplary HER2-specific antibody heavy chain of SEQ ID NO: 4 has mutations of Ser55 and Gly57 to histidine and glutamic acid (SG mutations), respectively. The cysteine residues (222 and 231) that usually pair with an immunoglobulin light chain and form a hinge sulfhydryl bridge have been mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 4 are those of the protein sequence, and do not refer to the EU numbering convention.

[00214] The DNA sequence of SEQ ID NO: 5 is of a polynucleotide encoding an exemplary light chain of a HER2-specific antibody, pertuzumab, (SEQ ID NO: 6) with mutation of Cys214 to serine and mutation of Tyr55 to histidine. The encoded light chain (SEQ ID NO: 6) is configured, for example, for heterodimer formation with the YS heavy chain variant of pertuzumab (SEQ ID NO: 8).

[00215] In particular, the amino acid sequence of the exemplary light chain of the HER2-specific antibody, pertuzumab, (SEQ ID NO: 6) has, in order from N- to C-terminus a HER2-specific VL domain at residues 1-108, and an immunoglobulin CL domain (human C κ) at residues 109-214. The exemplary HER2-specific antibody light chain of SEQ ID NO: 6 has mutation of residue 55 to histidine. The cysteine residue (214) that usually pairs with an immunoglobulin heavy chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 6 are those of the protein sequence, and do not refer to the EU numbering convention.

[00216] The DNA sequence of SEQ ID NO: 7 is of a polynucleotide encoding an exemplary heavy chain of the HER2-specific antibody, pertuzumab, (SEQ ID NO: 8) with mutation of Cys222 and Cys231 to serines, and mutation of Ser103 to histidine. The encoded heavy chain (SEQ ID NO: 8) is configured, for example, for heterodimer formation with the light chain variant of pertuzumab (SEQ ID NO: 6) to generate the YS variant of pertuzumab.

[00217] In particular, the amino acid sequence of the exemplary antibody heavy chain of SEQ ID NO: 8 has, in order from N- to C-terminus, a HER2-specific VH domain at residues 1-119, an immunoglobulin CH1 domain (human IgG1-derived) at residues 120-216, an immunoglobulin hinge (human IgG1-derived) at residues 217-232, an immunoglobulin CH2 domain (human IgG1-derived) at residues 233-342, an immunoglobulin CH3 domain (human IgG1-derived) at residues 343-449. The exemplary HER2-specific antibody heavy chain of SEQ ID NO: 8 has mutation of residue 103 to histidine. The cysteine residues (222 and 231) that usually pair with an immunoglobulin light chain and form a hinge sulfhydryl bridge have been mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 8 are those of the protein sequence, and do not refer to the EU numbering convention.

[00218] The DNA sequence of SEQ ID NO: 9 is of a polynucleotide encoding an exemplary single chain Fv of the HER2-specific antibody, pertuzumab, fused to the N-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 10) with mutation of Cys251 and Cys260 to serines, and the SG mutations. The encoded fusion protein (SEQ ID NO: 10) is configured to form homodimers.

[00219] In particular, the amino acid sequence of the exemplary antibody heavy chain of SEQ ID NO: 10 has, in order from N- to C-terminus, a HER2-specific VH domain at residues 1-119, a (G₄S)₃ linker peptide at residues 120-134, a HER2-specific VL domain at residues 135-242, a GGS linker peptide at residues 243-245, an immunoglobulin hinge (human IgG1-derived) at residues 246-261, an immunoglobulin CH2 domain (human IgG1-derived) at residues 262-371, an immunoglobulin CH3 domain (human IgG1-derived) at residues 372-478. The exemplary HER2-specific antibody scFv-heavy chain fusion of SEQ ID NO: 10 has mutations of Ser55 and Gly57 to histidine and glutamic acid (SG mutations), respectively. The cysteine residues (251 and 260) that usually pair with an immunoglobulin light chain and form a hinge sulfhydryl bridge have been mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 10 are those of the protein sequence, and do not refer to the EU numbering convention.

[00220] The DNA sequence of SEQ ID NO: 11 is of a polynucleotide encoding an exemplary single chain Fv of the HER2-specific antibody, pertuzumab, fused to the N-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 12) with mutation of Cys251 and Cys260 to serines and the YS mutations. The encoded fusion protein (SEQ ID NO: 12) is configured to form homodimers.

[00221] In particular, the amino acid sequence of the exemplary antibody heavy chain of SEQ ID NO: 12 has, in order from N- to C-terminus, a HER2-specific VH domain at residues 1-119, a (G₄S)₃ linker peptide at residues 120-134, a HER2-specific VL domain at residues 135-242, a GGS linker peptide at residues 243-245, an immunoglobulin hinge (human IgG1-derived) at residues 246-261, an immunoglobulin CH2 domain (human IgG1-derived) at residues 262-371, an immunoglobulin CH3 domain (human IgG1-derived) at residues 372-478. The exemplary HER2-specific antibody scFv-heavy chain fusion of SEQ ID NO: 12 has mutations of Ser103 and Tyr189 to histidines (YS mutations). The cysteine residues (251 and 260) that usually pair with an immunoglobulin light chain and form a hinge sulfhydryl bridge have been mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 12 are those of the protein sequence, and do not refer to the EU numbering convention.

[00222] The DNA sequence of SEQ ID NO: 13 is of a polynucleotide encoding an exemplary single chain Fv of the HER2-specific antibody, pertuzumab, fused to the C-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 14) with mutation of Cys6 and Cys15 to serines, and the SG mutations. The encoded fusion protein (SEQ ID NO: 14) is configured to form homodimers.

[00223] In particular, the amino acid sequence of the exemplary antibody heavy chain of SEQ ID NO: 14 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233, a GGS linker peptide at residues 234-236, a HER2-specific VH domain at residues 237-355, a (G₄S)₃ linker peptide at residues 356-370, a HER2-specific VL domain at residues 371-478. The exemplary HER2-specific antibody scFv-heavy chain fusion of SEQ ID NO: 14 has mutations of Ser291 and Gly293 to histidine and glutamic acid (SG mutations), respectively. The cysteine residues (6 and 15) that usually pair with an immunoglobulin light chain and form a hinge sulfhydryl bridge have been mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 14 are those of the protein sequence, and do not refer to the EU numbering convention.

[00224] The DNA sequence of SEQ ID NO: 15 is of a polynucleotide encoding an exemplary single chain Fv of the HER2-specific antibody, pertuzumab, fused to the C-terminus to an immunoglobulin Fc fragment (SEQ ID NO: 16) with mutation of Cys6 and Cys15 to serines, and the YS mutations. The encoded fusion protein (SEQ ID NO: 16) is configured to form homodimers.

[00225] In particular, the amino acid sequence of the exemplary antibody heavy chain of SEQ ID NO: 16 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233, a GGS linker peptide at residues 234-236, a HER2-specific VH domain at residues 237-355, a (G₄S)₃ linker peptide at residues 356-370, a HER2-specific VL domain at residues 371-478. The exemplary HER2-specific antibody scFv-heavy chain fusion of SEQ ID NO: 16 has mutations of Ser339 and Tyr425 to histidines (YS mutations). The cysteine residues (6 and 15) that usually pair with an immunoglobulin light chain and form a hinge sulfhydryl bridge have been mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 16 are those of the protein sequence, and do not refer to the EU numbering convention.

[00226] The DNA sequence of SEQ ID NO: 17 is of a polynucleotide encoding an exemplary CalD2 fusion protein comprising the VH domain and CH1 domain of the HER2-specific antibody, trastuzumab, fused to the N-terminus of calbindin domain 2 (CalD2) (SEQ ID NO: 18) via a linker peptide comprising part of the immunoglobulin hinge (with mutation of Cys223 to serine) and SGG. The encoded fusion protein (SEQ ID NO: 18) is configured,

for example, to form heterodimers with the light chain of trastuzumab (SEQ ID NO: 20) and associate in a Ca^{2+} -dependent way with SEQ ID NO: 22.

[00227] In particular, the amino acid sequence of the exemplary CalD2 fusion protein of SEQ ID NO: 18 has, in order from N- to C-terminus, a HER2-specific VH domain at residues 1-120, an immunoglobulin CH1 domain (human IgG1-derived) at residues 121-217, a linker sequence comprising part of the heavy chain hinge region at residues 218-228 followed by a SGG sequence at residues 229-231 and a CalD2 domain at residues 232-263. The cysteine residue (223) that usually pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 18 are those of the protein sequence, and do not refer to the EU numbering convention.

[00228] The DNA sequence of SEQ ID NO: 19 is of a polynucleotide encoding an exemplary light chain of a HER2-specific antibody, trastuzumab, (SEQ ID NO: 20) with mutation of Cys214 to serine. The encoded light chain (SEQ ID NO: 20) is configured, for example, for heterodimer formation with the trastuzumab VH-CH1: CalD2 fusion protein (SEQ ID NO: 18).

[00229] In particular, the amino acid sequence of the exemplary light chain of the HER2-specific antibody, trastuzumab, (SEQ ID NO: 20) has, in order from N- to C-terminus a HER2-specific VL domain at residues 1-108, and an immunoglobulin CL domain (human C κ) at residues 109-214 followed by a hexahistidine peptide tag at residues 215-220. The cysteine residue (214) that usually pairs with an immunoglobulin heavy chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 20 are those of the protein sequence, and do not refer to the EU numbering convention.

[00230] The DNA sequence of SEQ ID NO: 21 is of a polynucleotide encoding an exemplary CalD1 fusion protein comprising the calbindin domain 1 (CalD1) fused to the N-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 22) with mutation of Cys52 to serine. The encoded fusion protein (SEQ ID NO: 22) is configured, for example, to associate with the trastuzumab VH-CH1: CalD2 fusion protein (SEQ ID NO: 18) or 026 VH-CH1: CalD2 fusion protein (SEQ ID NO: 24) in a Ca^{2+} -dependent way.

[00231] In particular, the amino acid sequence of the exemplary CalD1 fusion protein of SEQ ID NO: 22 has, in order from N- to C-terminus, a CalD1 domain at residues 1-43, a GSS linker peptide at residues 44-46, an immunoglobulin hinge (human IgG1-derived) at residues 47-62, an immunoglobulin CH2 domain (human IgG1-derived) at residues 63-172, an immunoglobulin CH3 domain (human IgG1-derived) at residues 173-279. The cysteine residue (52) that usually pairs with an immunoglobulin light chain is

mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 22 are those of the protein sequence, and do not refer to the EU numbering convention.

[00232] The DNA sequence of SEQ ID NO: 23 is of a polynucleotide encoding an exemplary CalD2 fusion protein comprising the VH domain and CH1 domain of the PSMA-specific antibody, 026, fused to the N-terminus of calbindin domain 2 (CalD2) (SEQ ID NO: 24) via a linker peptide comprising part of the immunoglobulin hinge (with mutation of Cys227 to serine) and SGG. The encoded fusion protein (SEQ ID NO: 24) is configured, for example, to form heterodimers with the light chain of 026 (SEQ ID NO: 26) and associate in a Ca^{2+} -dependent way with SEQ ID NO: 22.

[00233] In particular, the amino acid sequence of the exemplary CalD2 fusion protein of SEQ ID NO: 24 has, in order from N- to C-terminus, a PSMA-specific VH domain at residues 1-124, an immunoglobulin CH1 domain (human IgG1-derived) at residues 125-221, a linker sequence comprising part of the heavy chain hinge region at residues 222-232 followed by a SGG sequence at residues 233-235 and a CalD2 domain at residues 236-267. The cysteine residue (227) that usually pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 24 are those of the protein sequence, and do not refer to the EU numbering convention.

[00234] The DNA sequence of SEQ ID NO: 25 is of a polynucleotide encoding an exemplary light chain of a PSMA-specific antibody, 026, (SEQ ID NO: 26) with mutation of Cys214 to serine. The encoded light chain (SEQ ID NO: 26) is configured, for example, for heterodimer formation with the 026 VH-CH1: CalD2 fusion protein (SEQ ID NO: 24).

[00235] In particular, the amino acid sequence of the exemplary light chain of the PSMA-specific antibody, 026, (SEQ ID NO: 26) has, in order from N- to C-terminus a HER2-specific VL domain at residues 1-108, and an immunoglobulin CL domain (human C κ) at residues 109-214 followed by a hexahistidine peptide tag at residues 215-220. The cysteine residue (214) that usually pairs with an immunoglobulin heavy chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 26 are those of the protein sequence, and do not refer to the EU numbering convention.

[00236] The DNA sequence of SEQ ID NO: 27 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the Syt1 C2A domain of synaptotagmin fused to the C-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 28) with mutation of Cys6 to serine. The encoded fusion protein (SEQ ID NO: 28) is configured to form homodimers.

[00237] In particular, the amino acid sequence of the exemplary Fc-Syt1 of SEQ ID NO: 28 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-

derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233. Residues 141-266 of the C2A PS-binding domain of synaptotagmin (Syt1) are fused as residues 239-364 to the C-terminus of the CH3 domain via a GGGGS linker peptide (residues 234-238). The cysteine residue (6) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 28 are those of the protein sequence, and do not refer to the EU numbering convention.

[00238] The DNA sequence of SEQ ID NO: 29 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the Syt1 C2A domain of synaptotagmin fused to both the N- and C-termini of an immunoglobulin Fc fragment (SEQ ID NO: 30) with mutation of Cys137 to serine. The encoded fusion protein (SEQ ID NO: 30) is configured to form homodimers.

[00239] In particular, the amino acid sequence of the exemplary Syt1-Fc-Syt1 of SEQ ID NO: 30 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 132-147, an immunoglobulin CH2 domain (human IgG1-derived) at residues 148-257, an immunoglobulin CH3 domain (human IgG1-derived) at residues 258-364. Residues 141-266 of the C2A PS-binding domain of synaptotagmin (Syt1) are fused as residues 1-126 and 370-495, respectively, to the N- and C-termini of the hinge and CH3 domain via GGGGS linker peptides (residues 127-131 and 365-369). The cysteine residue (137) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 30 are those of the protein sequence, and do not refer to the EU numbering convention.

[00240] The DNA sequence of SEQ ID NO: 31 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the C2 domain of PKC α fused to the C-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 32) with mutation of Cys6 to serine. The encoded fusion protein (SEQ ID NO: 32) is configured to form homodimers.

[00241] In particular, the amino acid sequence of the exemplary Fc-PKC α of SEQ ID NO: 32 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233. Residues 157-288 of the C2 domain of PKC α are fused to the C-terminus of the CH3 domain as residues 239-370 via a GGGGS linker peptide (residues 234-238). The cysteine residue (6) that pairs with an immunoglobulin light chain is mutated to serine. The amino

acid residue numbers referred to in SEQ ID NO: 32 are those of the protein sequence, and do not refer to the EU numbering convention.

[00242] The DNA sequence of SEQ ID NO: 33 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the AnxA1 PS-binding core domain fused to the C-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 34) with mutation of Cys6 to serine. The encoded fusion protein (SEQ ID NO: 34) is configured to form homodimers.

[00243] In particular, the amino acid sequence of the exemplary Fc-AnxA1 of SEQ ID NO: 34 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233. Residues 41-346 of the AnxA1 core domain are fused to the C-terminus of the CH3 domain as residues 239-544 via a GGGGS linker peptide (residues 234-238). The cysteine residue (6) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 34 are those of the protein sequence, and do not refer to the EU numbering convention.

[00244] The DNA sequence of SEQ ID NO: 35 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the Syt1 C2A domain of synaptotagmin fused to the N-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 36) with mutation of Cys137 to serine. The encoded fusion protein (SEQ ID NO: 36) is configured to form homodimers.

[00245] In particular, the amino acid sequence of the exemplary Syt1-Fc of SEQ ID NO: 36 has, in order from N- to C-terminus, residues 141-266 of the C2A PS-binding domain of synaptotagmin (Syt1) as residues 1-126, a GGGGS linker peptide at residues 127-131, an immunoglobulin hinge (human IgG1-derived) at residues 132-147, an immunoglobulin CH2 domain (human IgG1-derived) at residues 148-257, an immunoglobulin CH3 domain (human IgG1-derived) at residues 258-364. The cysteine residue (137) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 36 are those of the protein sequence, and do not refer to the EU numbering convention.

[00246] The DNA sequence of SEQ ID NO: 37 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the C2 domain of PKC α fused to the N-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 38) with mutation of Cys143 to serine. The encoded fusion protein (SEQ ID NO: 38) is configured to form homodimers.

[00247] In particular, the amino acid sequence of the exemplary PKC α -Fc of SEQ ID NO: 38 has, in order from N- to C-terminus, residues 157-288 of the C2 domain of PKC α as residues 1-132, a GGGGS linker peptide at residues 133-137, an immunoglobulin hinge (human IgG1-derived) at residues 138-153, an immunoglobulin CH2 domain (human IgG1-derived) at residues 154-263, an immunoglobulin CH3 domain (human IgG1-derived) at residues 264-370. The cysteine residue (143) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 38 are those of the protein sequence, and do not refer to the EU numbering convention.

[00248] The DNA sequence of SEQ ID NO: 39 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the AnxA1 PS-binding core domain fused to the N-terminus of an immunoglobulin Fc fragment (SEQ ID NO: 40) with mutation of Cys317 to serine. The encoded fusion protein (SEQ ID NO: 40) is configured to form homodimers.

[00249] In particular, the amino acid sequence of the exemplary AnxA1-Fc of SEQ ID NO: 40 has, in order from N- to C-terminus, residues 41-346 of the AnxA1 core domain as residues 1-306, a GGGGS linker peptide at residues 307-311, an immunoglobulin hinge (human IgG1-derived) at residues 312-327, an immunoglobulin CH2 domain (human IgG1-derived) at residues 328-437, an immunoglobulin CH3 domain (human IgG1-derived) at residues 438-544. The cysteine residue (317) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 40 are those of the protein sequence, and do not refer to the EU numbering convention.

[00250] The DNA sequence of SEQ ID NO: 41 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the Syt1 C2A domain of synaptotagmin fused to the N- and C-termini of an immunoglobulin Fc fragment (SEQ ID NO: 42) with knobs-into-holes mutations and mutation of Cys137 to serine. The encoded fusion protein (SEQ ID NO: 42) is configured, for example, to form heterodimers with an exemplary Fc fragment (SEQ ID NO: 44).

[00251] In particular, the amino acid sequence of the exemplary Syt1-Fc-Syt1 of SEQ ID NO: 42 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 132-147, an immunoglobulin CH2 domain (human IgG1-derived) at residues 148-257, an immunoglobulin CH3 domain (human IgG1-derived) at residues 258-364. Residues 141-266 of the C2A PS-binding domain of synaptotagmin (Syt1) are fused as residues 1-126 and 370-495, respectively, to the N- and C-termini of the hinge and CH3 domain via GGGGS linker peptides (residues 127-131 and 365-369). The exemplary Syt1-

Fc-Syt1 of SEQ ID NO: 42 has 'knobs-into-holes' mutations at residues 266 and 311. The cysteine residue (137) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 42 are those of the protein sequence, and do not refer to the EU numbering convention.

[00252] The DNA sequence of SEQ ID NO: 43 is of a polynucleotide encoding an exemplary Fc fragment (SEQ ID NO: 44) having knobs-into-holes mutations and mutation of Cys6 to serine. The Fc fragment of SEQ ID NO: 44 is configured, for example, to form heterodimers with an exemplary Syt1-Fc-Syt1 fusion (SEQ ID NO: 42).

[00253] In particular, the amino acid sequence of the exemplary Fc fragment of SEQ ID NO: 44 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233. The exemplary Fc fragment of SEQ ID NO: 44 has knobs-into-holes mutations at residues 150 and 191. The cysteine residue (6) that usually pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 44 are those of the protein sequence, and do not refer to the EU numbering convention.

[00254] The DNA sequence of SEQ ID NO: 45 is of a polynucleotide encoding an exemplary Fc fusion protein comprising the Syt1 C2A domain of synaptotagmin fused to both the N- and C-termini of an immunoglobulin Fc fragment (SEQ ID NO: 46) with knobs-into-holes mutations, electrostatic steering mutations and mutation of Cys137 to serine. The encoded fusion protein (SEQ ID NO: 46) is configured, for example, to form heterodimers with an exemplary Fc fragment (SEQ ID NO: 48).

[00255] In particular, the amino acid sequence of the exemplary Syt1-Fc-Syt1 of SEQ ID NO: 46 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 132-147, an immunoglobulin CH2 domain (human IgG1-derived) at residues 148-257, an immunoglobulin CH3 domain (human IgG1-derived) at residues 258-364. Residues 141-266 of the C2A PS-binding domain of synaptotagmin (Syt1) are fused as residues 1-126 and 370-495, respectively, to the N- and C-termini of the hinge and CH3 domain via GGGGS linker peptides (residues 127-131 and 365-369). The exemplary Syt1-Fc-Syt1 of SEQ ID NO: 46 has 'knobs-into-holes' mutations at residues 266 and 311 and electrostatic steering mutations at residues 309 and 326. The cysteine residue (137) that pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 46 are those of the protein sequence, and do not refer to the EU numbering convention.

[00256] The DNA sequence of SEQ ID NO: 47 is of a polynucleotide encoding an exemplary Fc fragment (SEQ ID NO: 48) having knobs-into-holes mutations, electrostatic steering mutations and mutation of Cys6 to serine. The Fc fragment of SEQ ID NO: 48 is configured, for example, to form heterodimers with an exemplary Syt1-Fc-Syt1 fusion (SEQ ID NO: 46).

[00257] In particular, the amino acid sequence of the exemplary Fc fragment of SEQ ID NO: 48 has, in order from N- to C-terminus, an immunoglobulin hinge (human IgG1-derived) at residues 1-16, an immunoglobulin CH2 domain (human IgG1-derived) at residues 17-126, an immunoglobulin CH3 domain (human IgG1-derived) at residues 127-233. The exemplary Fc fragment of SEQ ID NO: 48 has knobs-into-holes mutations at residues 150 and 191 and electrostatic steering mutations at residues 143 and 185. The cysteine residue (6) that usually pairs with an immunoglobulin light chain is mutated to serine. The amino acid residue numbers referred to in SEQ ID NO: 48 are those of the protein sequence, and do not refer to the EU numbering convention.

[00258] The above disclosed subject matter is to be considered illustrative, and not restrictive, and the appended claims are intended to cover all such modifications, enhancements, and other embodiments which fall within the true spirit and scope of the present disclosure. Thus, to the maximum extent allowed by law, the scope of the present disclosure is to be determined by the broadest permissible interpretation of the following claims and their equivalents, and shall not be restricted or limited by the foregoing detailed description.

[00259] The various methods and techniques described above provide a number of ways to carry out the application. Of course, it is to be understood that not necessarily all objectives or advantages described can be achieved in accordance with any particular embodiment described herein. Thus, for example, those skilled in the art will recognize that the methods can be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as taught or suggested herein. A variety of alternatives are mentioned herein. It is to be understood that some preferred embodiments specifically include one, another, or several features, while others specifically exclude one, another, or several features, while still others mitigate a particular feature by inclusion of one, another, or several advantageous features.

[00260] Furthermore, the skilled artisan will recognize the applicability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step,

can be employed in various combinations by one of ordinary skill in this art to perform methods in accordance with the principles described herein. Among the various elements, features, and steps some will be specifically included and others specifically excluded in diverse embodiments.

[00261] Although the application has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the embodiments of the application extend beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and modifications and equivalents thereof.

[00262] In some embodiments, the numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth, used to describe and claim certain embodiments of the application are to be understood as being modified in some instances by the term “about.” Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. In some embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the application are approximations, the numerical values set forth in the specific examples are reported as precisely as practicable.

[00263] In some embodiments, the terms “a” and “an” and “the” and similar references used in the context of describing a particular embodiment of the application (especially in the context of certain of the following claims) can be construed to cover both the singular and the plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (for example, “such as”) provided with respect to certain embodiments herein is intended merely to better illuminate the application and does not pose a limitation on the scope of the application otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the application.

[00264] Preferred embodiments of this application are described herein, including the best mode known to the inventors for carrying out the application. Variations on those

preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans can employ such variations as appropriate, and the application can be practiced otherwise than specifically described herein. Accordingly, many embodiments of this application include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the application unless otherwise indicated herein or otherwise clearly contradicted by context.

[00265] All patents, patent applications, publications of patent applications, and other material, such as articles, books, specifications, publications, documents, things, and/or the like, referenced herein are hereby incorporated herein by this reference in their entirety for all purposes, excepting any prosecution file history associated with same, any of same that is inconsistent with or in conflict with the present document, or any of same that may have a limiting affect as to the broadest scope of the claims now or later associated with the present document. By way of example, should there be any inconsistency or conflict between the description, definition, and/or the use of a term associated with any of the incorporated material and that associated with the present document, the description, definition, and/or the use of the term in the present document shall prevail.

[00266] It is to be understood that the embodiments of the application disclosed herein are illustrative of the principles of the embodiments of the application. Other modifications that can be employed can be within the scope of the application. Thus, by way of example, but not of limitation, alternative configurations of the embodiments of the application can be utilized in accordance with the teachings herein. Accordingly, embodiments of the present application are not limited to that precisely as shown and described.

CLAIMS

1. An endolysosomal targeting conjugate, comprising:
 - a targeting component comprising an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain, wherein the targeting component is configured to bind to a cell surface molecule of a target cell with a lower dissociation constant in an extracellular space than in an endolysosomal compartment of the target cell; and
 - a cargo component comprising a cargo molecule conjugated to an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain;wherein
 - the targeting component is fused directly or indirectly to the cargo component;
 - upon entry to the endolysosomal compartment, the targeting component is configured to dissociate from the cell surface molecule; and
 - wherein the endolysosomal targeting conjugate is configured to deliver the cargo molecule to the endolysosomal compartment of the target cell.
2. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to the cell surface molecule in the extracellular space with a dissociation constant less than 500 nM.
3. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to the cell surface molecule with a lower dissociation constant at a near neutral pH than at an acidic endolysosomal pH.
4. The endolysosomal targeting conjugate of claim 3 wherein the near neutral pH is from about 6.8 to about 7.5 and the acidic endolysosomal pH is from about 5.0 to about 6.5.

5. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to a cell surface molecule with a lower dissociation constant at an extracellular Ca^{2+} concentration than at an endolysosomal Ca^{2+} concentration.
6. The endolysosomal targeting conjugate of claim 5 wherein the extracellular Ca^{2+} concentration is about 2 mM and the endolysosomal Ca^{2+} concentration is about 2 μM .
7. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule in the extracellular space with a dissociation constant less than 500 nM.
8. The endolysosomal targeting conjugate of claim 1 wherein the targeting component is a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at a near neutral pH than at an acidic endolysosomal pH.
9. The endolysosomal targeting conjugate of claim 8 wherein the near neutral pH is from about 6.8 to about 7.5 and the acidic endolysosomal pH is from about 5.0 to about 6.5.
10. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at an extracellular Ca^{2+} concentration than at an endolysosomal Ca^{2+} concentration.
11. The endolysosomal targeting conjugate of claim 10 wherein the extracellular Ca^{2+} concentration is about 2 mM and the endolysosomal Ca^{2+} concentration is about 2 μM .

12. The endolysosomal targeting conjugate of claim 1 wherein the cargo component comprises an antibody, an antibody fragment or an antibody domain comprising an antibody Fc region or a domain of the antibody Fc fragment.
13. The endolysosomal targeting conjugate of claim 12 wherein the antibody Fc region or the domain of the antibody Fc fragment is derived from human IgG1.
14. The endolysosomal targeting conjugate of claim 1 wherein the cargo component comprises an albumin molecule or a domain of albumin.
15. The endolysosomal targeting conjugate of claim 3 wherein the targeting component comprises a Fab fragment or a scFv fragment of a HER2-specific antibody, wherein a heavy chain variable domain of the Fab fragment or the scFv fragment comprises mutations of Ser55 to histidine and Gly57 to glutamic acid.
16. The endolysosomal targeting conjugate of claim 3 wherein the targeting component comprises a Fab fragment or a scFv fragment of a HER2-specific antibody, wherein in the Fab fragment or the scFv fragment a heavy chain variable domain comprises a mutation of Ser103 to histidine and a light chain variable domain comprises a mutation of Tyr55 to histidine.
17. The endolysosomal targeting conjugate of claim 1 comprising at least a first targeting component and a second targeting component, wherein the first targeting component is configured to bind to a different cell surface molecule than the second targeting component.
18. The endolysosomal targeting conjugate of claim 17, wherein the first and second targeting components are fused to a heterodimer of two immunoglobulin Fc fragments.
19. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises a phosphatidylserine-binding protein and the target cell is a cell having a phosphatidylserine on the cell surface.

20. The endolysosomal targeting conjugate of claim 19 wherein the phosphatidylserine-binding protein is selected from the group consisting of a core domain of AnxA1, a C2A domain of Syt1, and a C2 domain of PKC α .
21. The endolysosomal targeting conjugate of claim 20 wherein the phosphatidylserine-binding protein is the C2A domain of Syt1.
22. The endolysosomal targeting conjugate of claim 21 wherein the targeting component comprises two of the C2A domains of Syt1.
23. The endolysosomal targeting conjugate of claim 21 wherein the targeting component comprises four of the C2A domains of Syt1.
24. The endolysosomal targeting conjugate of claim 21 wherein the targeting component comprises more than four of the C2A domains of Syt1.
25. The endolysosomal targeting conjugate of claim 19, wherein:
 - the targeting component comprises a phosphatidylserine-binding protein;
 - the cargo component comprises an Fc portion of human IgG1; and
 - the targeting component is covalently fused to the cargo component by a linker protein.
26. The endolysosomal targeting conjugate of claim 25, wherein the linker protein is a Gly4Ser linker.
27. The endolysosomal targeting conjugate of claim 1, wherein the cargo molecule is a cytotoxic drug.
28. The endolysosomal targeting conjugate of claim 27 wherein the cytotoxic drug is MMAE.
29. The endolysosomal targeting conjugate of claim 1 wherein the cargo molecule is an imaging label.

30. An endolysosomal targeting conjugate, comprising:

a targeting component comprising an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain, wherein the targeting component is configured to bind to a cell surface molecule of a target cell; and

a cargo component comprising a cargo molecule conjugated to an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain;

wherein

the targeting component is configured to bind to the cargo component with a lower dissociation constant in an extracellular space than in an endolysosomal compartment of the target cell;

upon entry to the endolysosomal compartment, the targeting component is configured to dissociate from the cargo component; and

wherein the endolysosomal targeting conjugate is configured to deliver the cargo molecule to the endolysosomal compartment of the target cell.

31. The endolysosomal targeting conjugate of claim 30 wherein the targeting component is configured to bind to the cargo compartment with a lower dissociation constant at near neutral pH than at acidic endosomal pH.

32. The endolysosomal targeting conjugate of claim 31 wherein the near neutral pH is from about 6.8 to about 7.5 and the acidic endolysosomal pH is from about 5.0 to about 6.5.

33. The endolysosomal targeting conjugate of claim 30 wherein the targeting component is configured to bind to the cargo component with a lower dissociation constant at an extracellular Ca^{2+} concentration than at an endolysosomal Ca^{2+} concentration.

34. The endolysosomal targeting conjugate of claim 33 wherein the extracellular Ca^{2+} concentration is about 2 mM and the endolysosomal Ca^{2+} concentration is about 2 μM .

35. The endolysosomal targeting conjugate of claim 33 wherein the targeting component comprises a calbindin D9K domain 2 and the cargo component comprises a calbindin D9K domain 1.
36. The endolysosomal targeting conjugate of claim 35 wherein the calbindin D9K domain 1 is fused to the cargo component by a linker peptide and/or the calbindin D9K domain 2 is fused to the targeting component by a linker peptide.
37. The endolysosomal targeting conjugate of claim 30 wherein the cargo molecule is a cytotoxic drug.
38. The endolysosomal targeting conjugate of claim 37 wherein the cytotoxic drug is MMAE.
39. The endolysosomal targeting conjugate of claim 30 wherein the cargo molecule is an imaging label.
40. A composition comprising the endolysosomal targeting conjugate of claim 1 or claim 30 and a pharmaceutically acceptable vehicle.
41. A method of treating cancer comprising:
administering a subject with an effective dose of the composition of claim 40 to a subject;
wherein
the cargo molecule is a cytotoxic drug; and
the administering of the composition suppresses growth of a tumor in the subject.
42. The method of claim 41 wherein the composition comprises at least one endolysosomal targeting conjugate configured to target tumors comprising one or more types of target cells.
43. A method of imaging a tumor in a subject, comprising:

administering a subject with an effective dose of the composition of claim 40 to a subject, wherein the cargo molecule is an imaging label; and

performing an imaging method suitable for detecting the imaging label in the subject;

wherein

the administering of the composition provides a sufficient concentration of the imaging label detectable by the imaging method.

44. A method of providing an endolysosomal targeting conjugate for the treatment of cancer comprising the steps of:

selecting the targeting component of claim 1, wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, a nanobody, a protein, a protein fragment, or a protein domain configured to selectively bind to a cell surface molecule on a selected type of tumor target cell, wherein the targeting component is configured to bind to a cell surface molecule with a lower dissociation constant in an extracellular space than in an endolysosomal compartment;

selecting the cargo component of claim 1, wherein the cargo molecule comprises a cytotoxic drug having efficacy for suppressing growth of the selected type of tumor target cell; and

providing the endolysosomal targeting conjugate comprising the targeting component fused directly or indirectly to the cargo component.

45. A method of providing an endolysosomal targeting conjugate for the treatment of cancer comprising the steps of:

selecting the targeting component of claim 30, wherein the targeting component comprises an antibody, an antibody fragment, a nanobody, a protein, a protein fragment, or a protein domain configured to selectively bind to a cell surface molecule on a selected type of tumor target cell;

selecting the cargo component of claim 30, wherein the cargo molecule comprises a cytotoxic drug having efficacy for suppressing growth of the selected type of tumor target cell;

wherein

the targeting component is engineered to further comprise a first protein domain;

the cargo component is engineered to further comprise a second protein domain; and

the first protein domain is configured to bind to the second domain with a lower dissociation constant in an extracellular space than in an endolysosomal compartment.

46. The endolysosomal targeting conjugate of claims 1 or 30, wherein the cargo component comprises a dimer of two immunoglobulin Fc fragments.
47. The endolysosomal targeting conjugate of claims 1 or 30, wherein the cargo molecule is conjugated to the antibody fragment, the antibody domain, the nanobody, the protein, the protein fragment, or the protein domain by a polypeptide linker or through a chemical conjugation reaction.
48. The endolysosomal targeting conjugate of claims 1 or 30, wherein the imaging label is a radiolabel or a fluorescent or a near infrared label.
49. The endolysosomal targeting conjugate of claims 1 or 30, wherein an N-terminus of the targeting component is fused to a C-terminus of the cargo component.
50. The endolysosomal targeting conjugate of claims 1 or 30, wherein a C-terminus of the targeting component is fused to an N-terminus of the cargo component.
51. The endolysosomal targeting conjugate of claims 1 or 30, wherein the targeting component is fused to the cargo component at a non-terminal location of the cargo component.
52. The endolysosomal targeting conjugate of claims 1 or 30, wherein the cargo component comprises an immunoglobulin Fc fragment; and

the targeting component is fused to the immunoglobulin Fc fragment of the cargo component at an N-terminus or a C-terminus of a hinge-CH2-CH3 domain of the immunoglobulin Fc fragment.

53. The endolysosomal targeting conjugate of claims 1 or 30, wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to human epidermal growth factor receptor 2.
54. The endolysosomal targeting conjugate of claims 1 or 30, wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to prostate-specific membrane antigen.
55. The endolysosomal targeting conjugate of claims 1 or 30 comprising one or more proteins having an amino acid sequence of at least one of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, or a homolog thereof.
56. The endolysosomal targeting conjugate of claims 1 or 30 comprising a heterodimer of proteins having amino acid sequences of SEQ ID NO: 2 plus SEQ ID NO: 4, SEQ ID NO: 6 plus SEQ ID NO: 8, SEQ ID NO: 42 plus SEQ ID NO: 44, or SEQ ID NO: 46 plus SEQ ID NO: 48, or homologs thereof.
57. The endolysosomal targeting conjugate of claims 1 or 30 comprising a heterotrimer of proteins having amino acid sequence of SEQ ID NO: 18 plus SEQ ID NO: 20 plus SEQ ID NO: 22, or SEQ ID NO: 22 plus SEQ ID NO: 24 plus SEQ ID NO: 26, or homologs thereof.
58. The endolysosomal targeting conjugate of claim 1, wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that

is configured to bind to the cell surface molecule at an acidic pH with a dissociation constant greater than 1.5 μM .

59. The endolysosomal targeting conjugate of claim 58, wherein the acidic pH is about 5.8.
60. The endolysosomal targeting conjugate of claim 1, wherein the targeting component comprises a protein, a protein fragment or a protein domain that is configured to bind to the cell surface molecule at an acidic pH with a dissociation constant greater than 1.5 μM .
61. The endolysosomal targeting conjugate of claim 60, wherein the acidic pH is about 5.8.
62. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to the cell surface molecule with a lower dissociation constant at a pH from about 6.8 to about 7.5 than at a pH from about 5.0 to about 6.5.
63. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises an antibody, an antibody fragment, an antibody domain, or a nanobody that is configured to bind to a cell surface molecule with a lower dissociation constant at a Ca^{2+} concentration of about 2mM than at a Ca^{2+} concentration of about 2 μM .
64. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises a protein, a protein fragment or a protein domain that is configured to bind to a cell surface molecule with a lower dissociation constant at a pH from about 6.8 to about 7.5 than at a pH from about 5.0 to about 6.5.
65. The endolysosomal targeting conjugate of claim 1 wherein the targeting component comprises a protein, a protein fragment or a protein domain that is configured to bind

to a cell surface molecule with a lower dissociation constant at a Ca^{2+} concentration of about 2mM than at a Ca^{2+} concentration of about 2 μM .

66. The endolysosomal targeting conjugate of claim 30 wherein the targeting component is configured to bind to the cargo compartment with a lower dissociation constant at a pH from about 6.8 to about 7.5 than at a pH from about 5.0 to about 6.5.
67. The endolysosomal targeting conjugate of claim 30 wherein the targeting component is configured to bind to the cargo component with a lower dissociation constant at a Ca^{2+} concentration of about 2mM than at a Ca^{2+} concentration of about 2 μM .
68. The endolysosomal targeting conjugate of claim 1 or 30, wherein the cargo molecule is a cytotoxic radiolabel.
69. The endolysosomal targeting conjugate of claim 1 or 30, wherein the cargo molecule is a drug or other agent that modifies a behavior of the target cell.

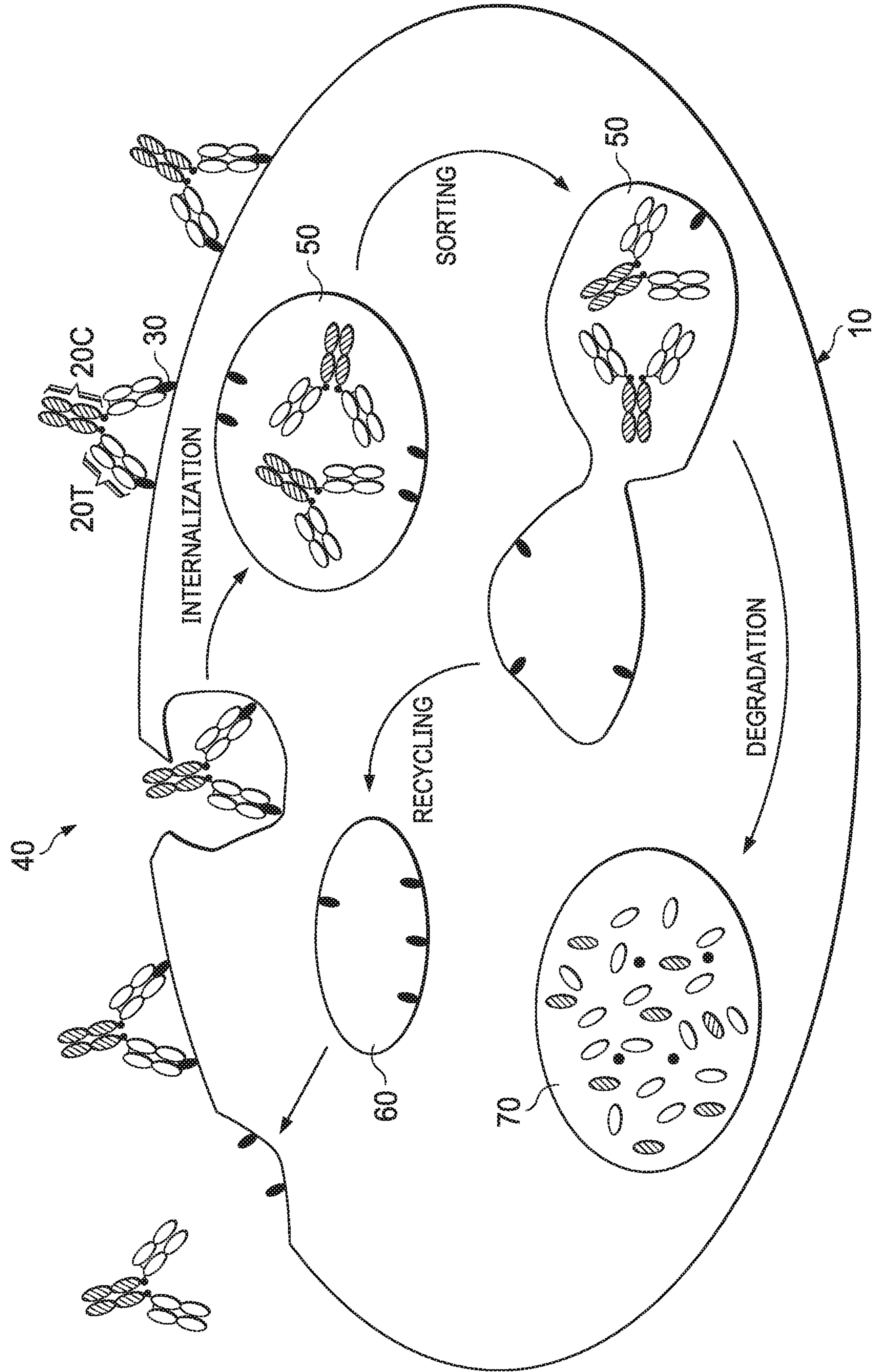


FIG. 1

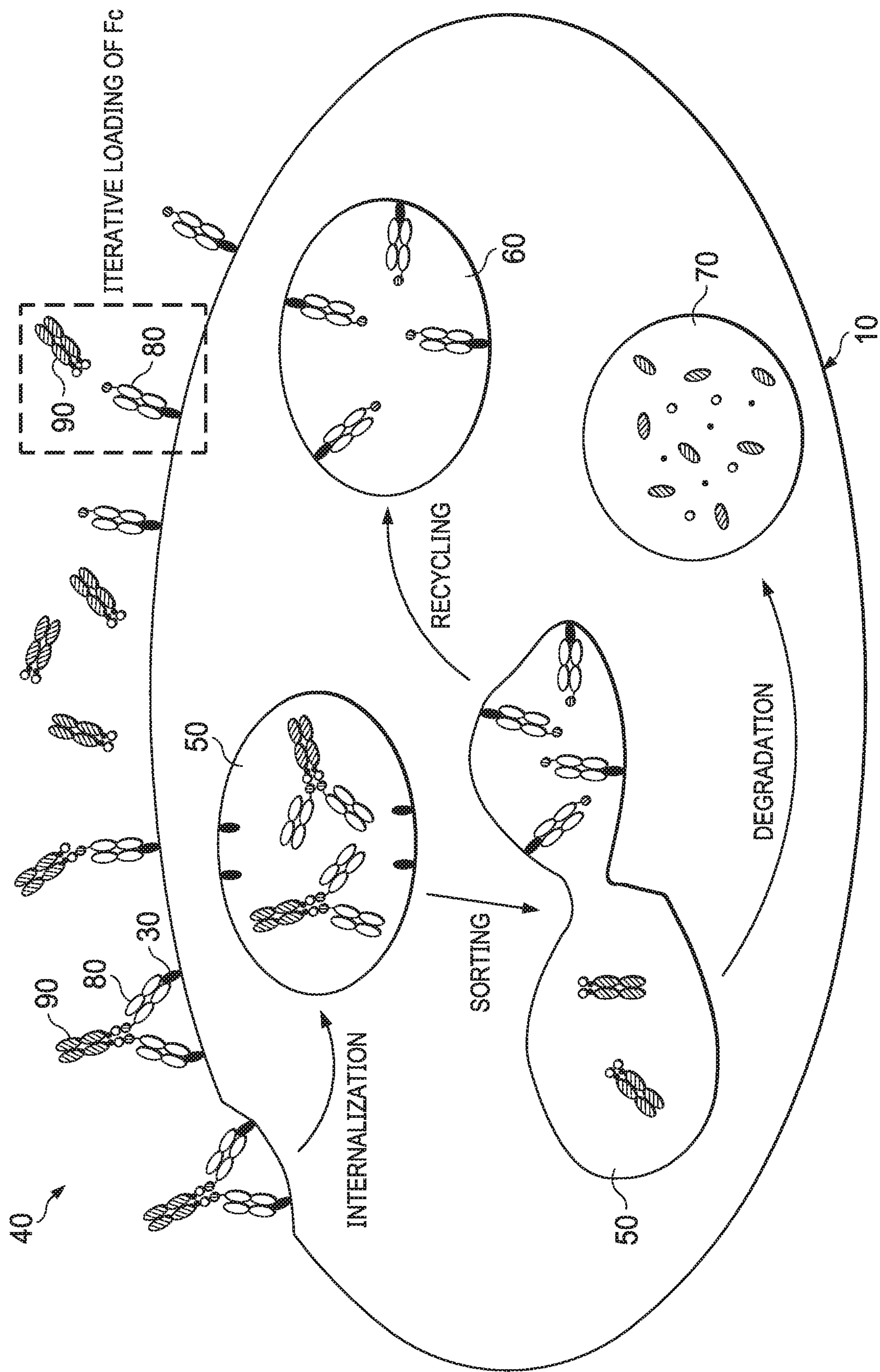


FIG. 2

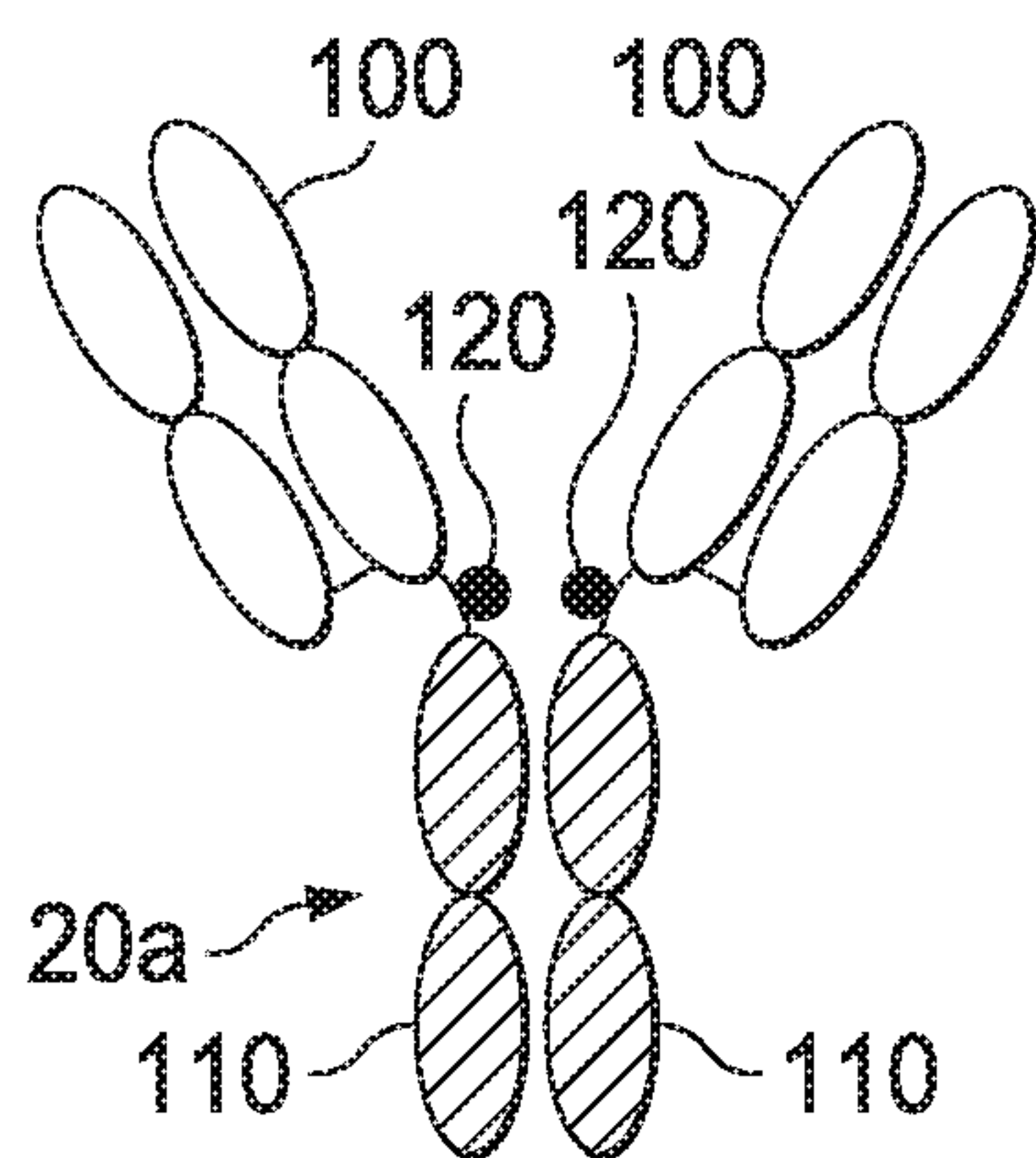


FIG. 3A

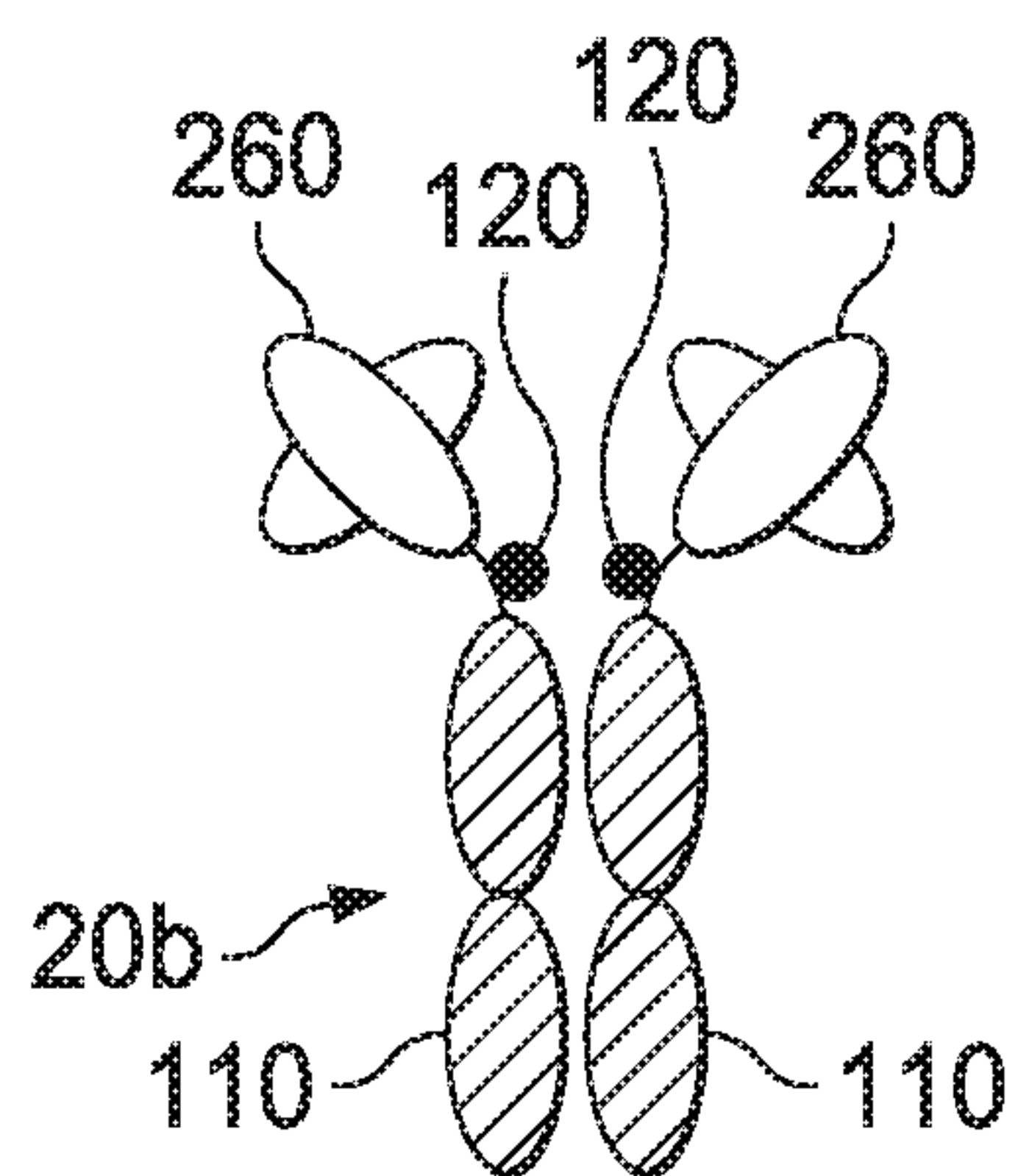


FIG. 3B

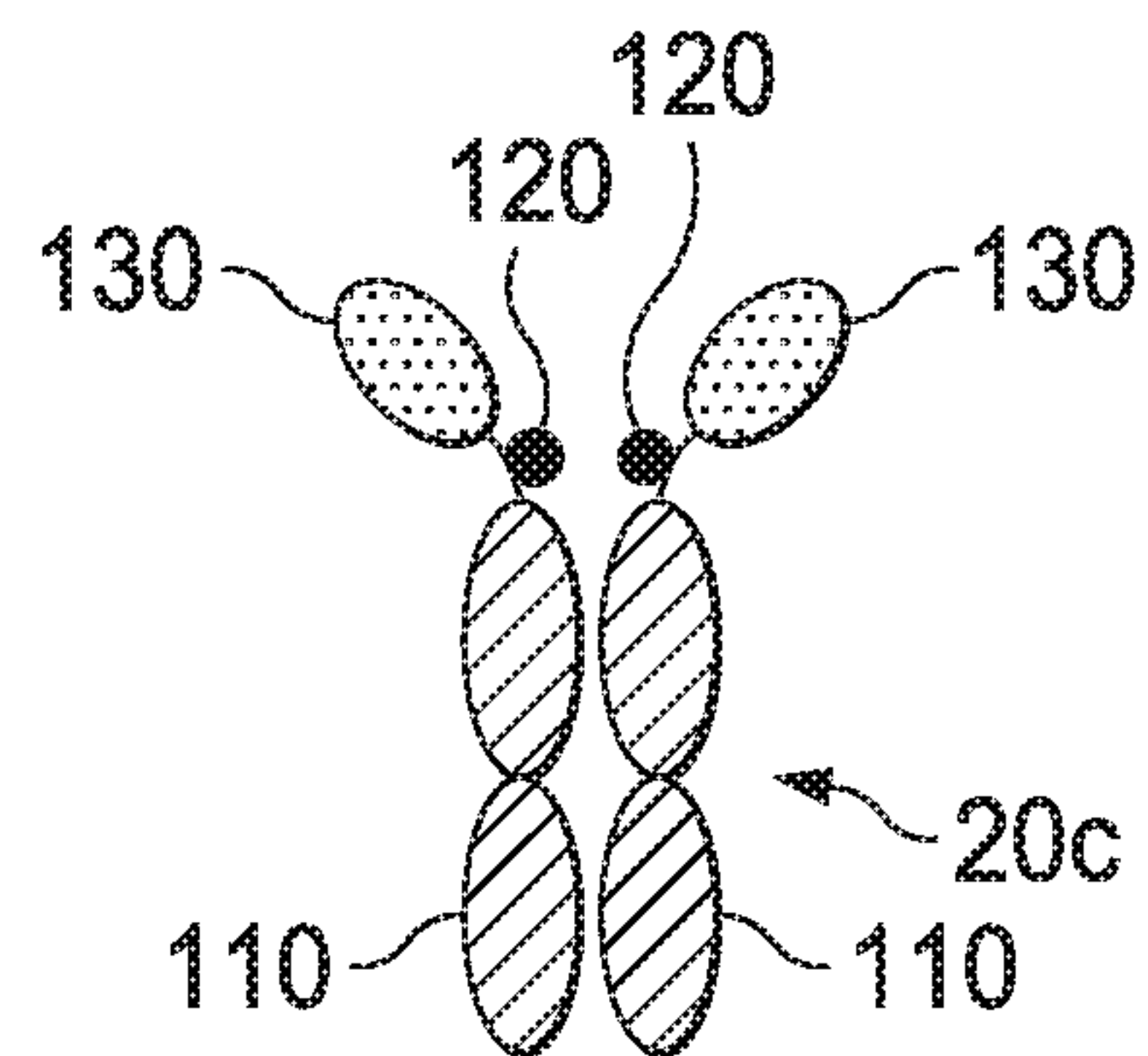


FIG. 3C

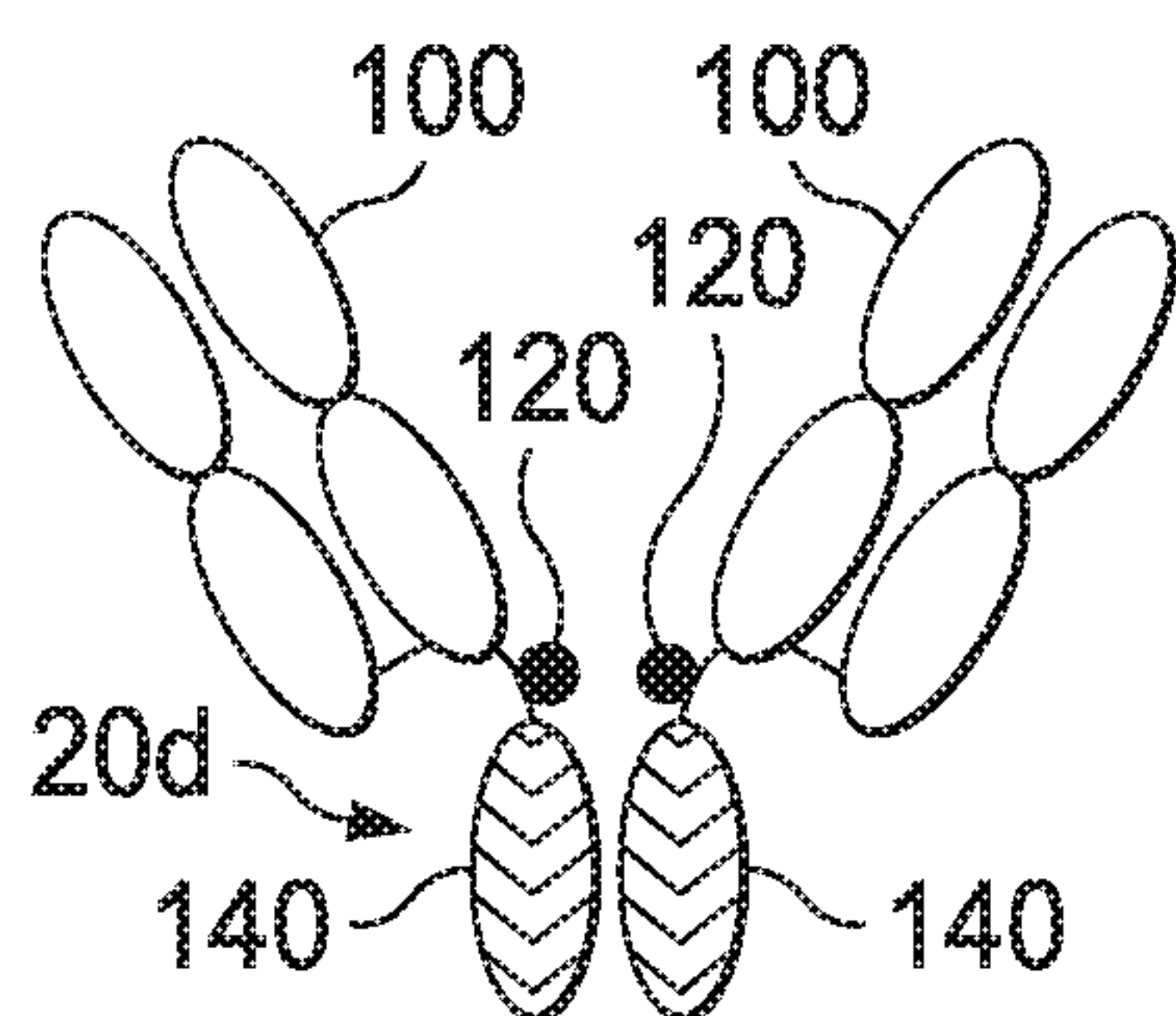


FIG. 3D

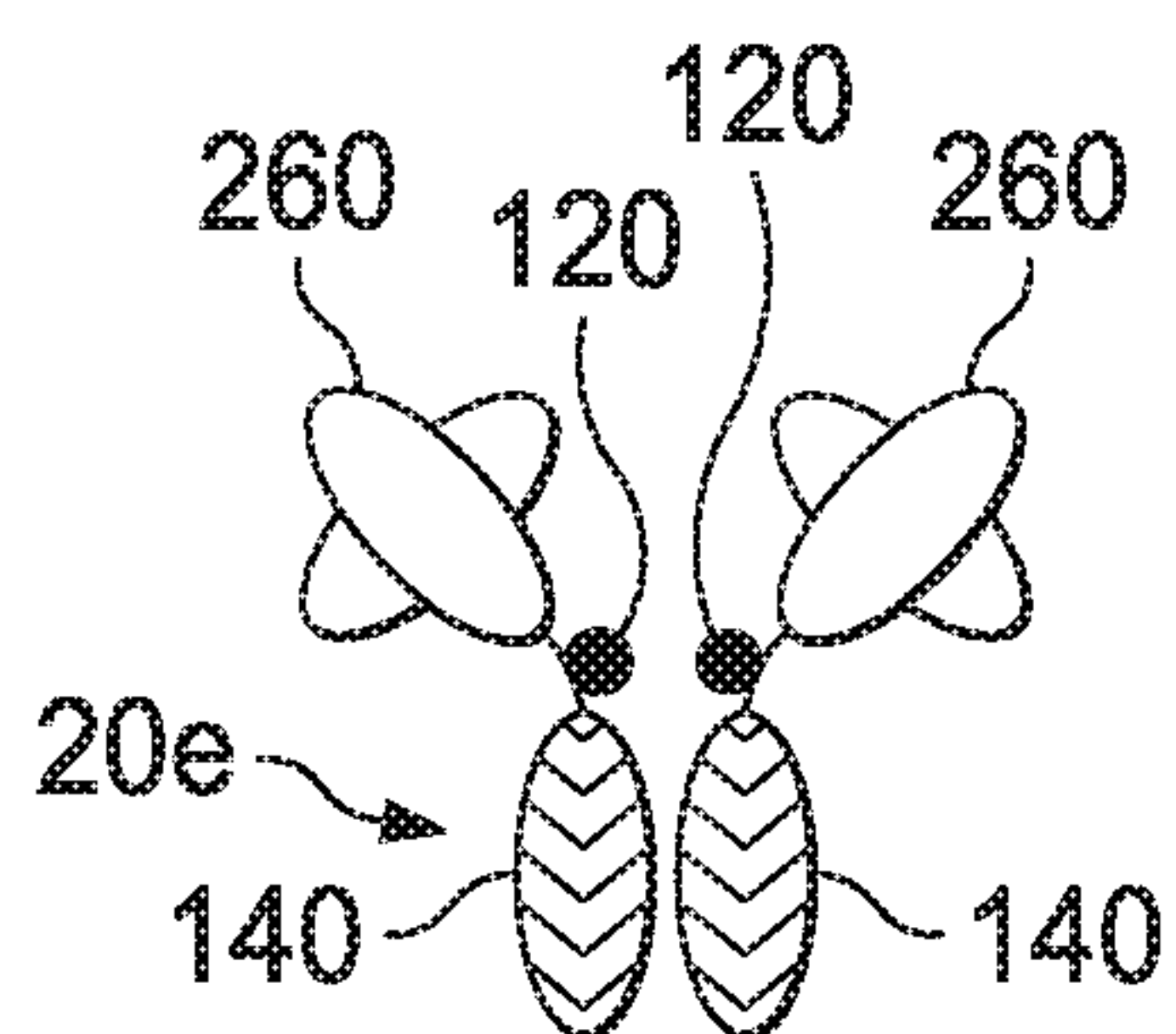


FIG. 3E

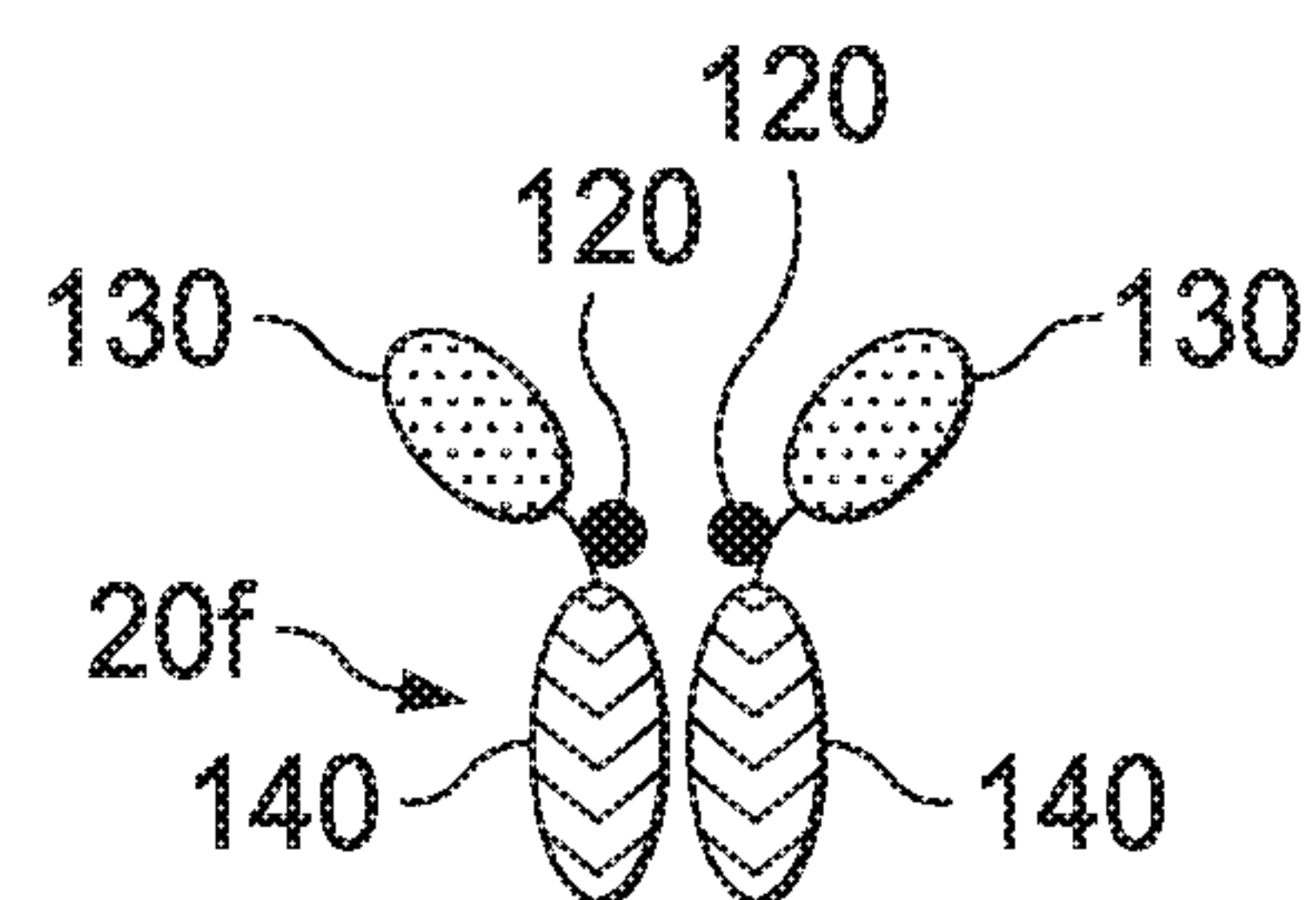


FIG. 3F

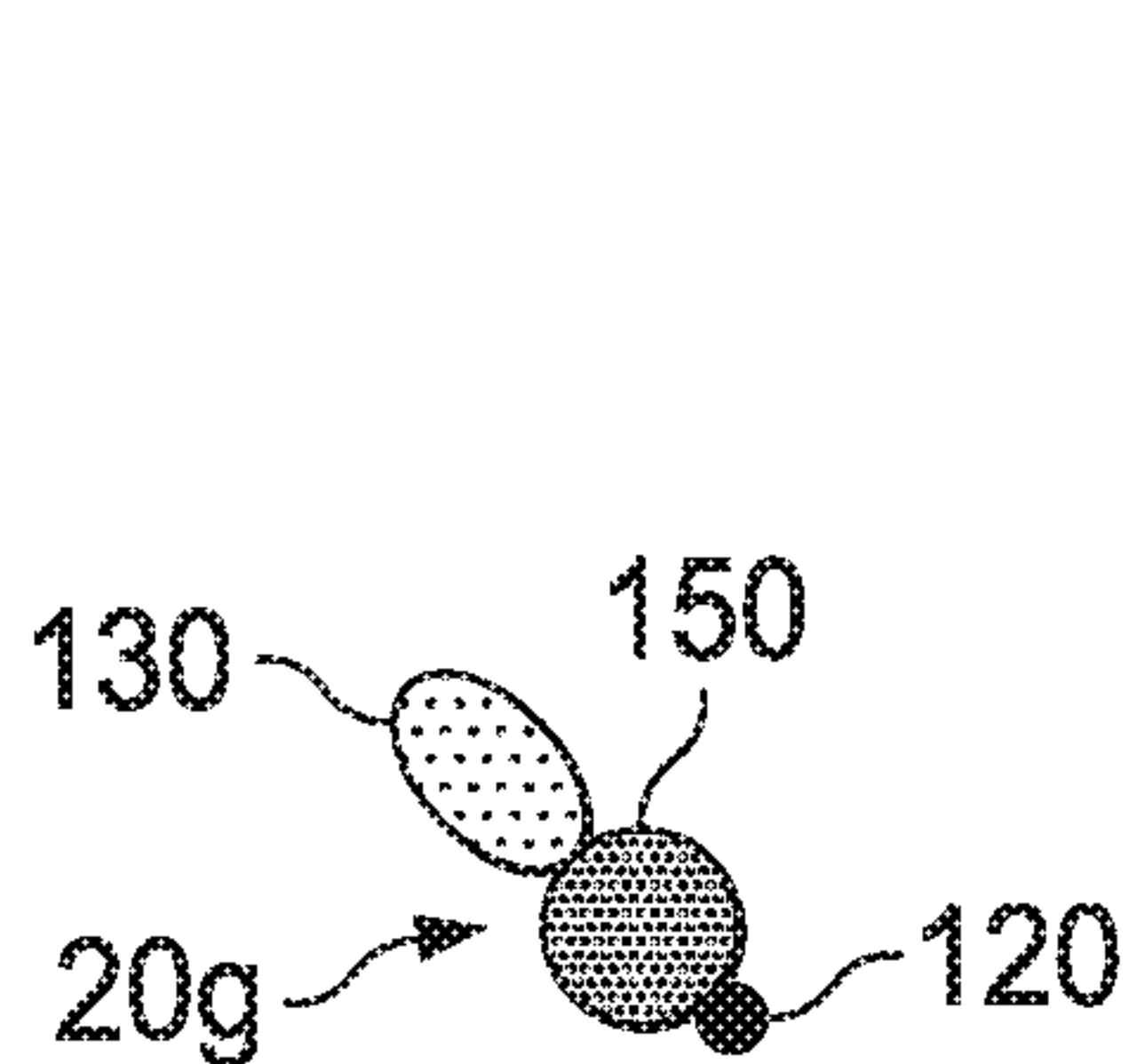


FIG. 3G

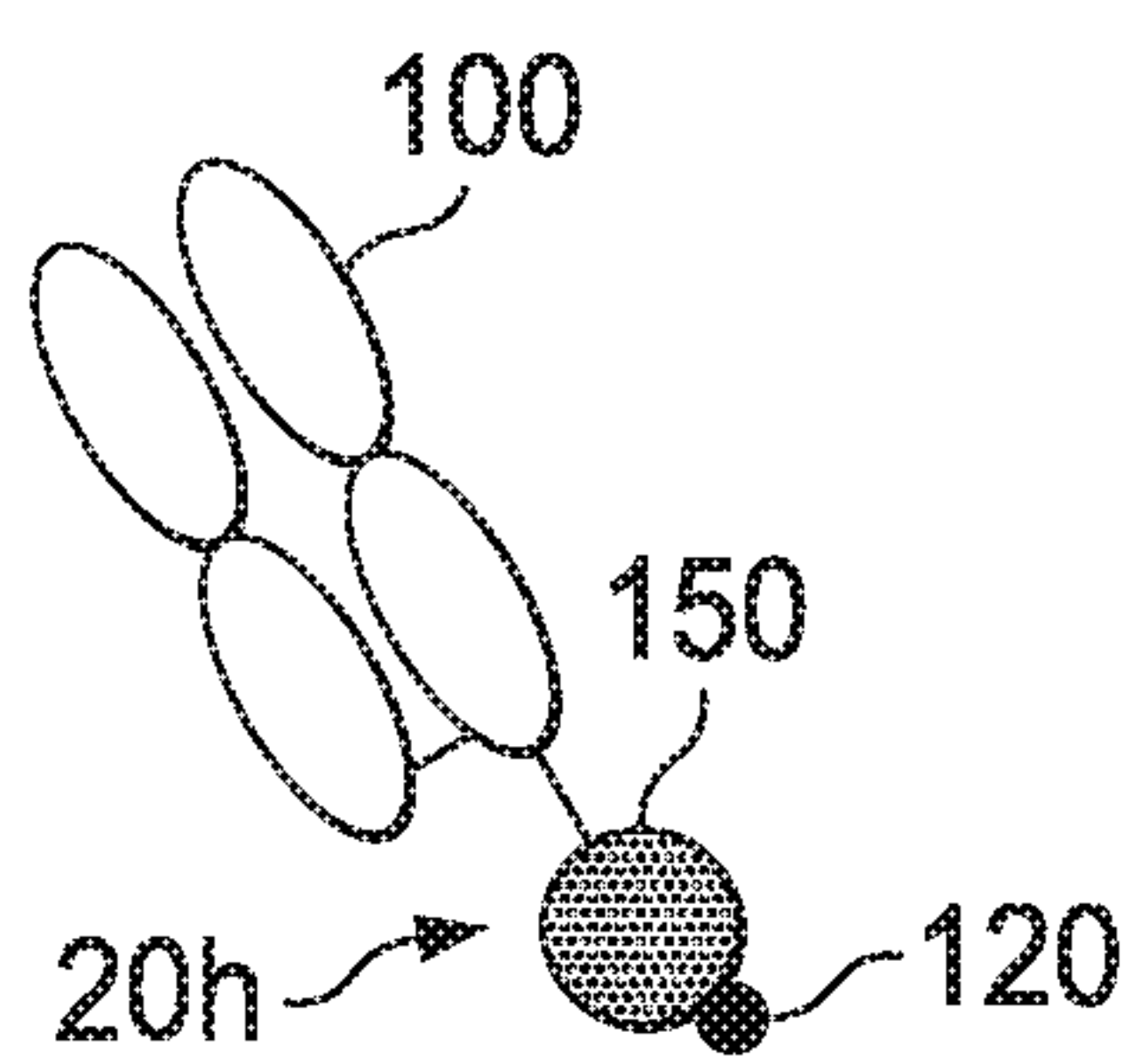


FIG. 3H

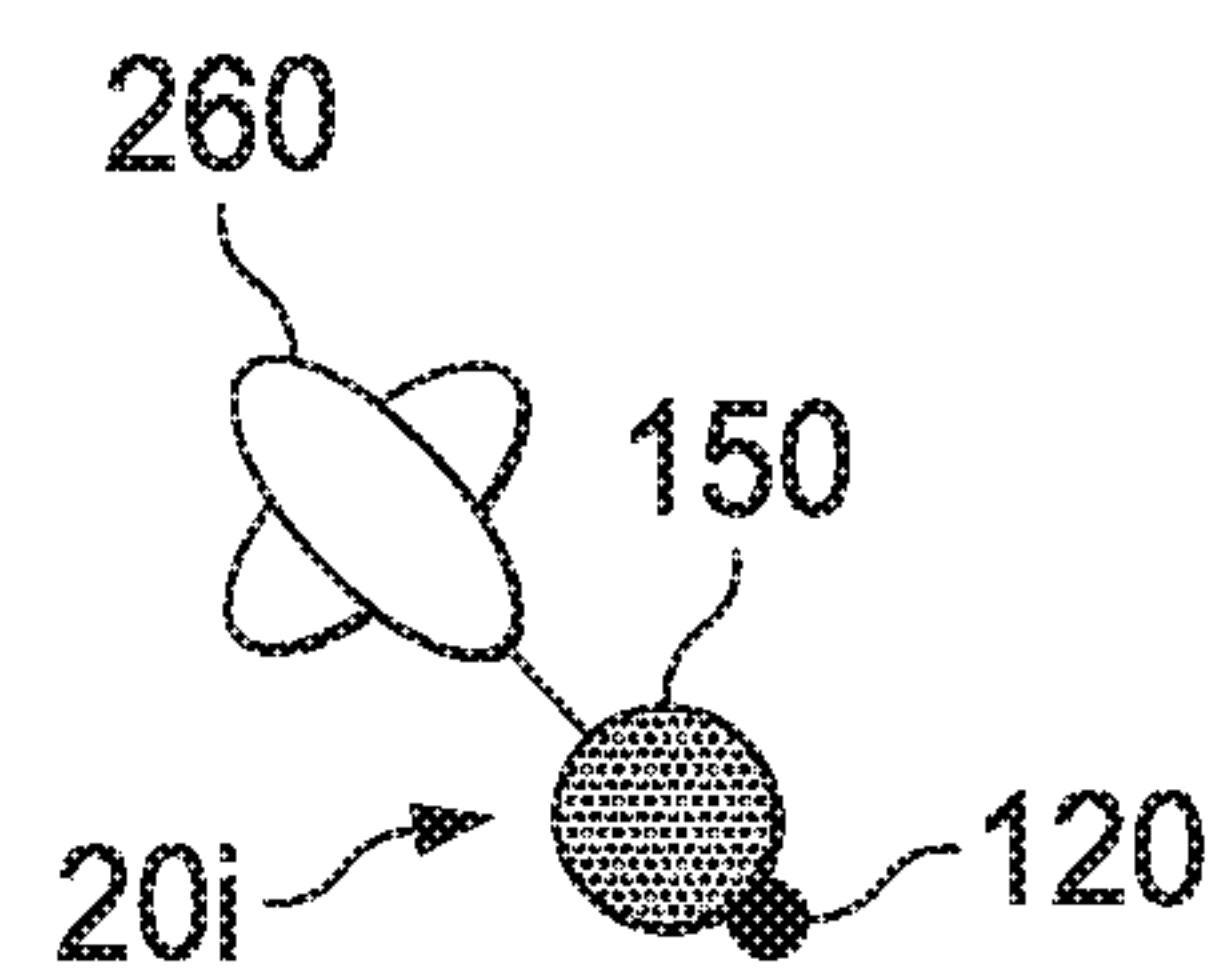


FIG. 3I

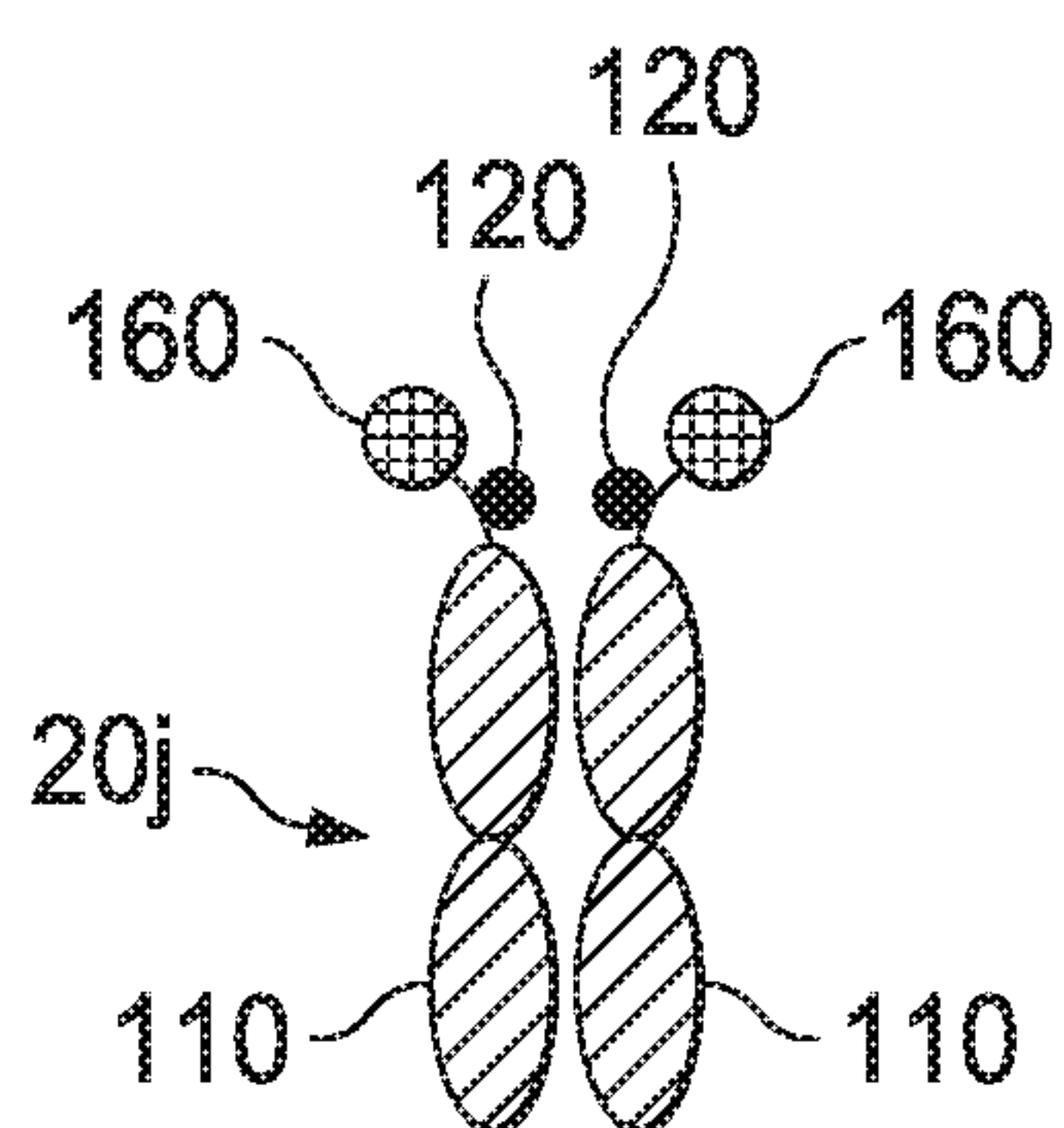


FIG. 3J

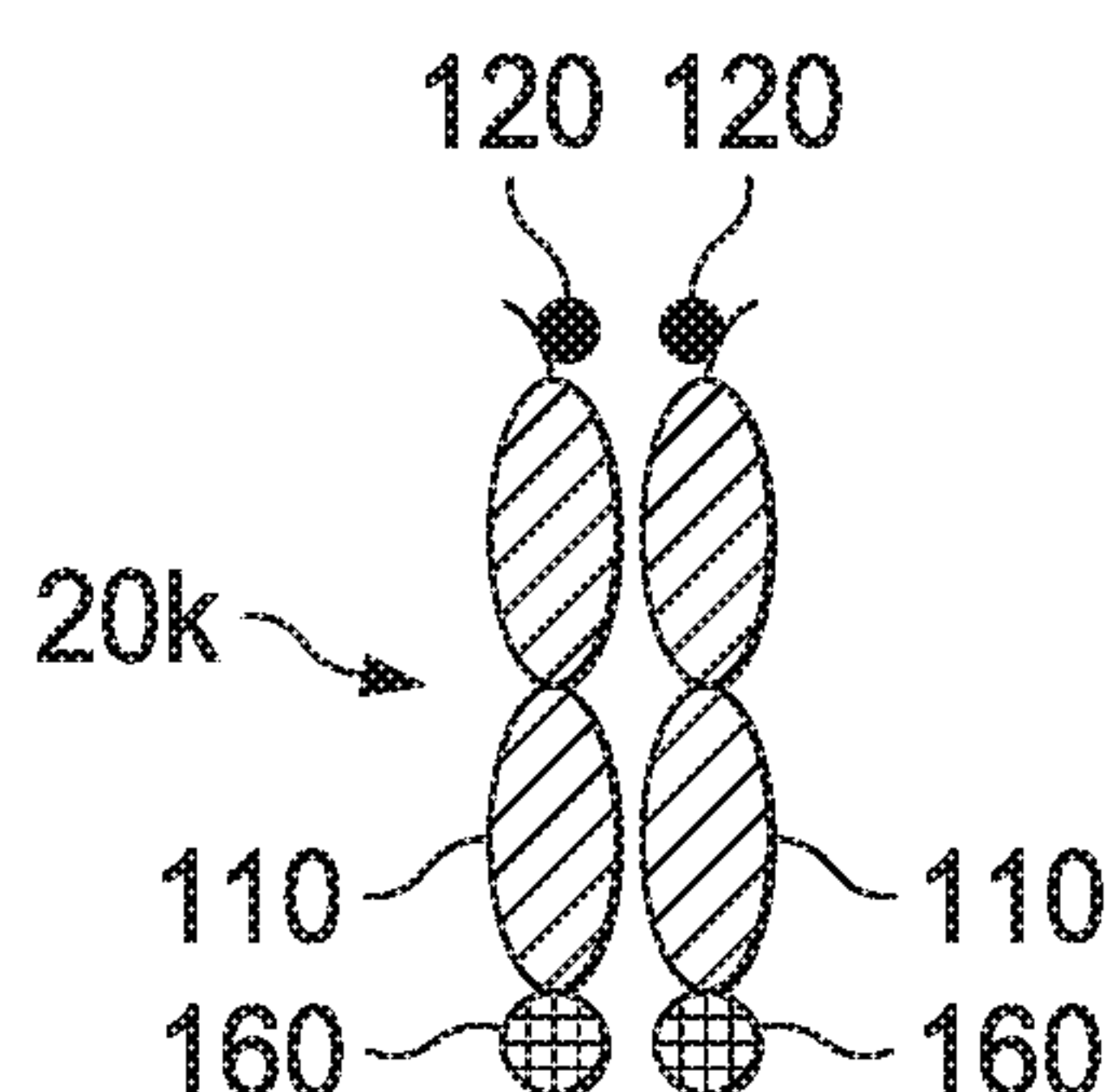


FIG. 3K

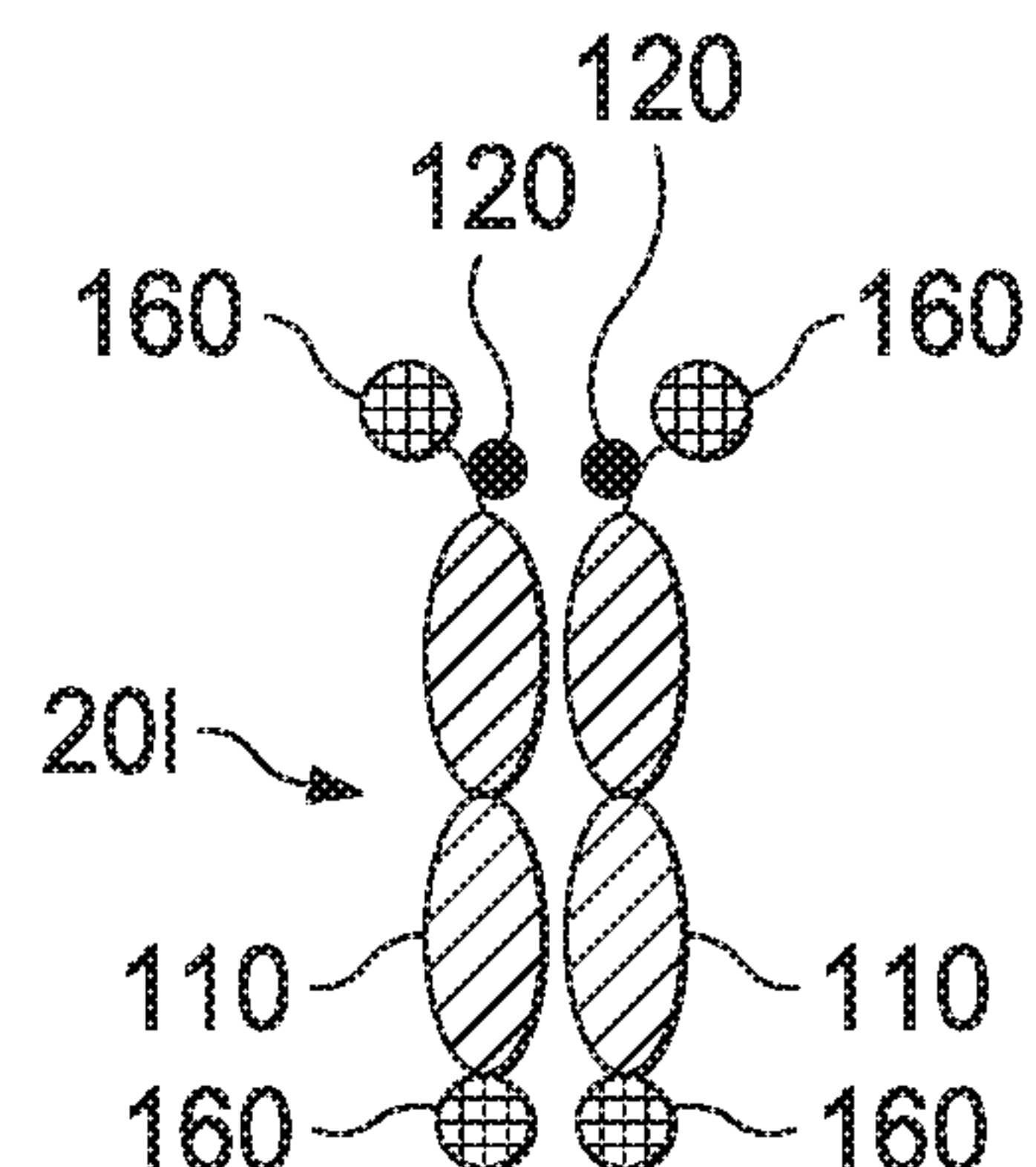


FIG. 3L

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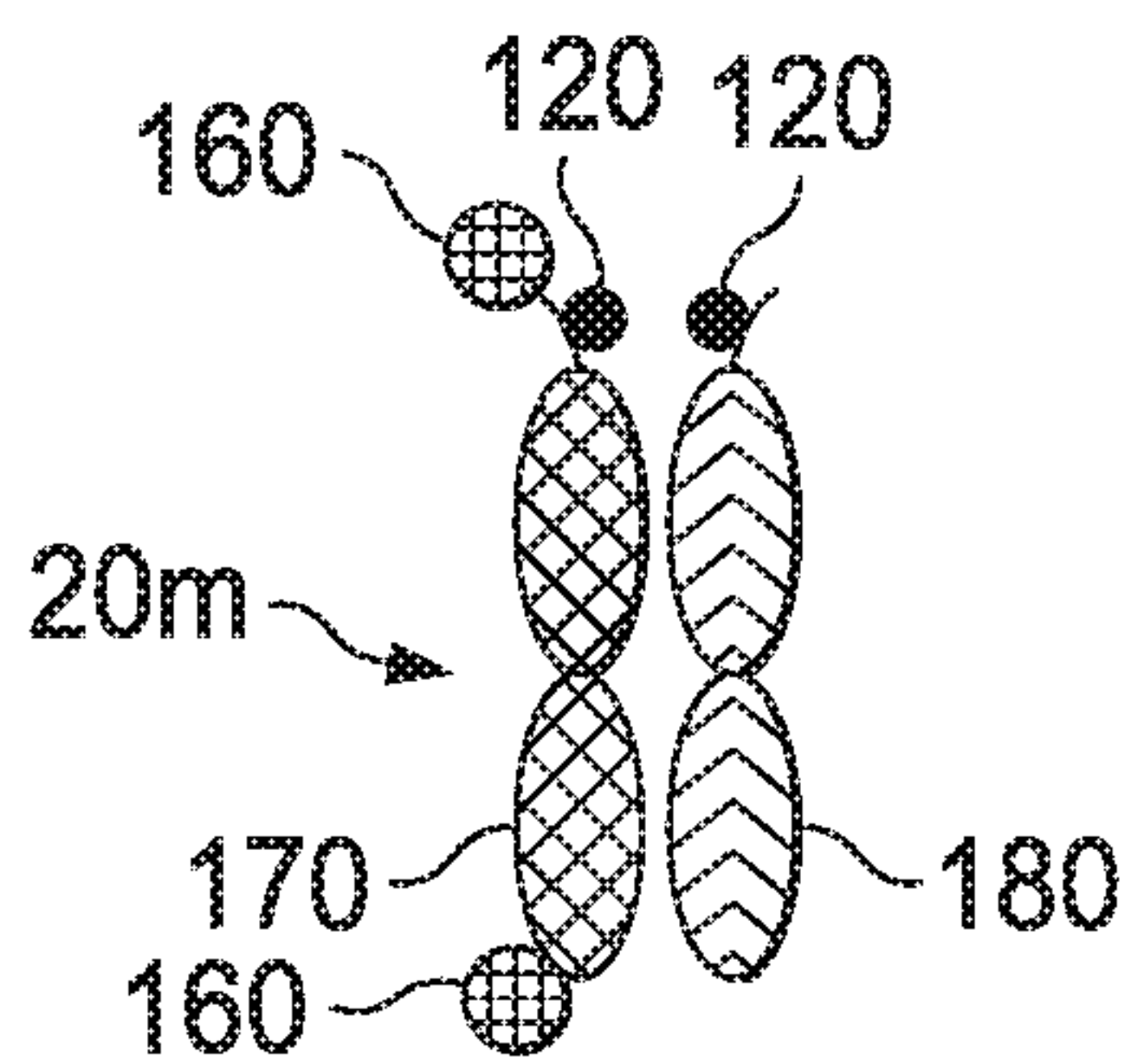


FIG. 3M

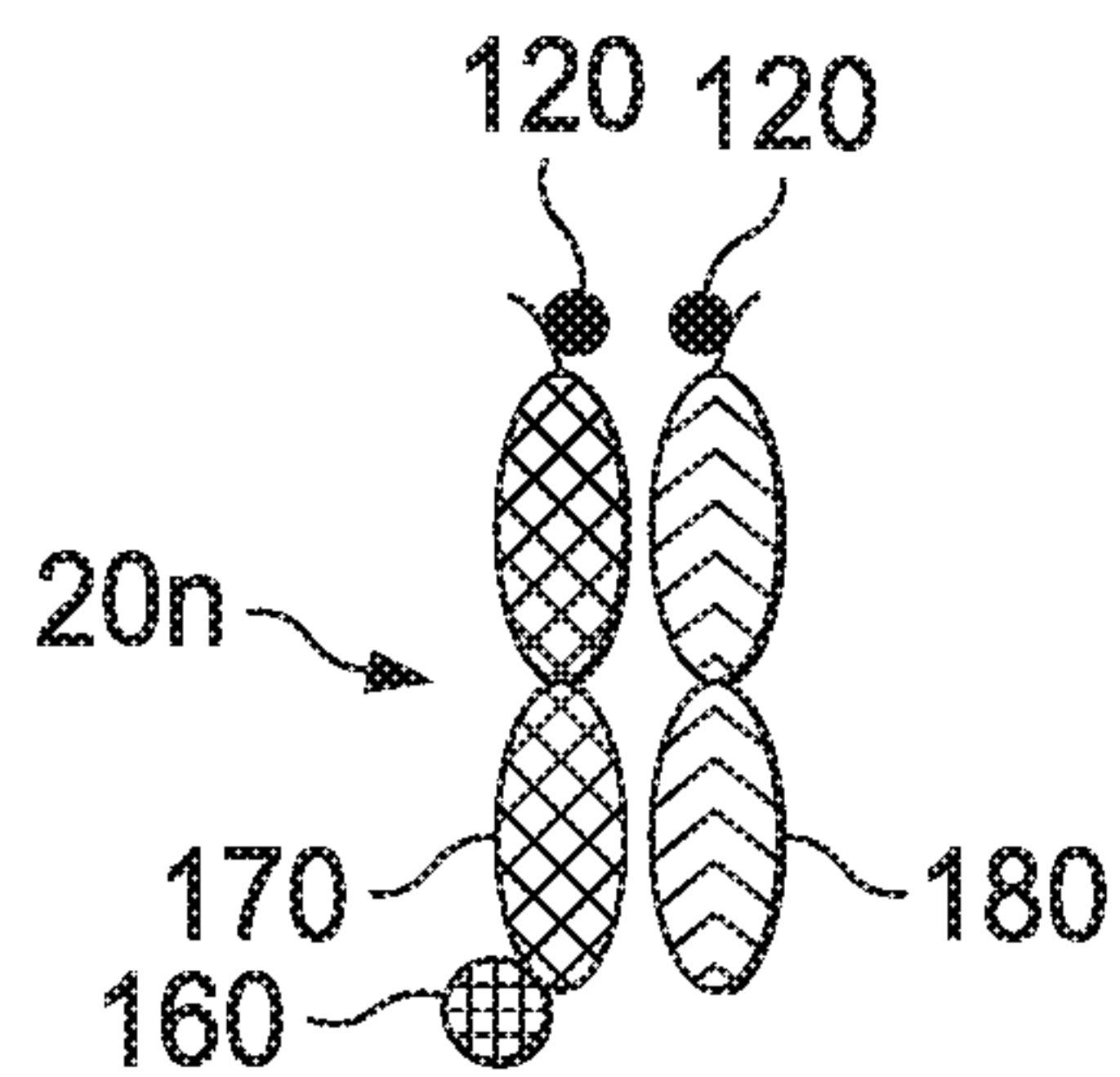


FIG. 3N

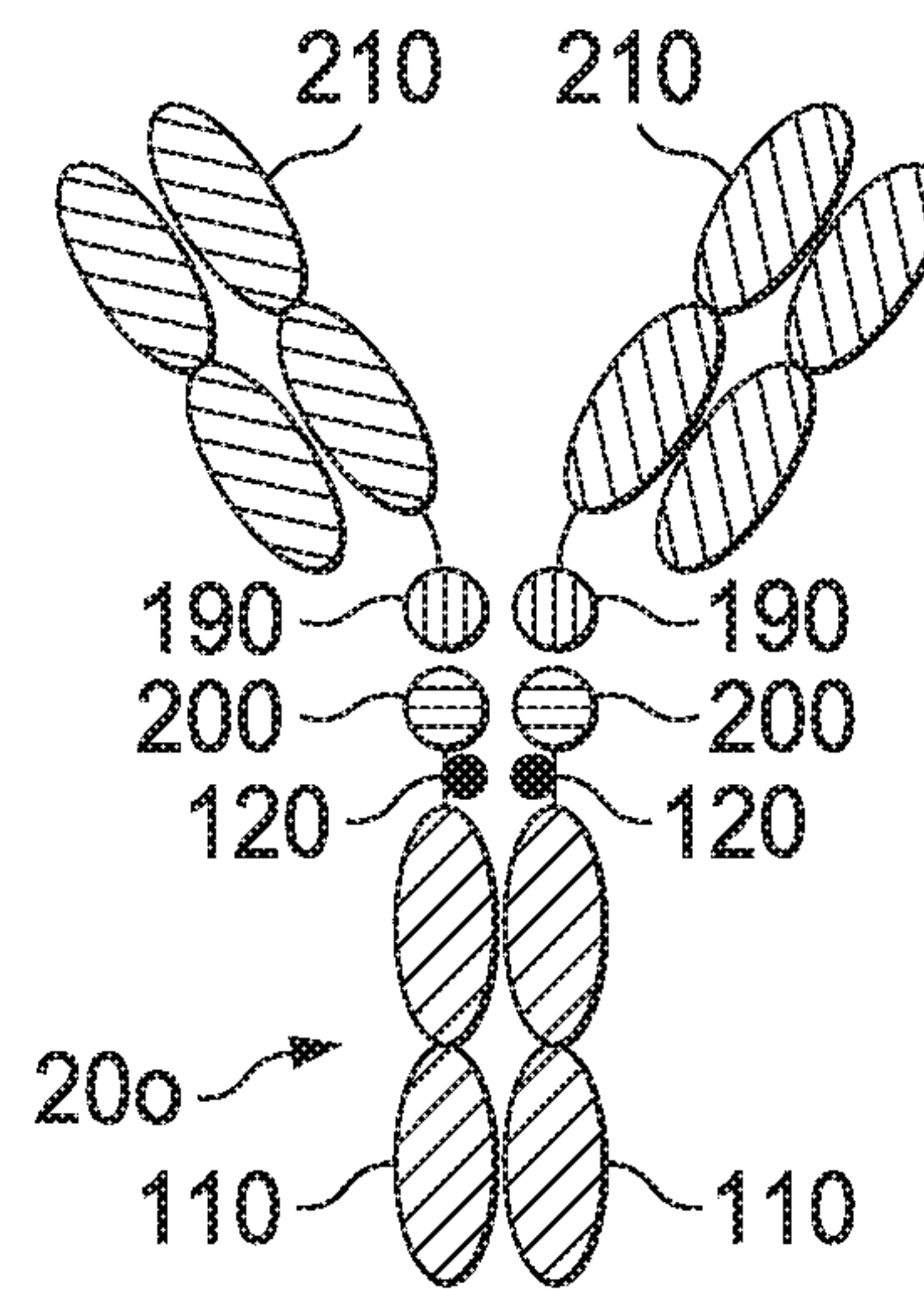


FIG. 3O

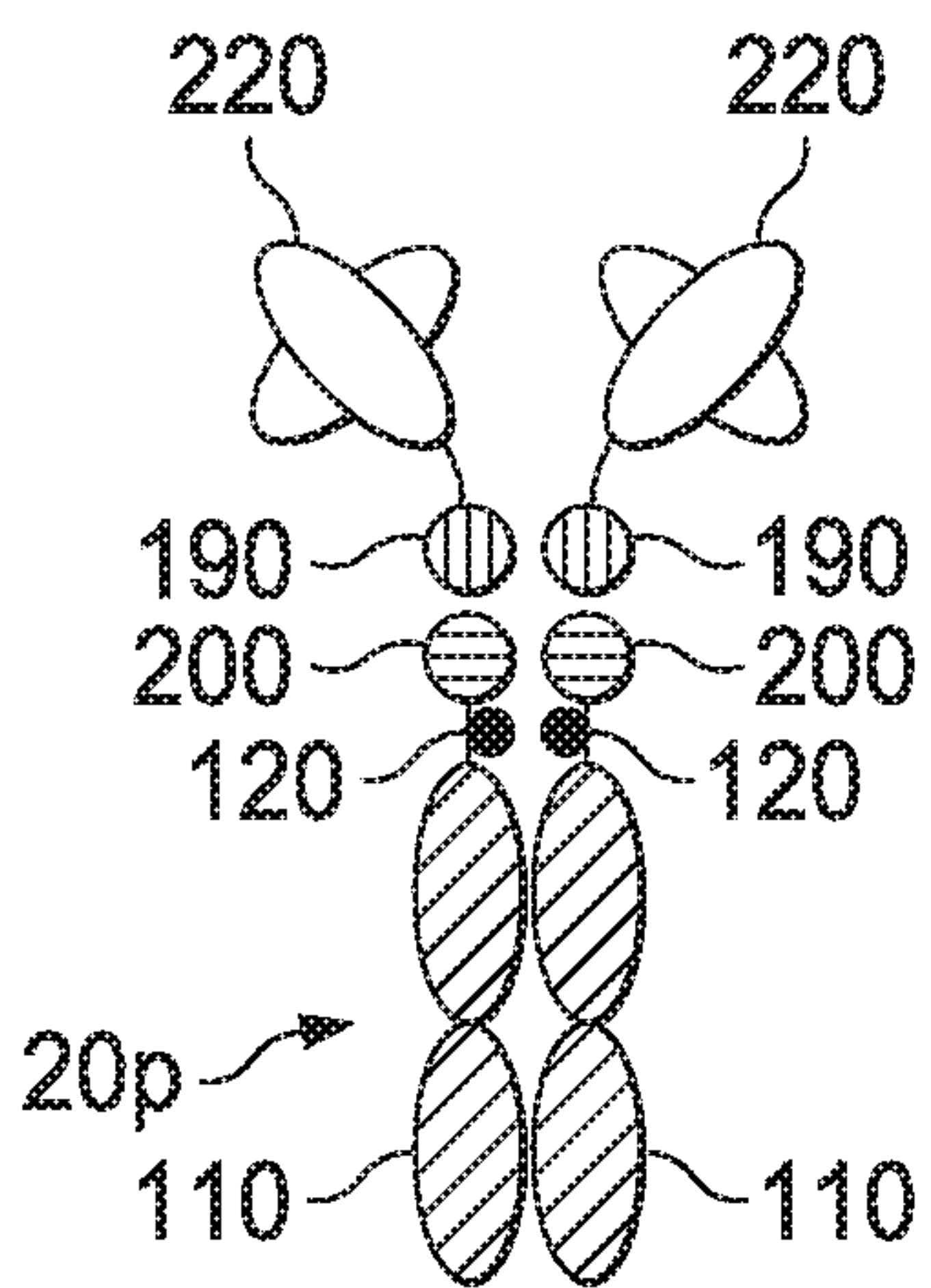


FIG. 3P

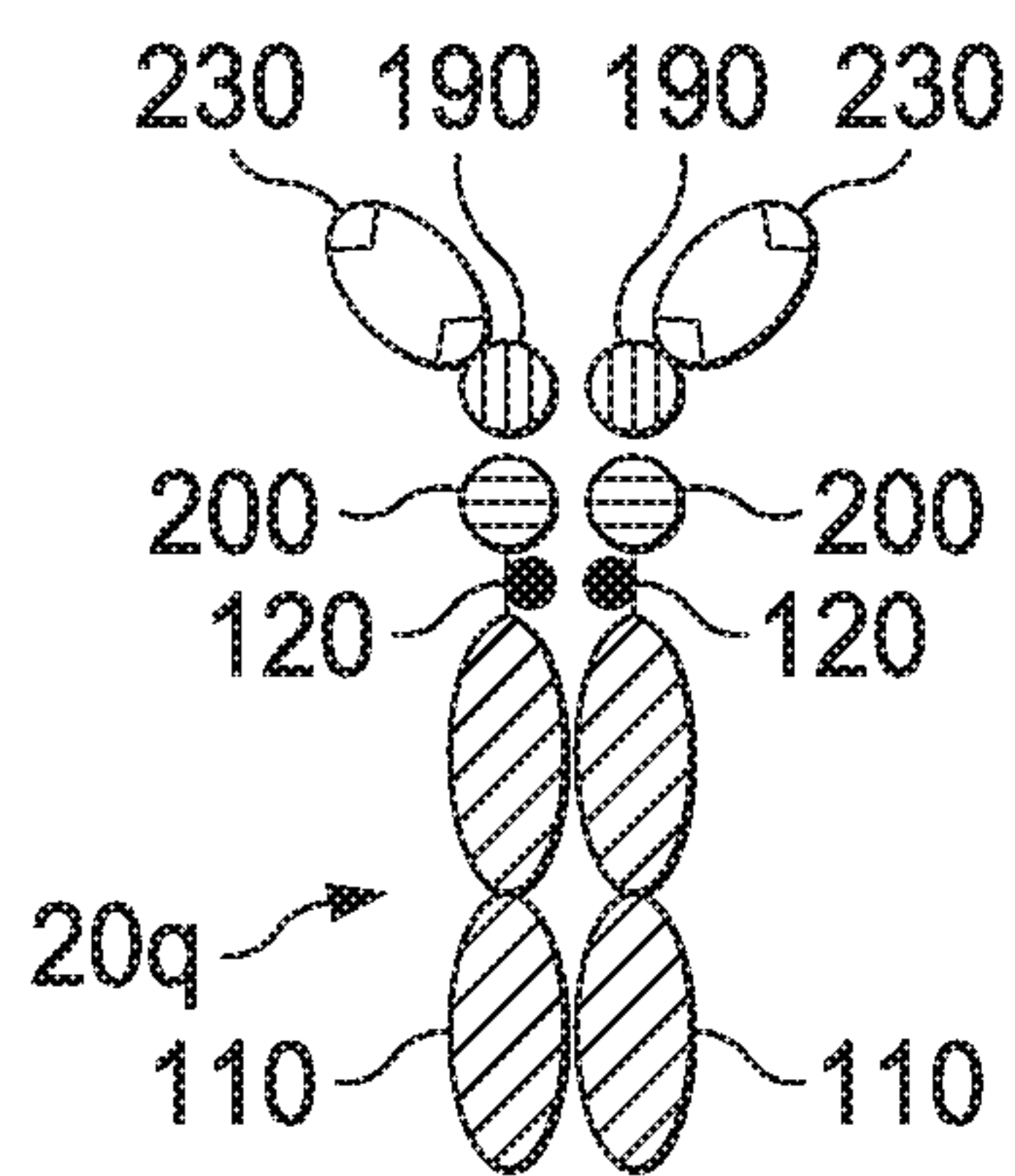


FIG. 3Q

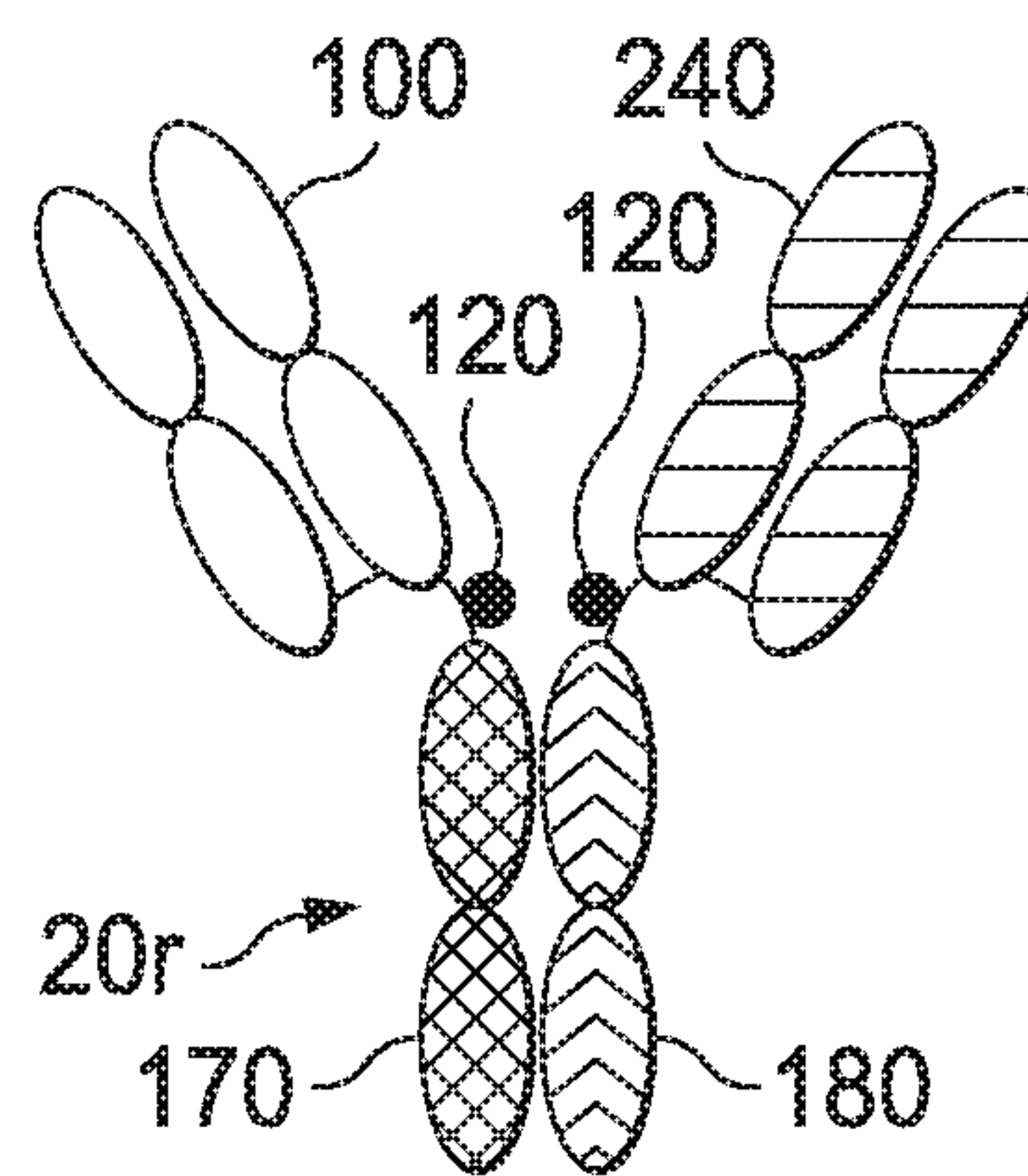


FIG. 3R

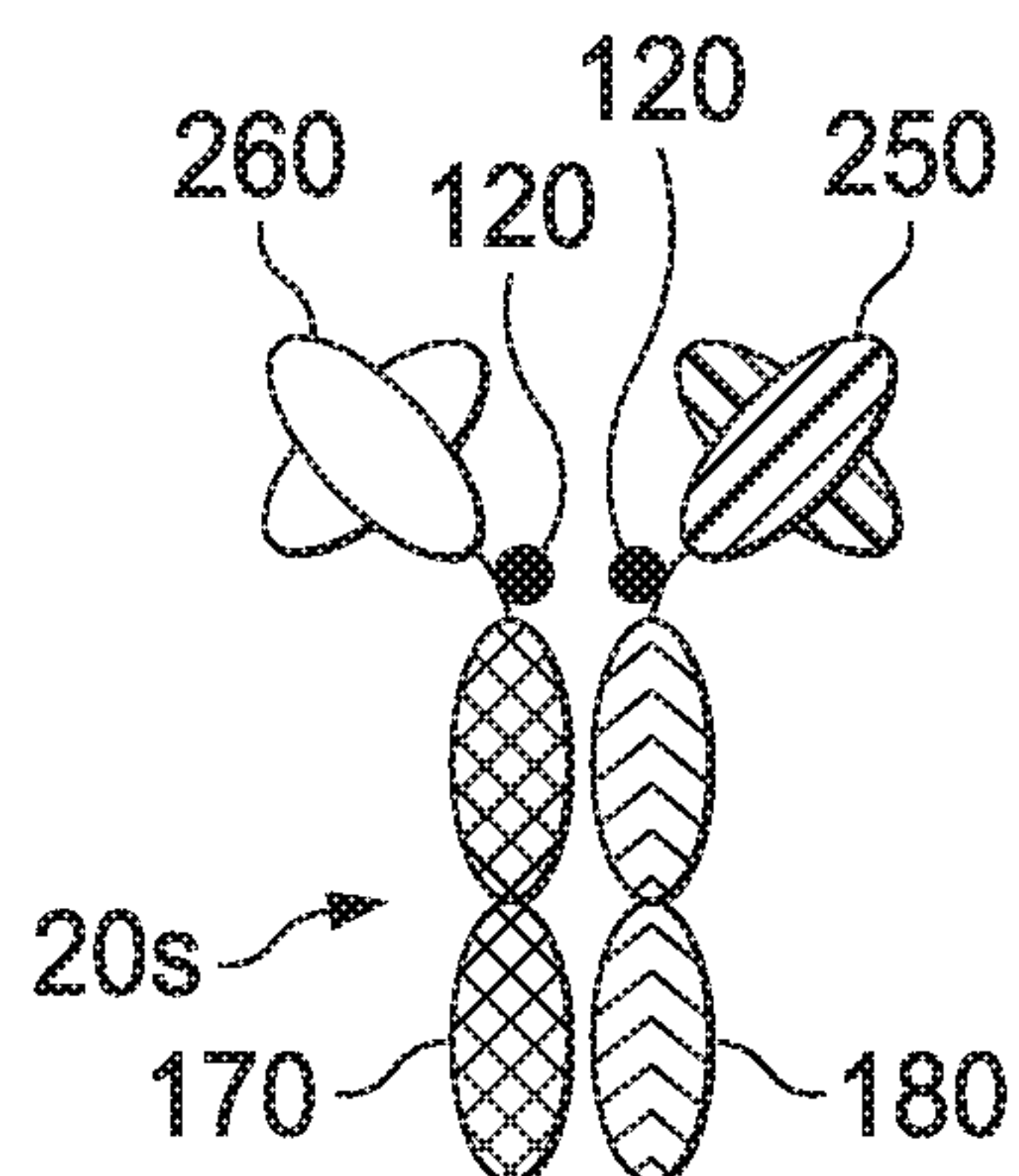


FIG. 3S

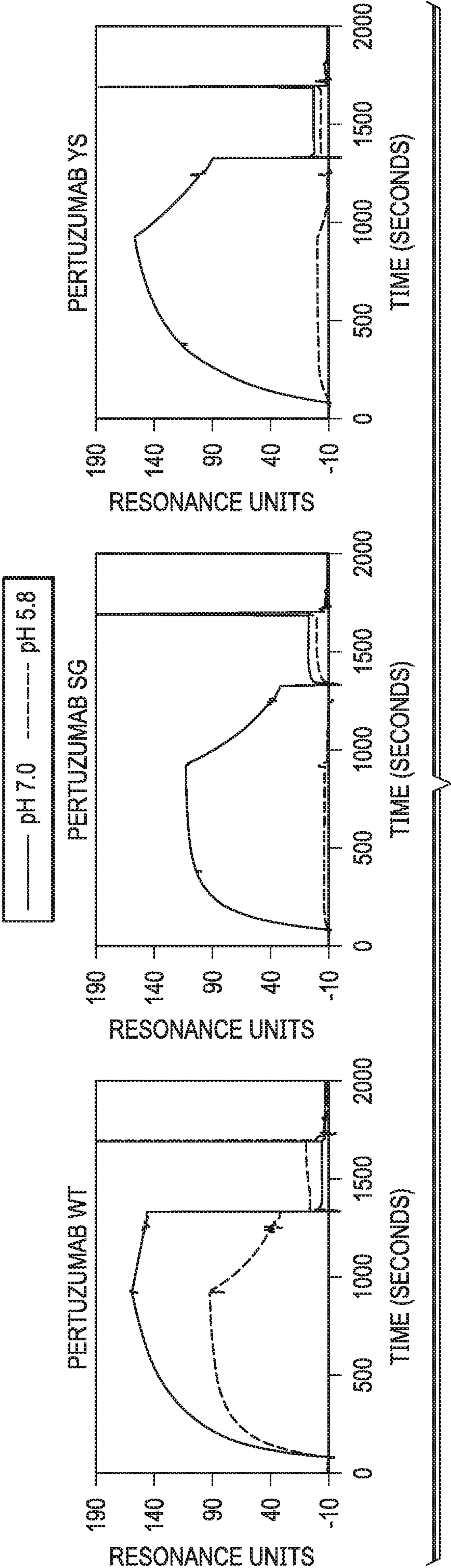


FIG. 4A

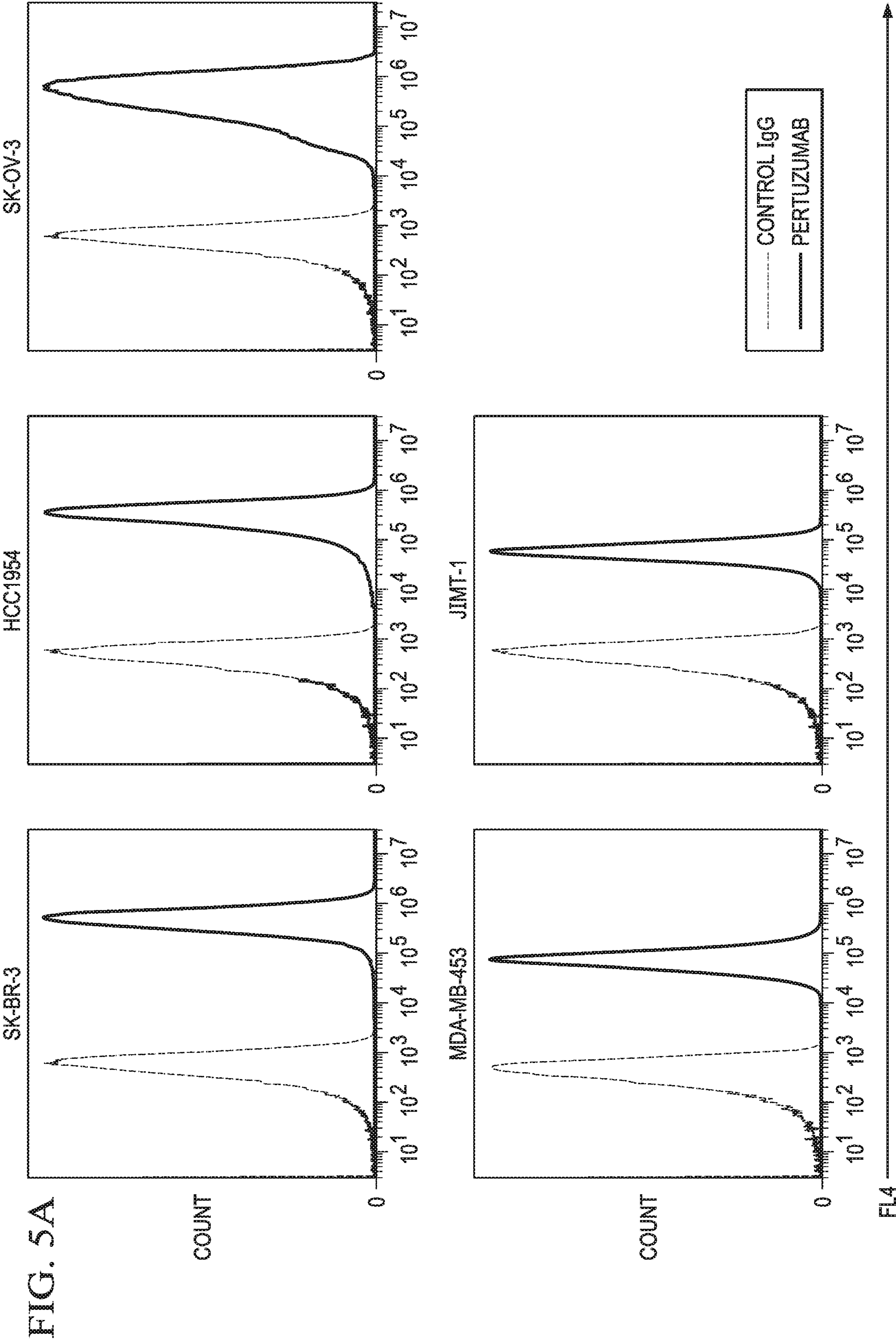
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PERTUZUMAB (WT OR VARIANTS)	pH			
	7.4	7.0	6.5	5.8
WT	2.9	5.9	27.2	137.6
SG	8.2	46.4	150.1	N.D.*
YS	6.3	22.1	67.9	1627.2

*N.D. INDICATES AFFINITY TOO LOW TO DETERMINE AN ACCURATE DISSOCIATION CONSTANT.

FIG. 4B

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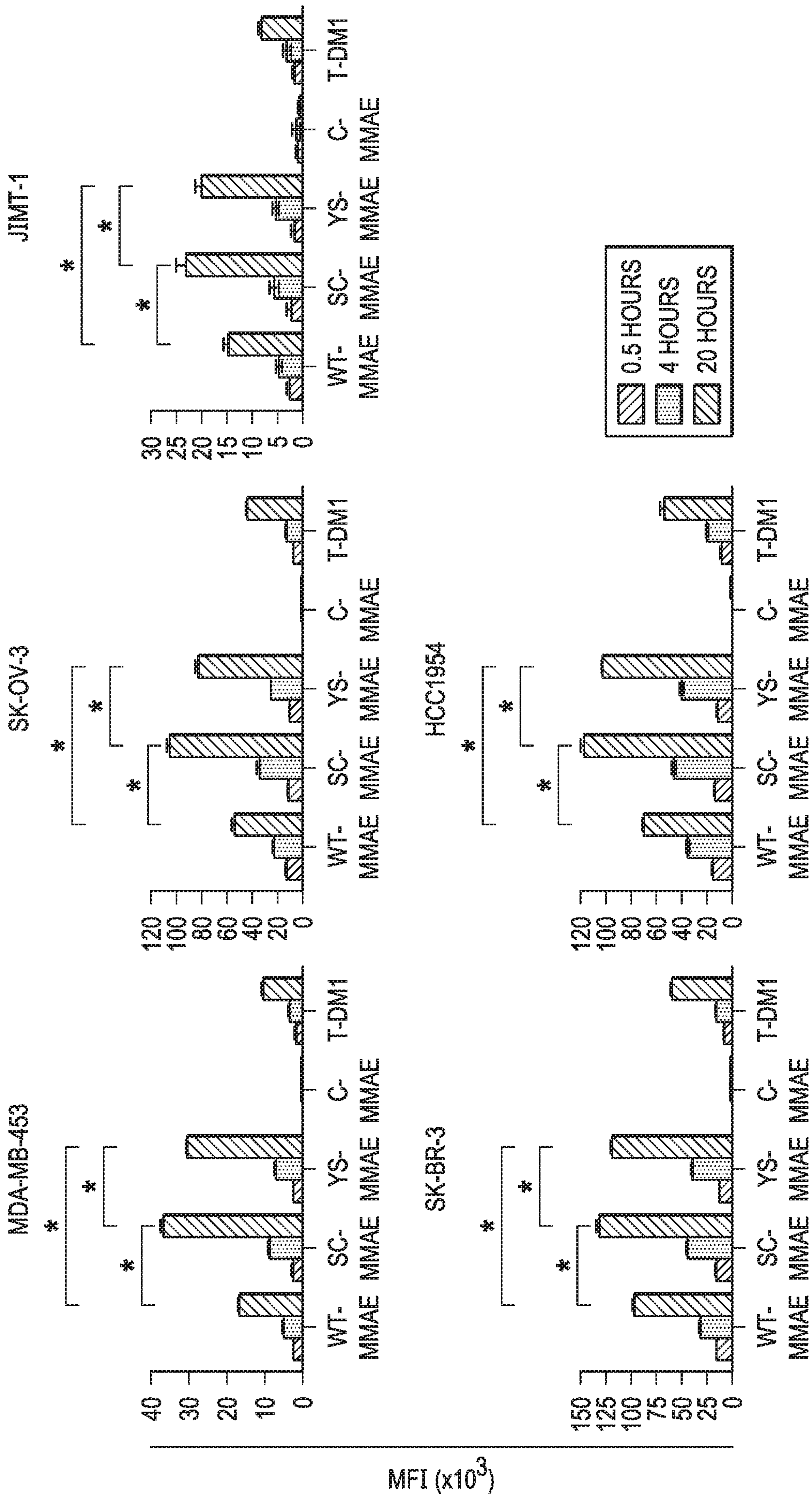


FIG. 5B

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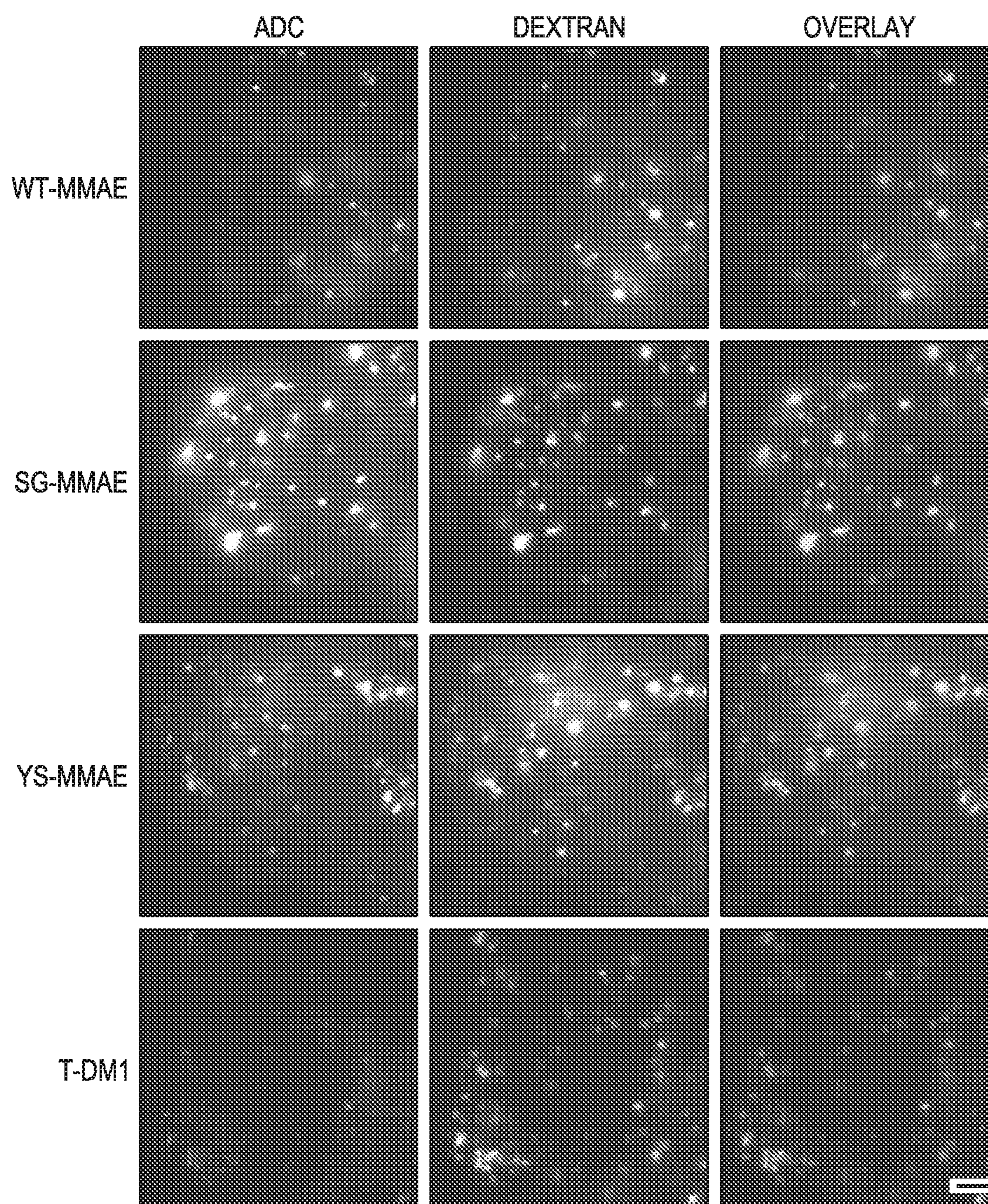


FIG. 5C

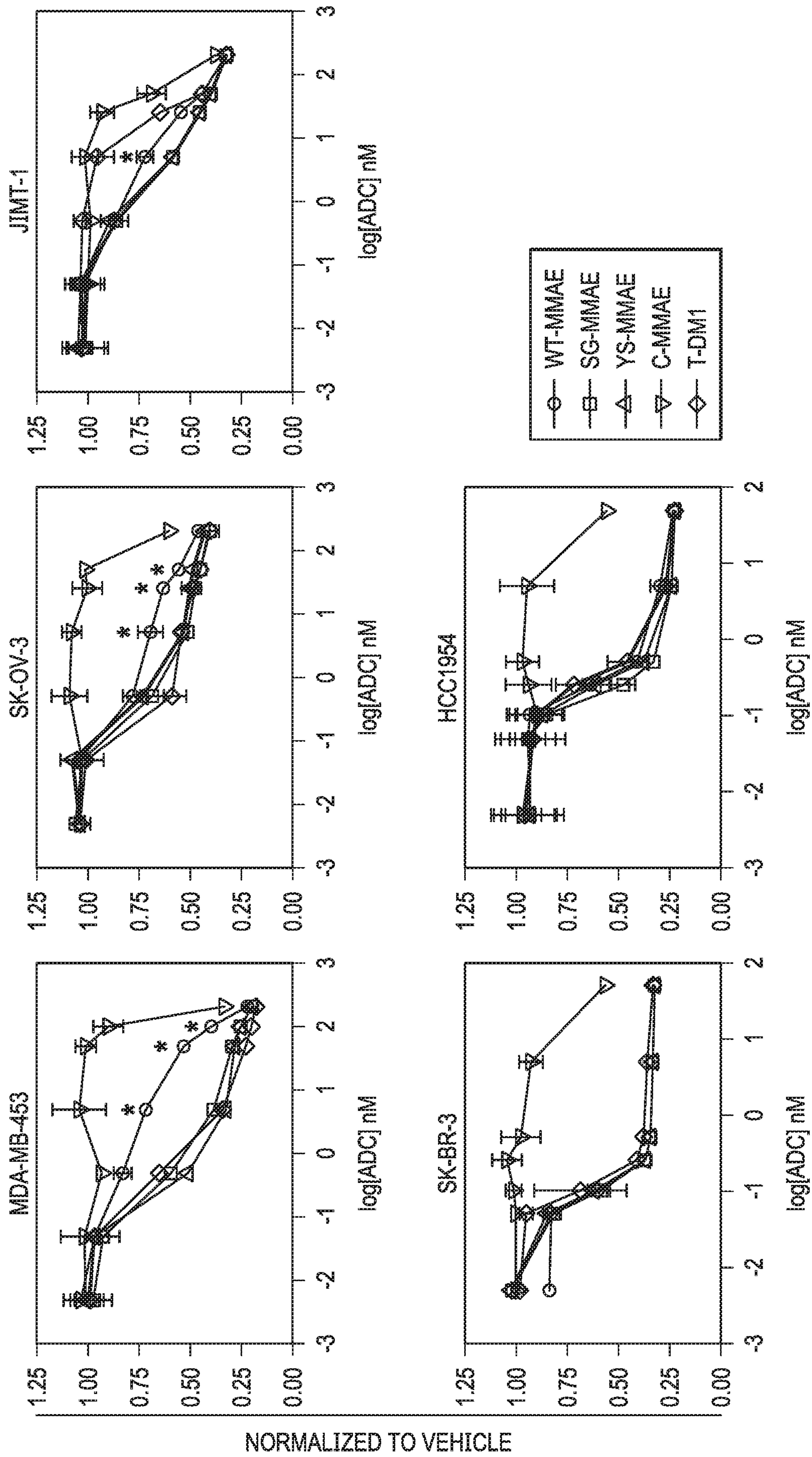


FIG. 6

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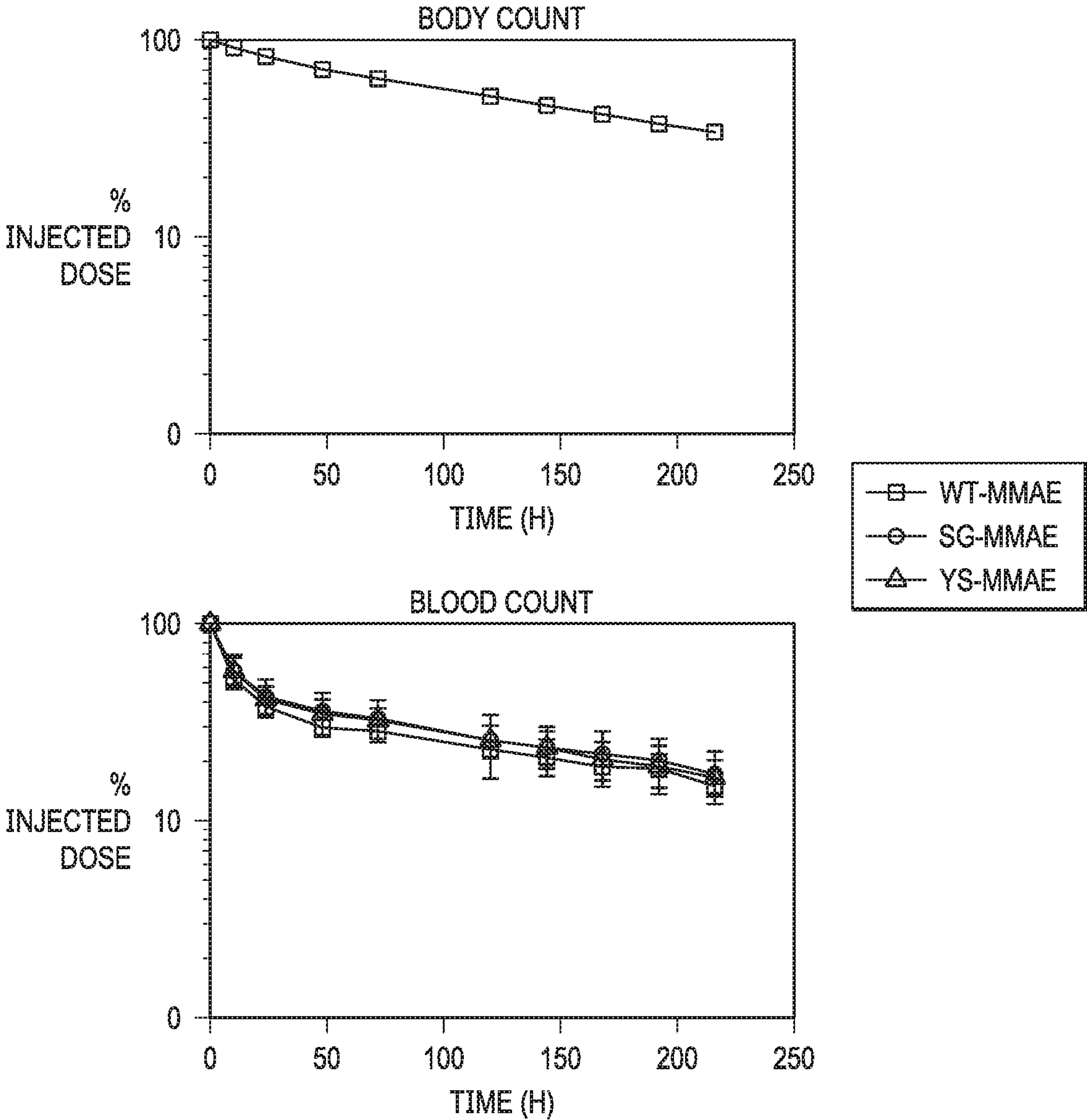


FIG. 7A

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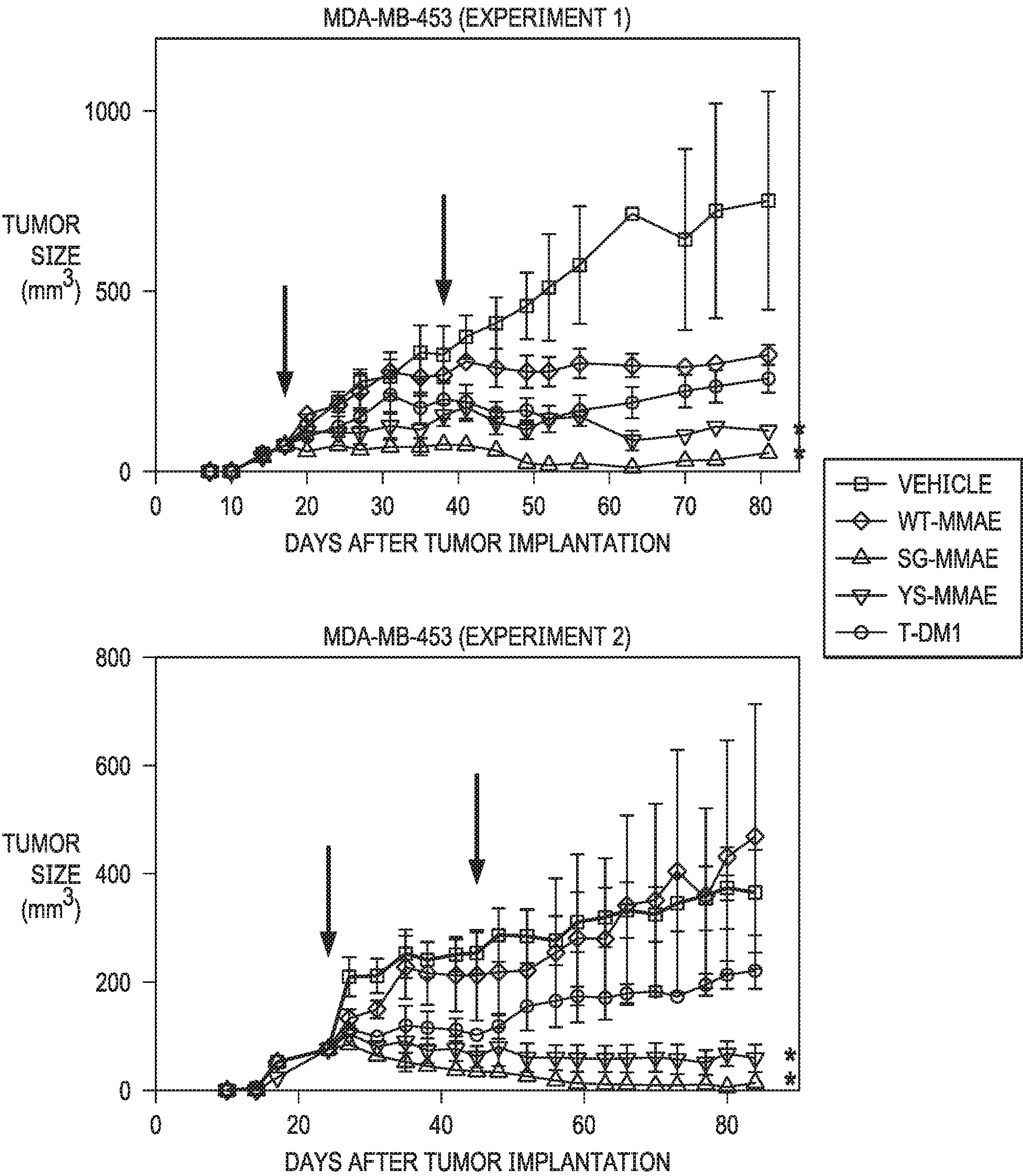


FIG. 7B

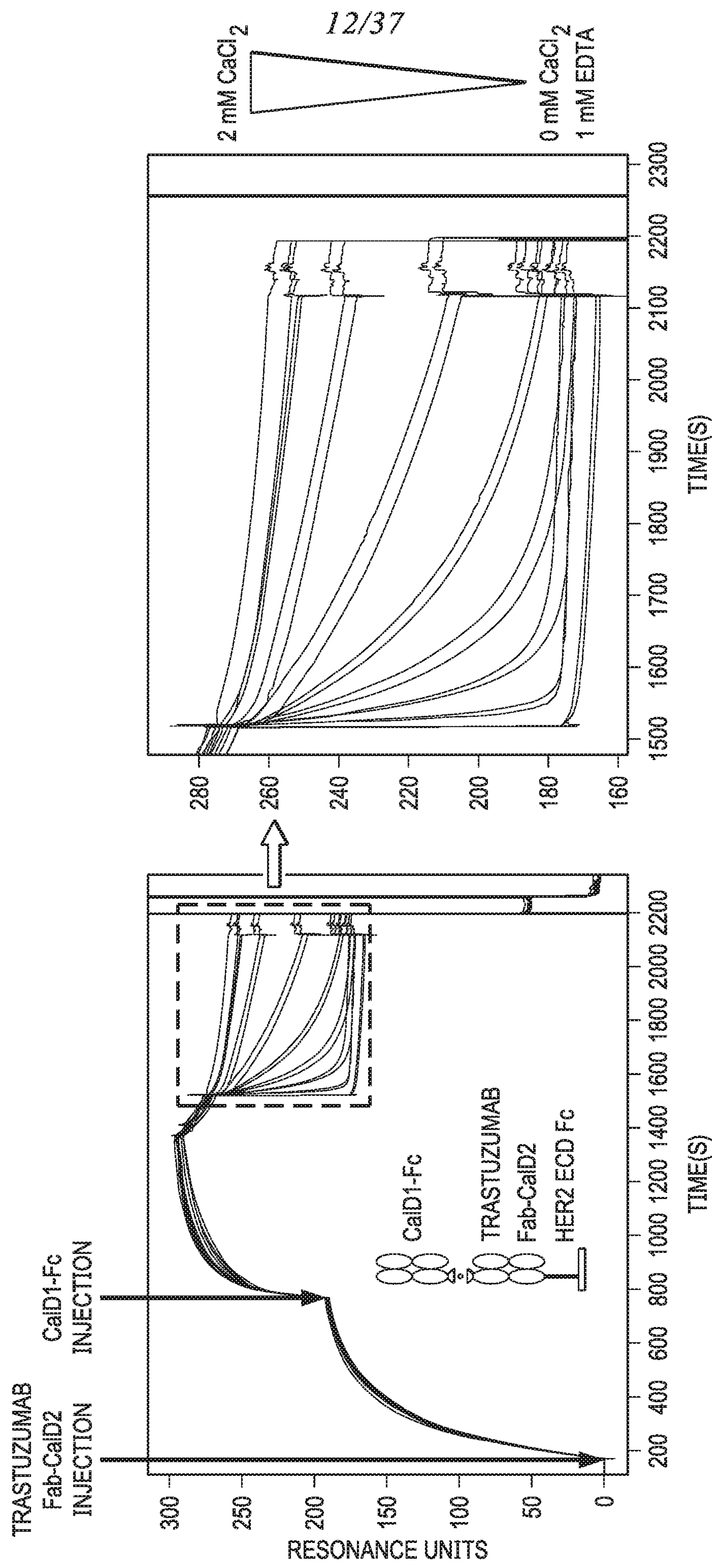


FIG. 8A

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FIG. 8B

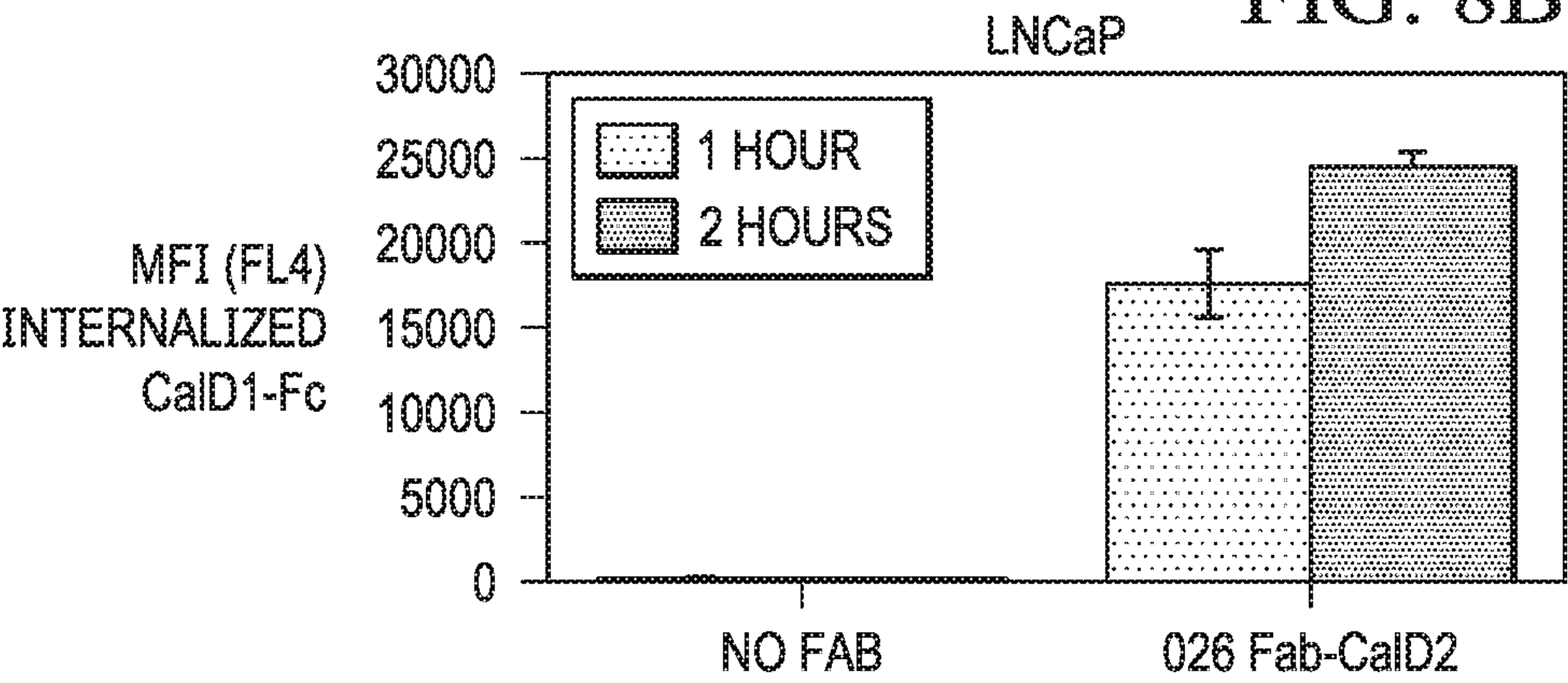
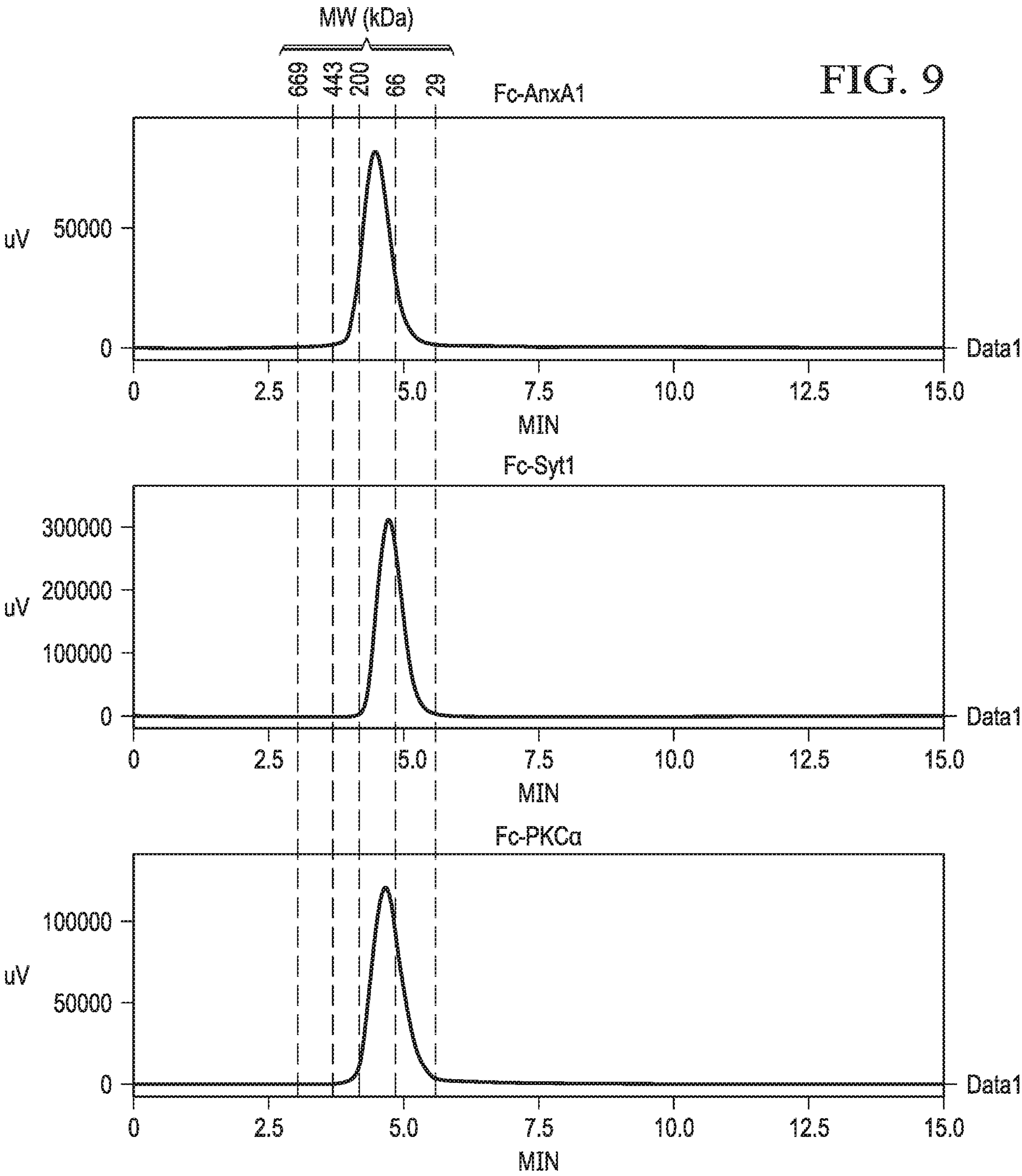


FIG. 9



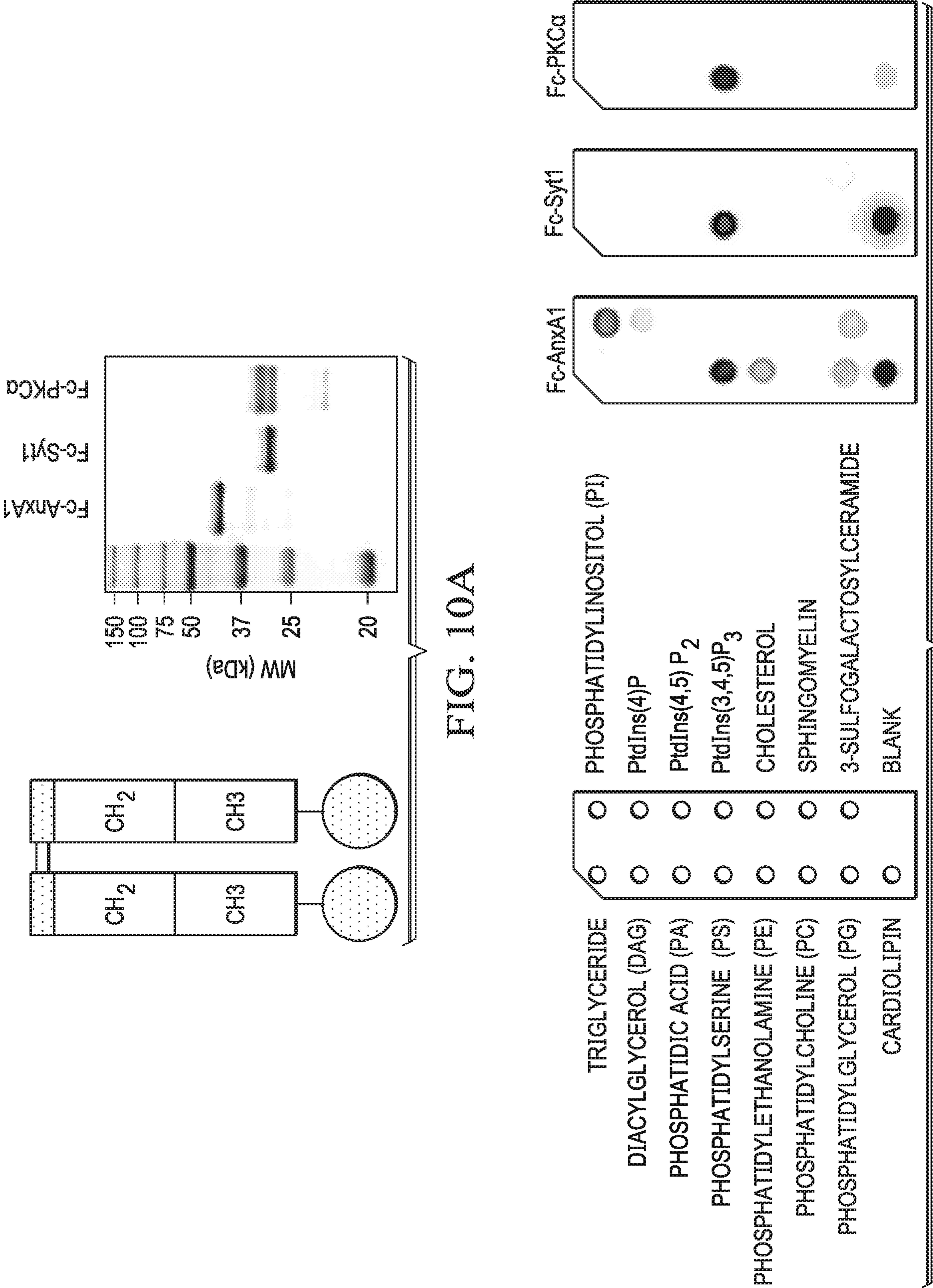
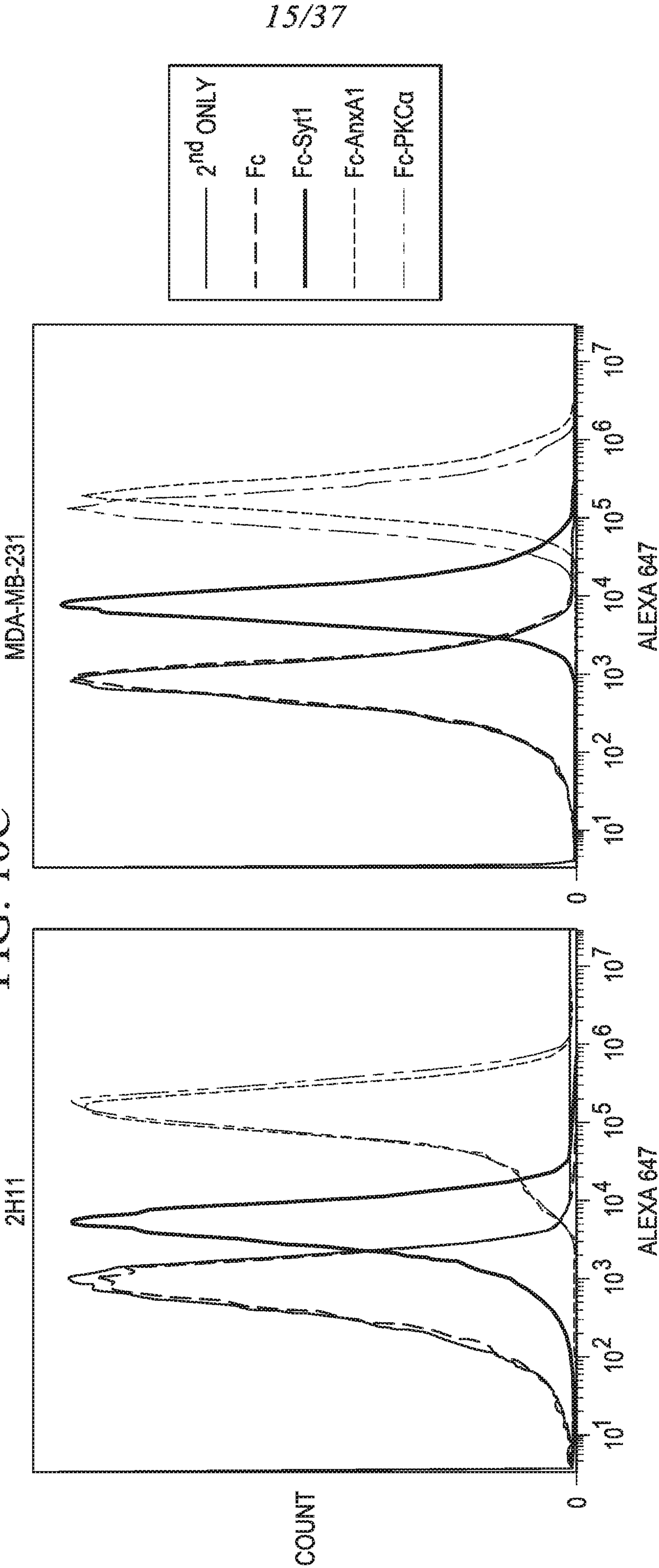


FIG. 10C



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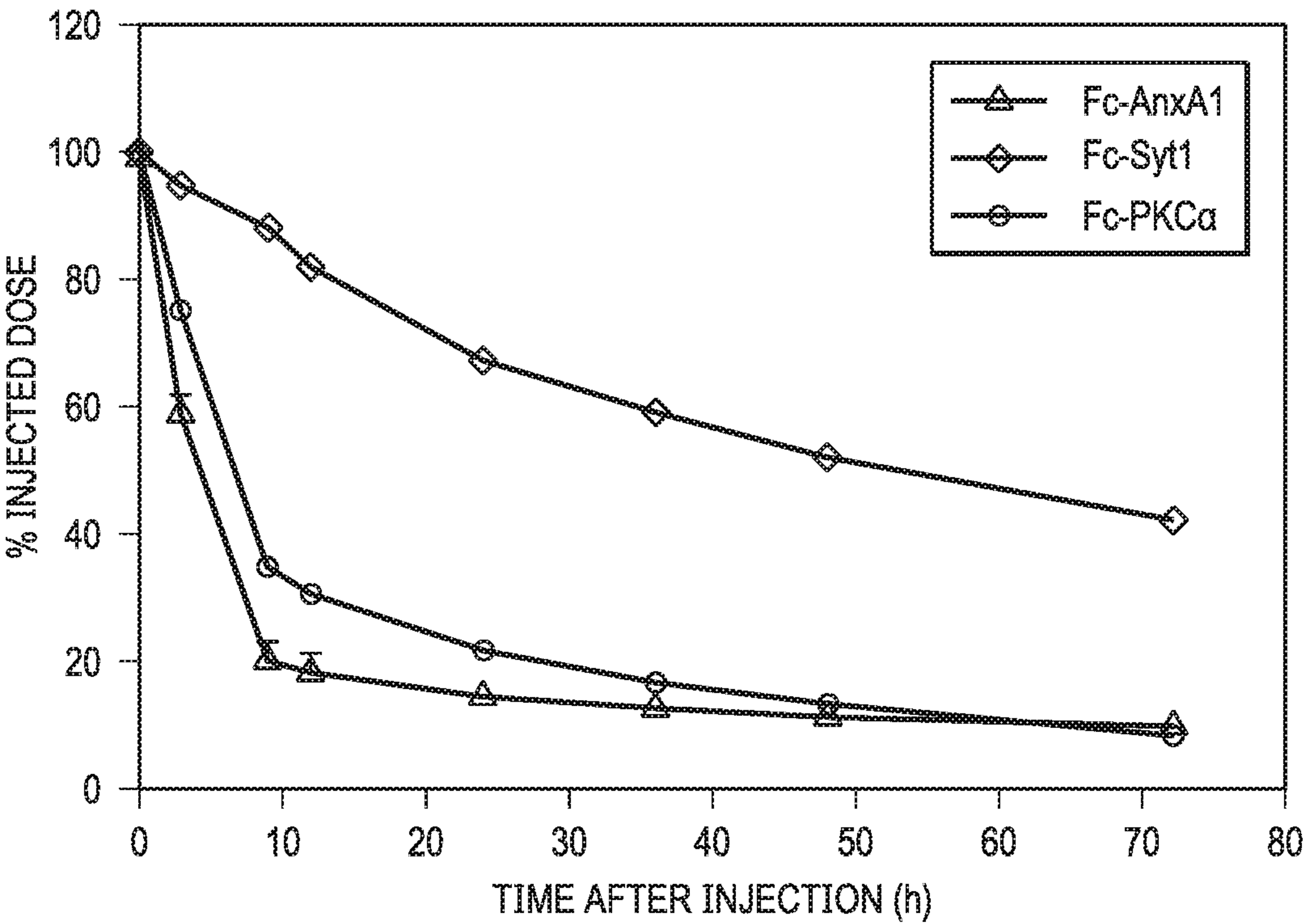


FIG. 10D

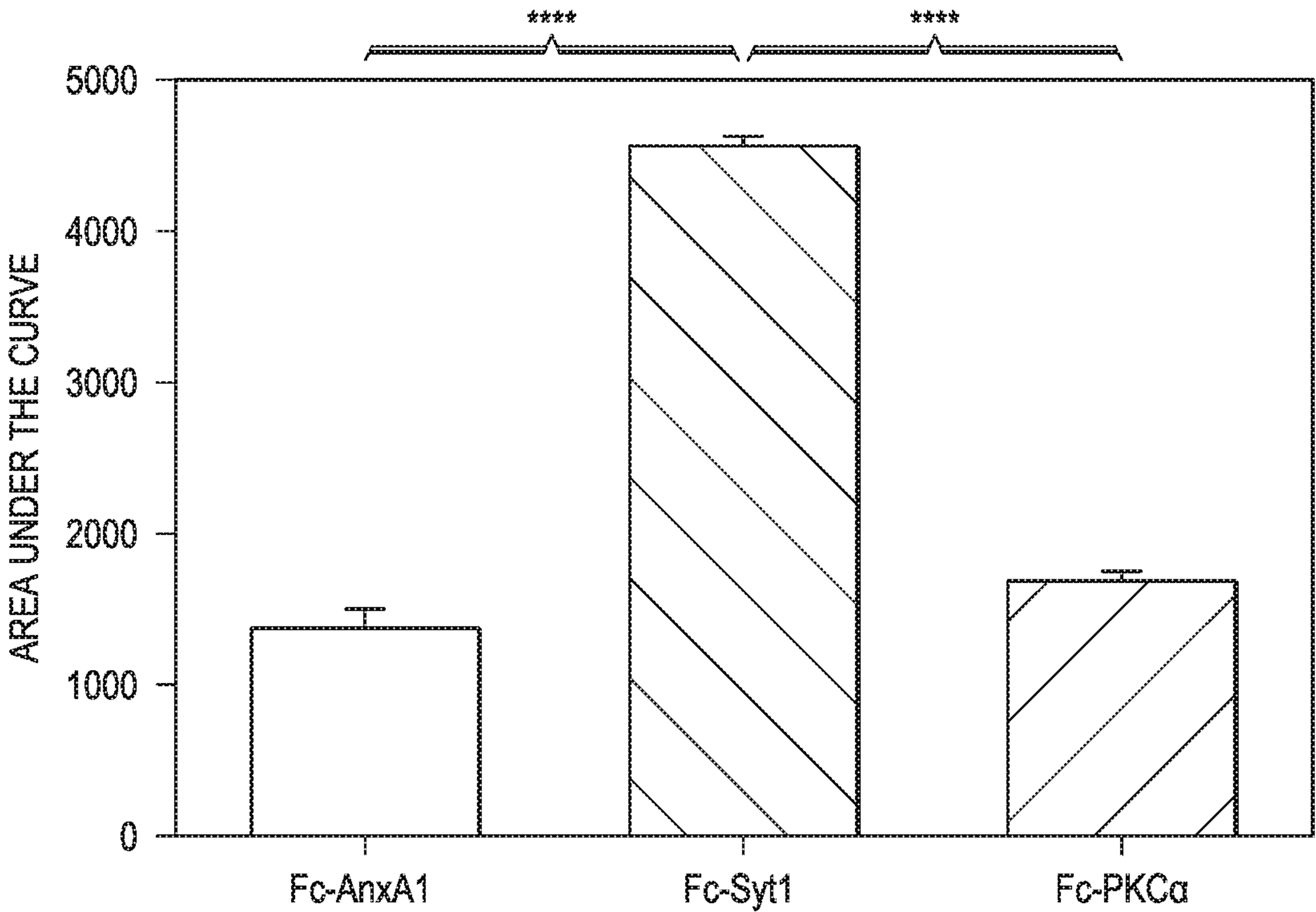


FIG. 10E

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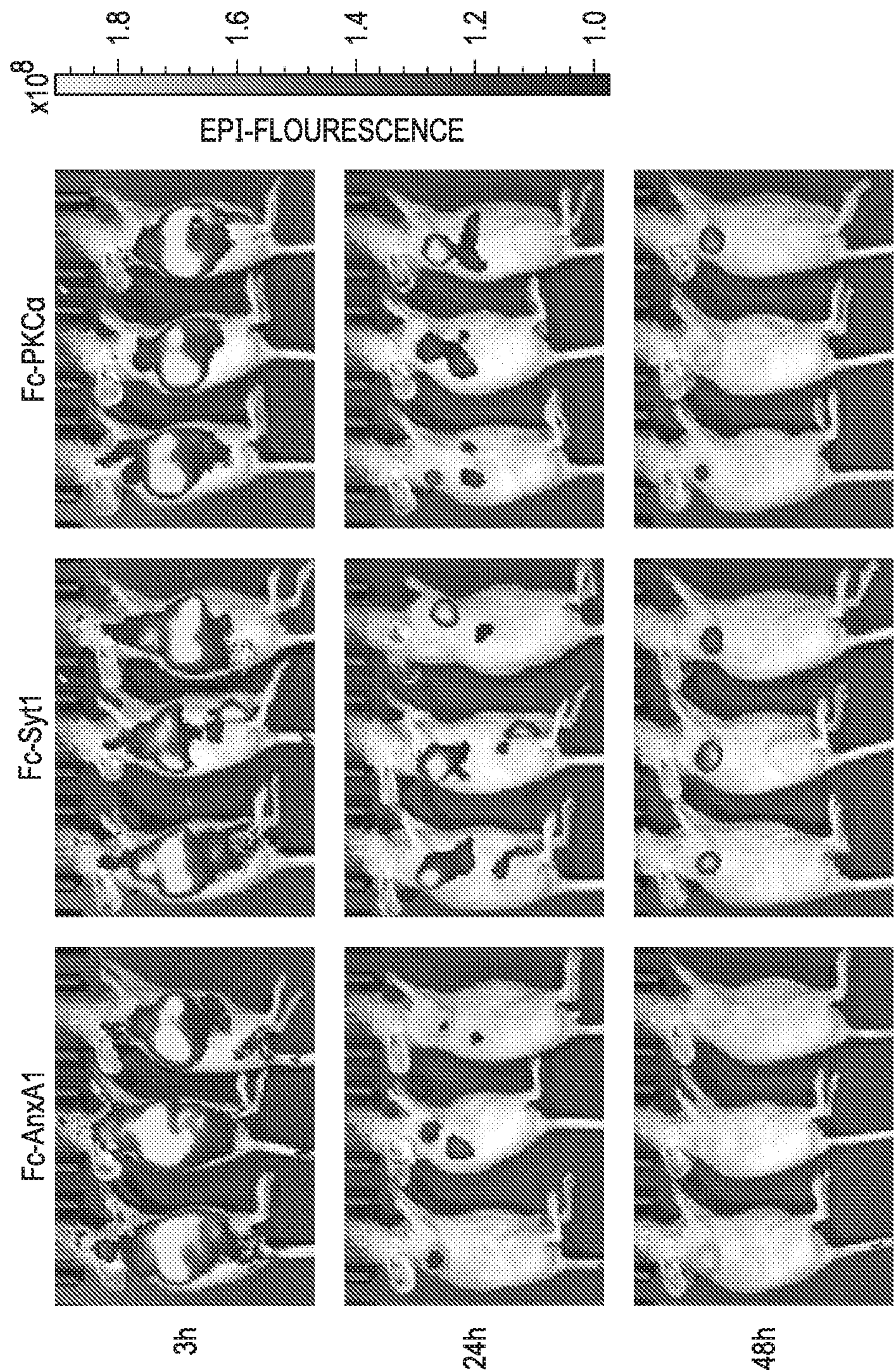
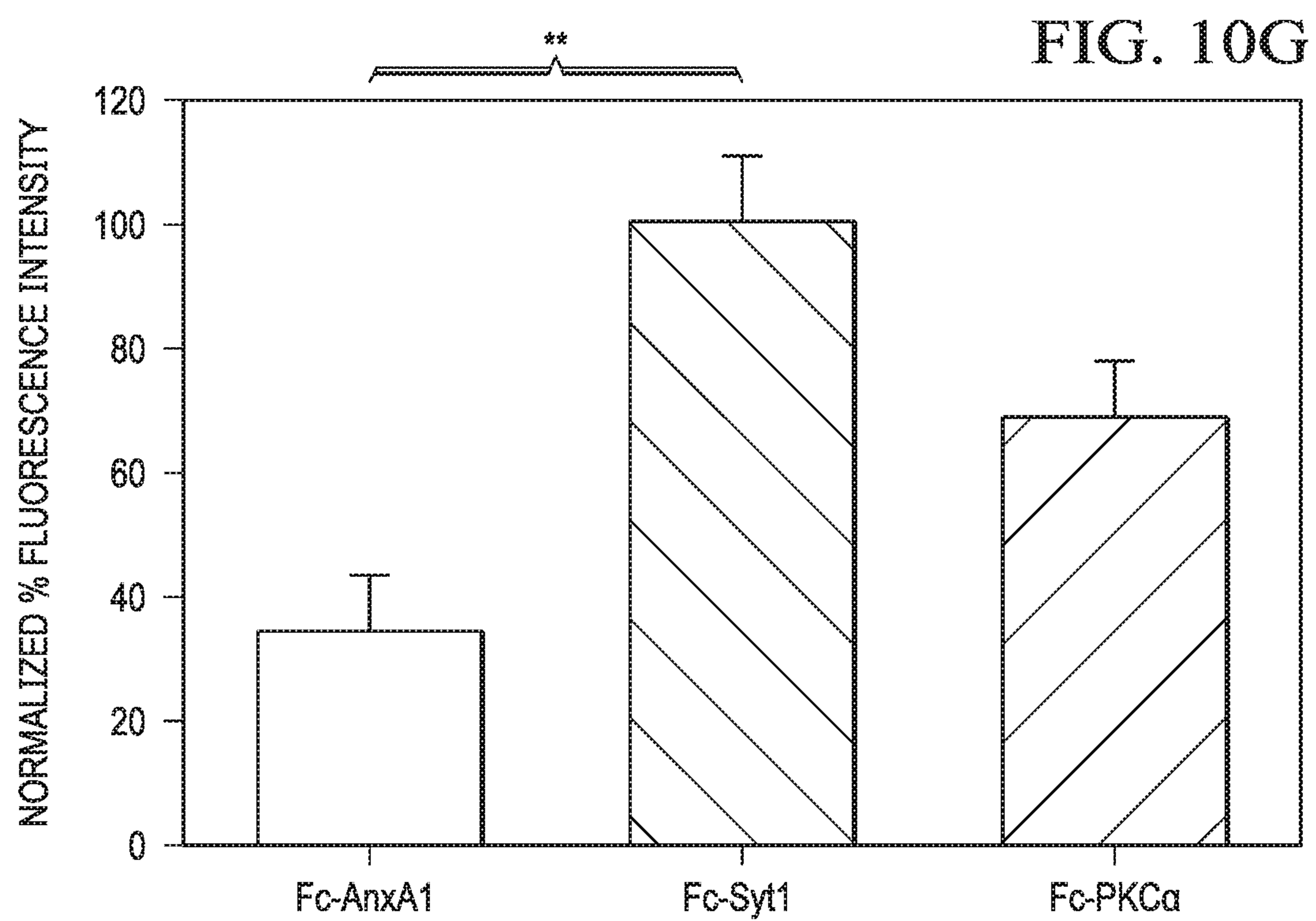


FIG. 10F

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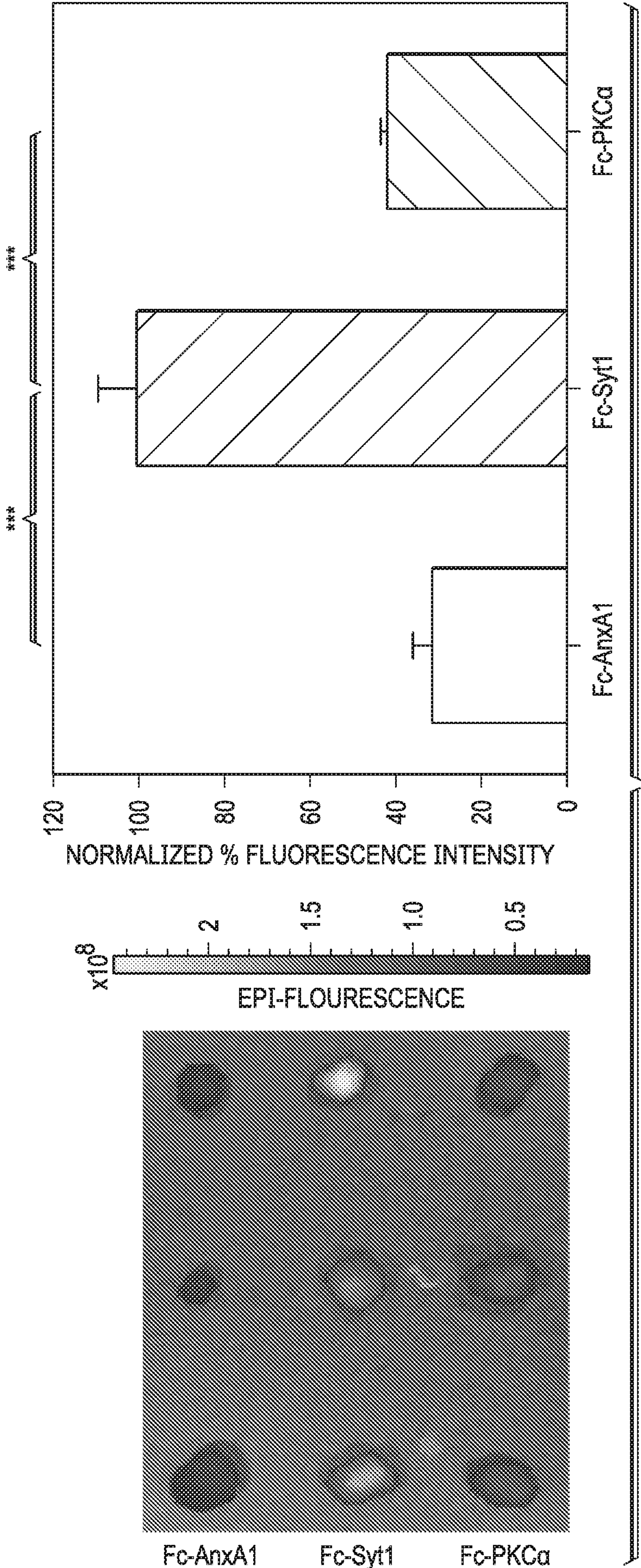


FIG. 10H

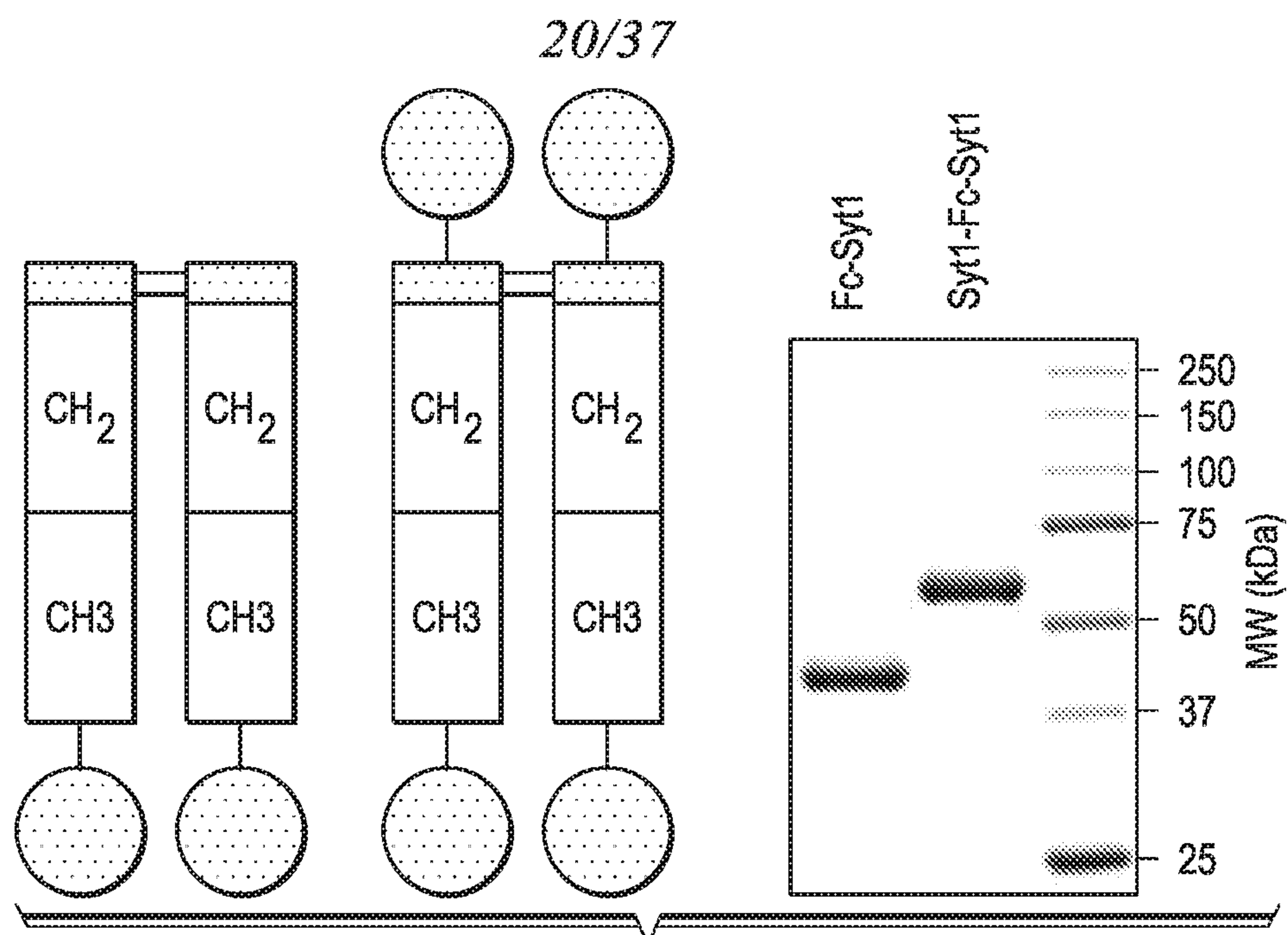


FIG. 11A

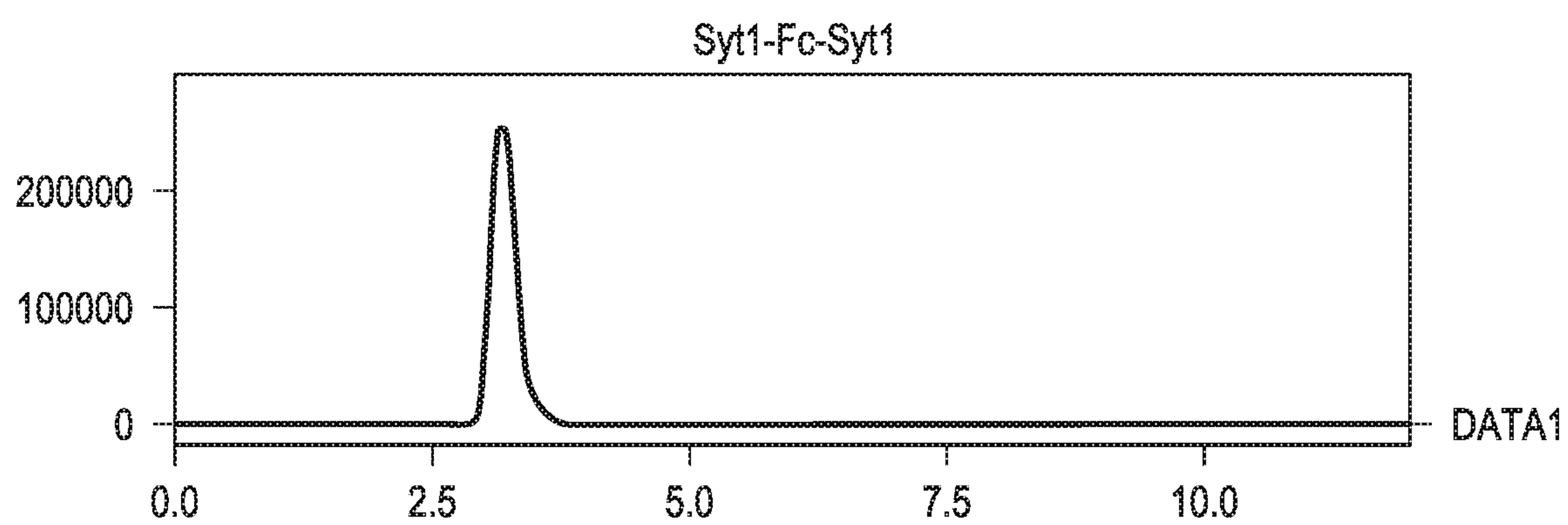


FIG. 11B

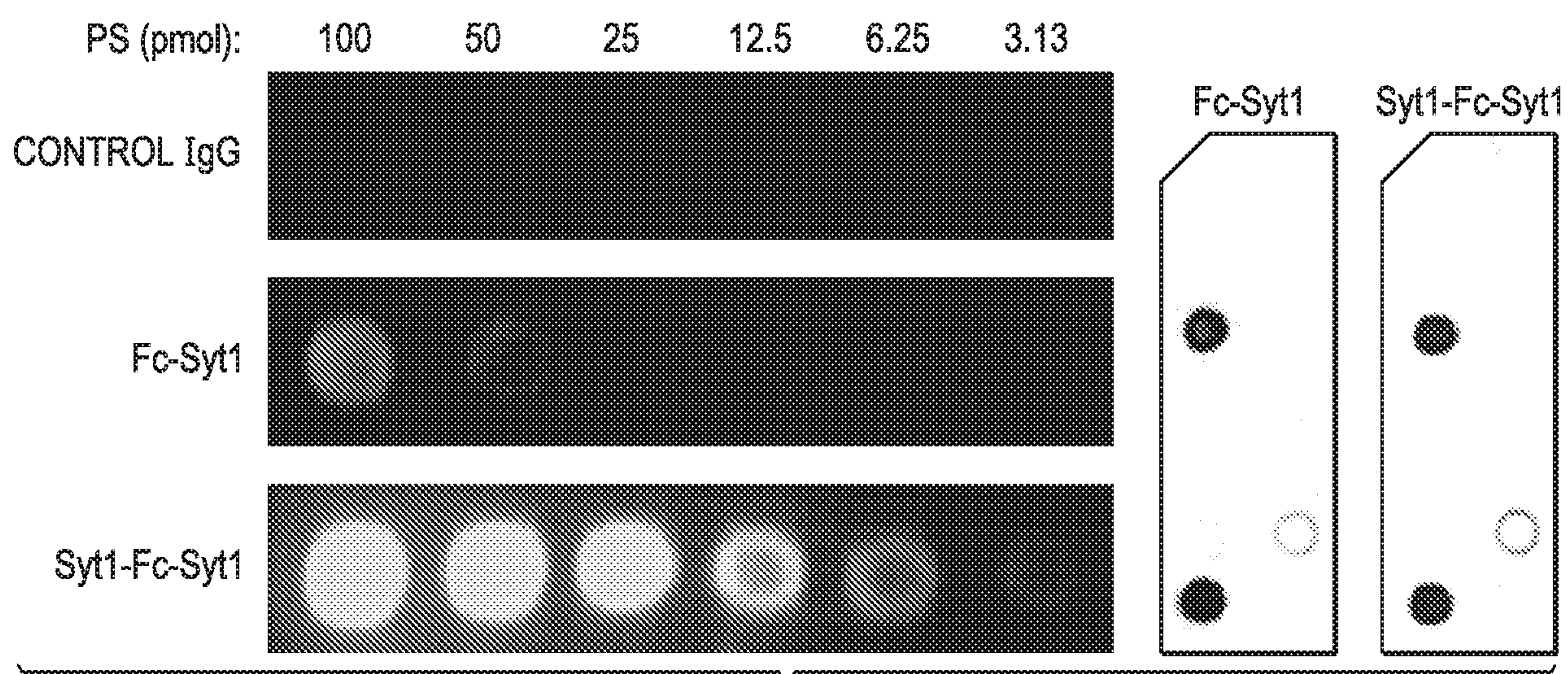


FIG. 11C

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FIG. 11D

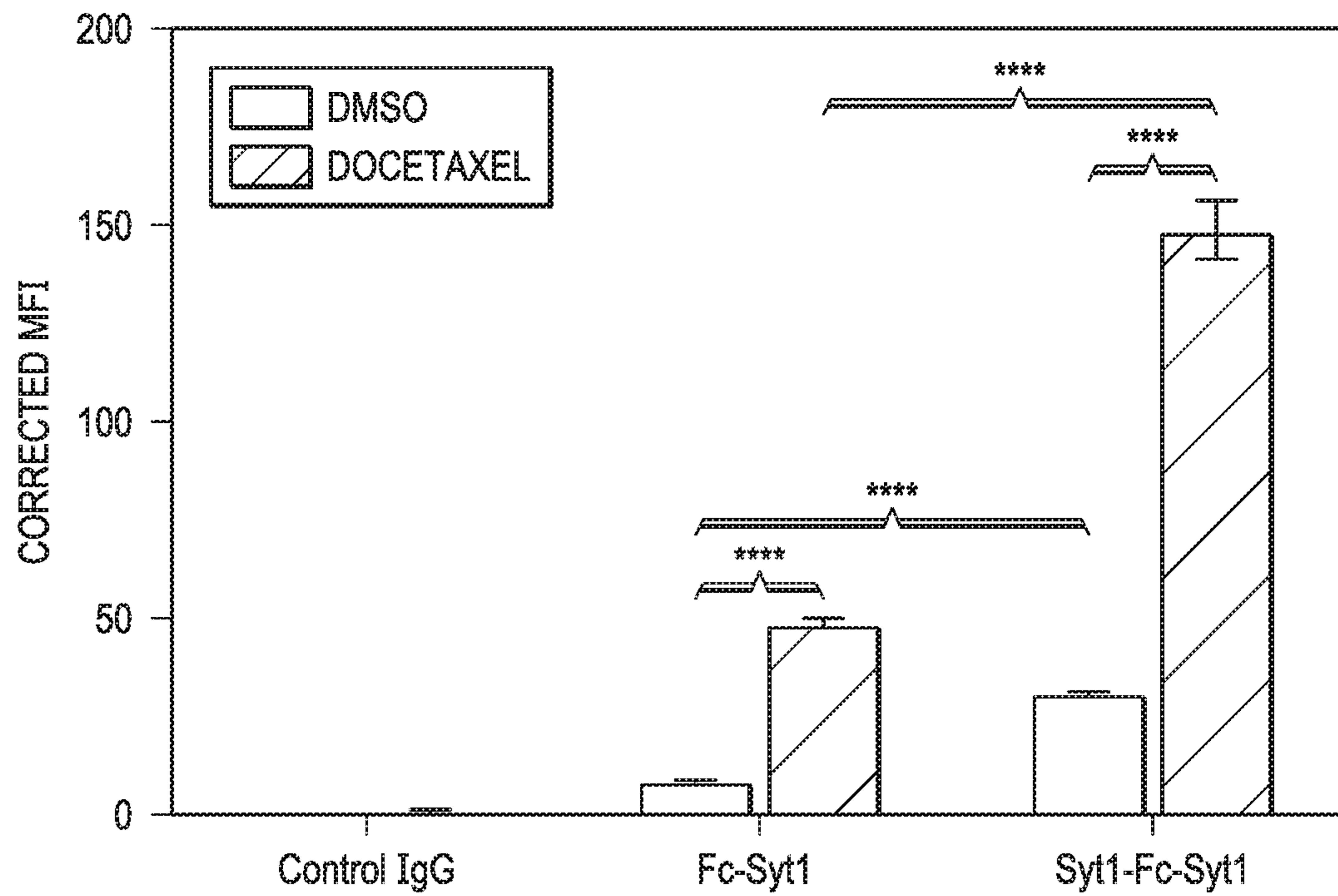
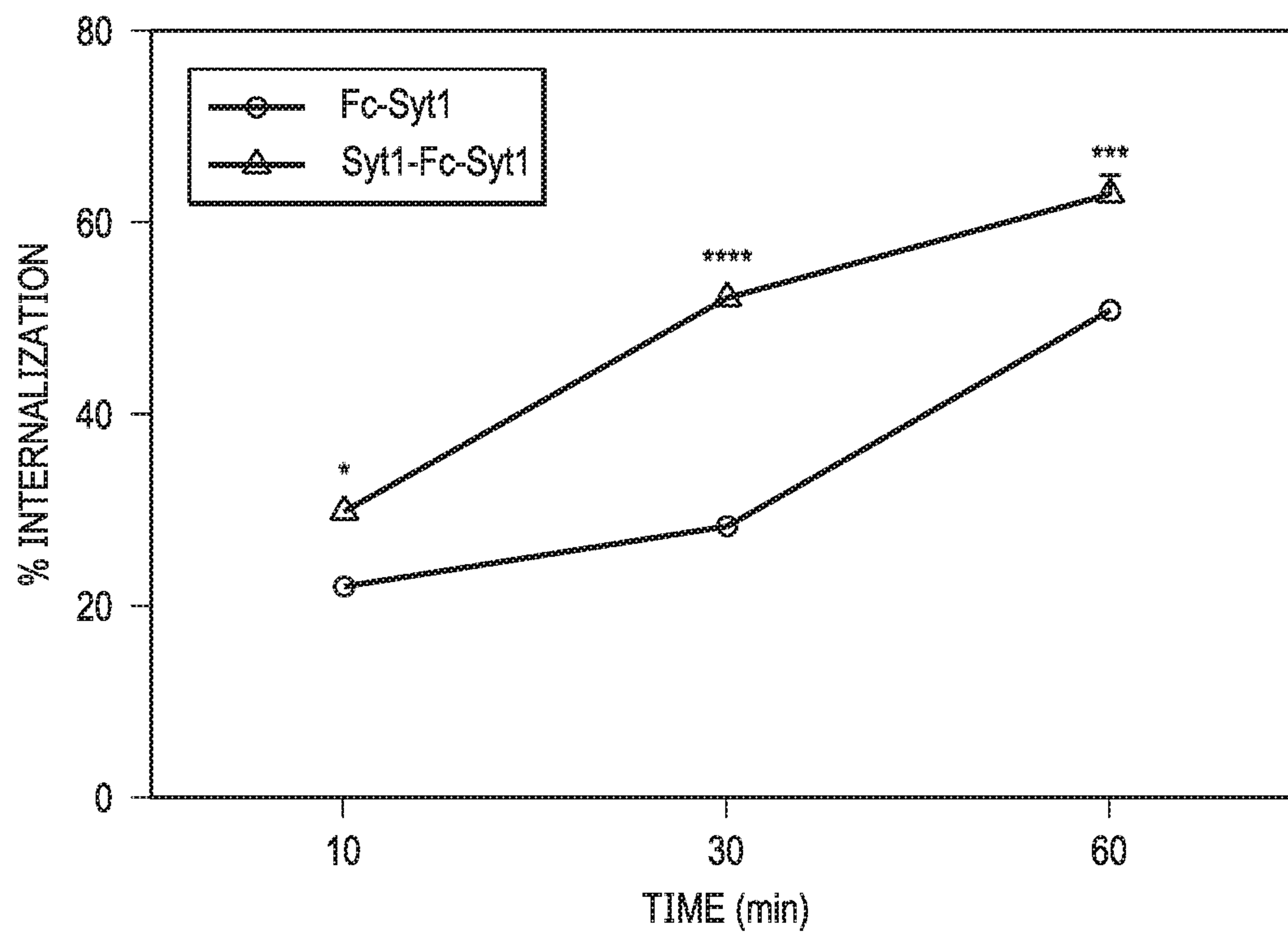


FIG. 11E



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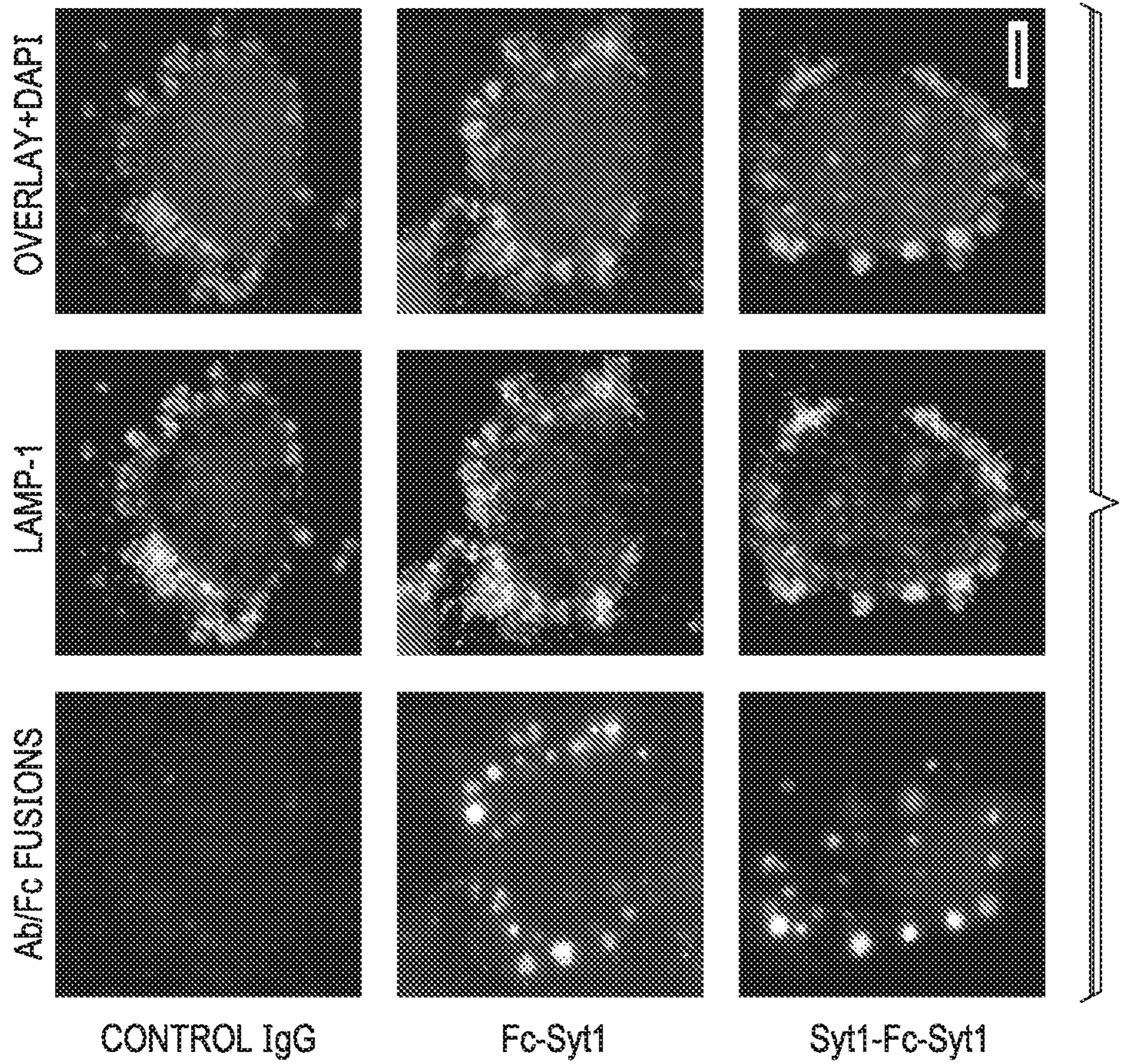


FIG. 11G

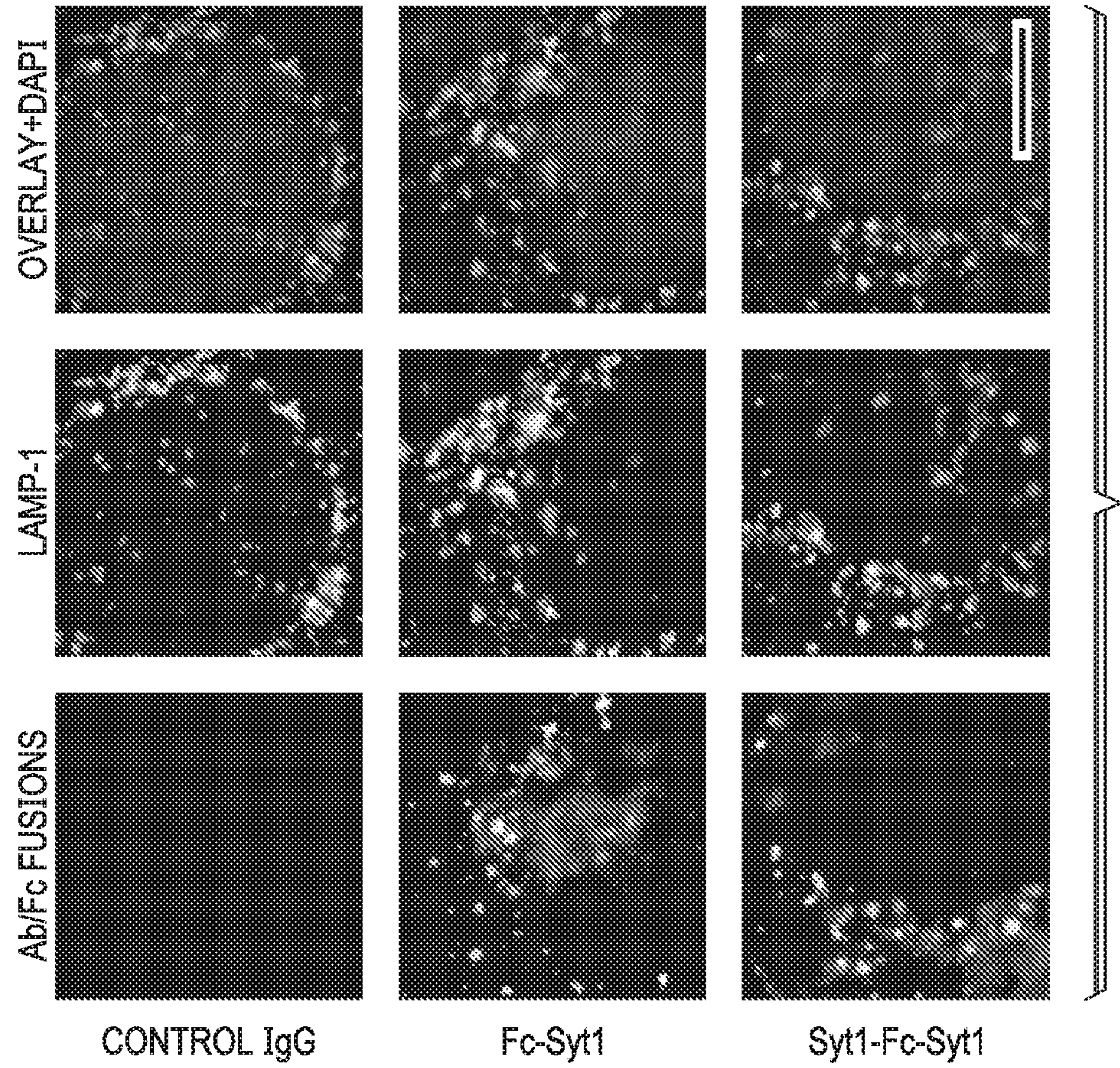


FIG. 11F

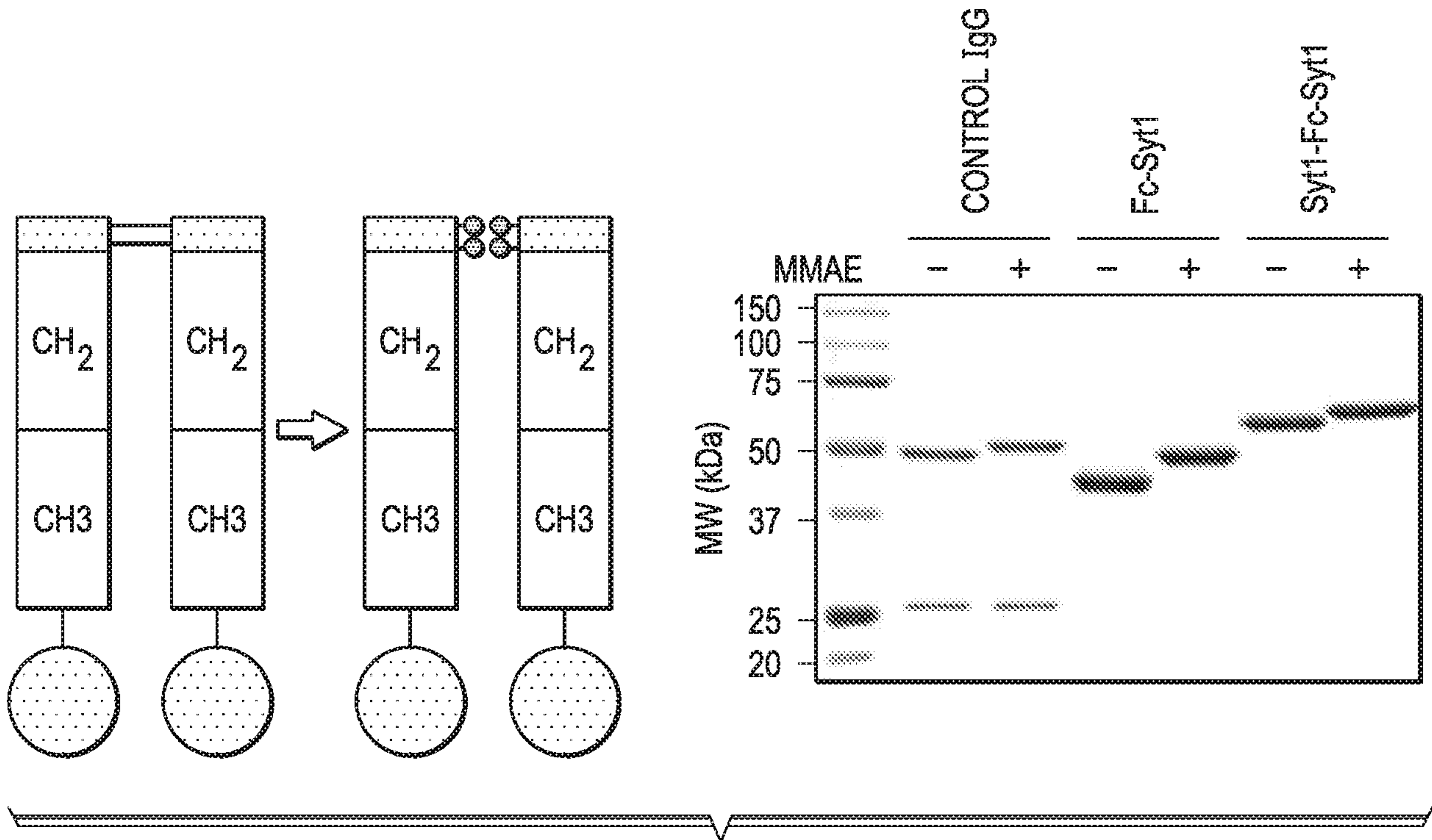


FIG. 12A

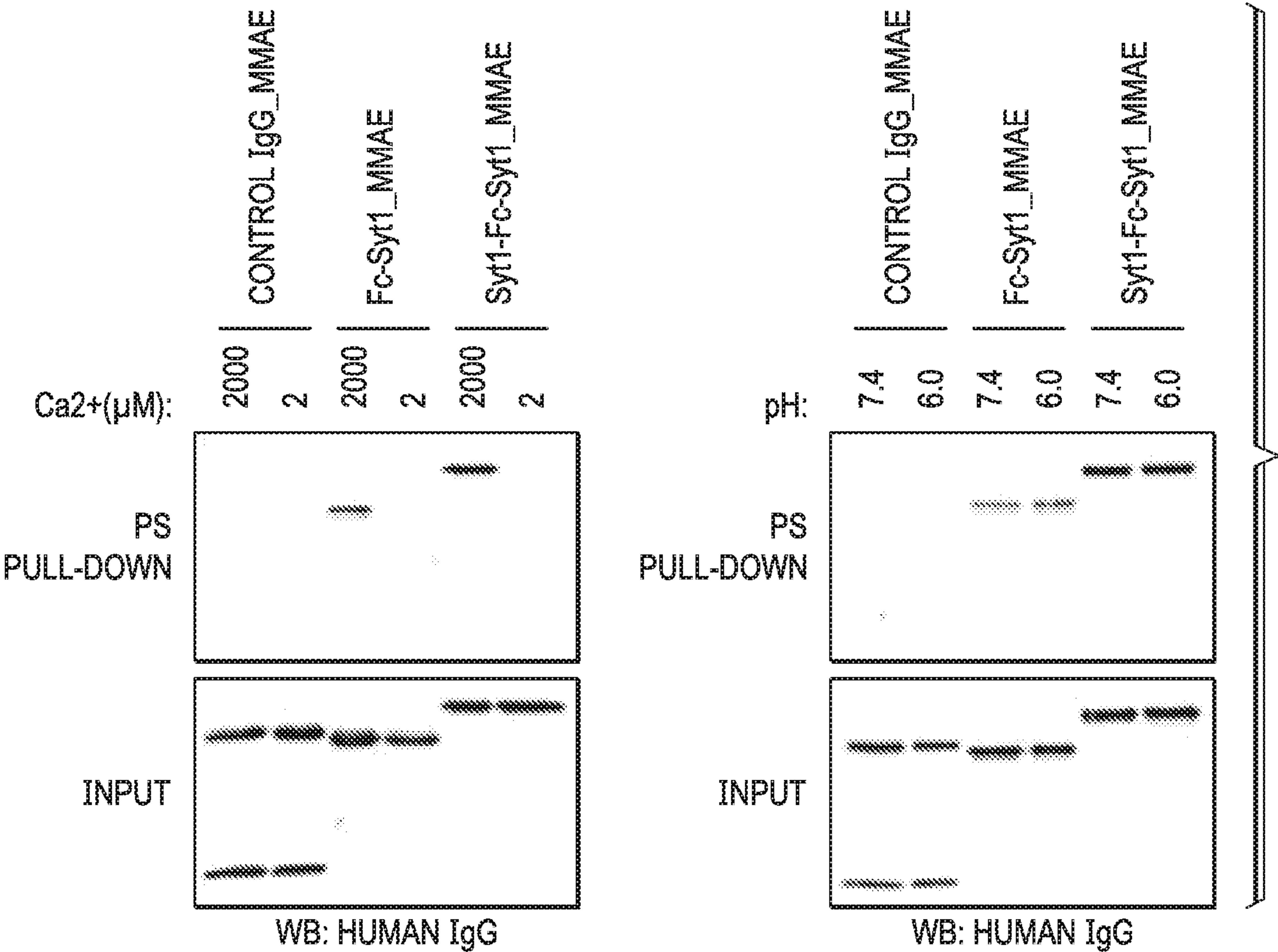


FIG. 12D

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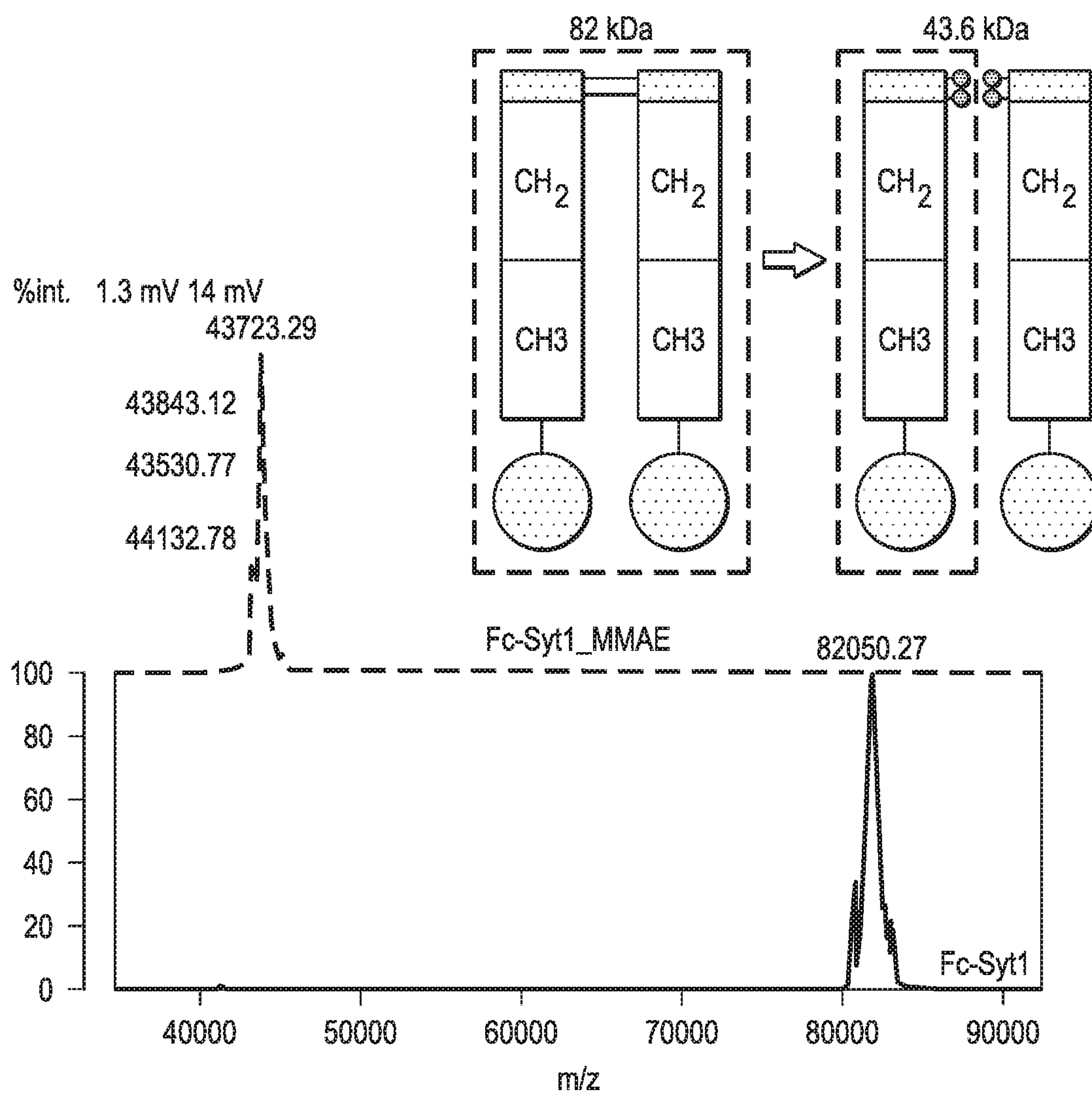
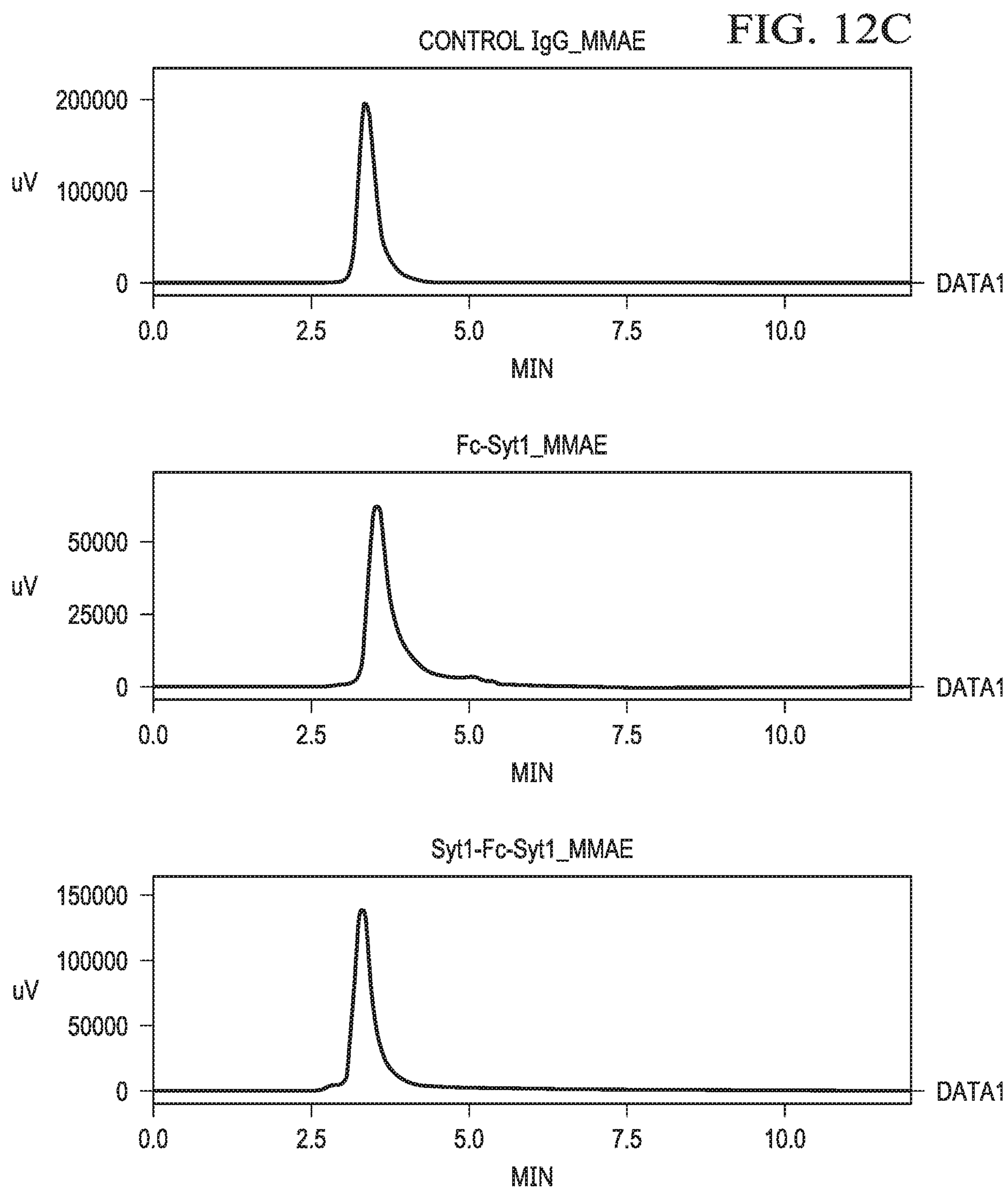
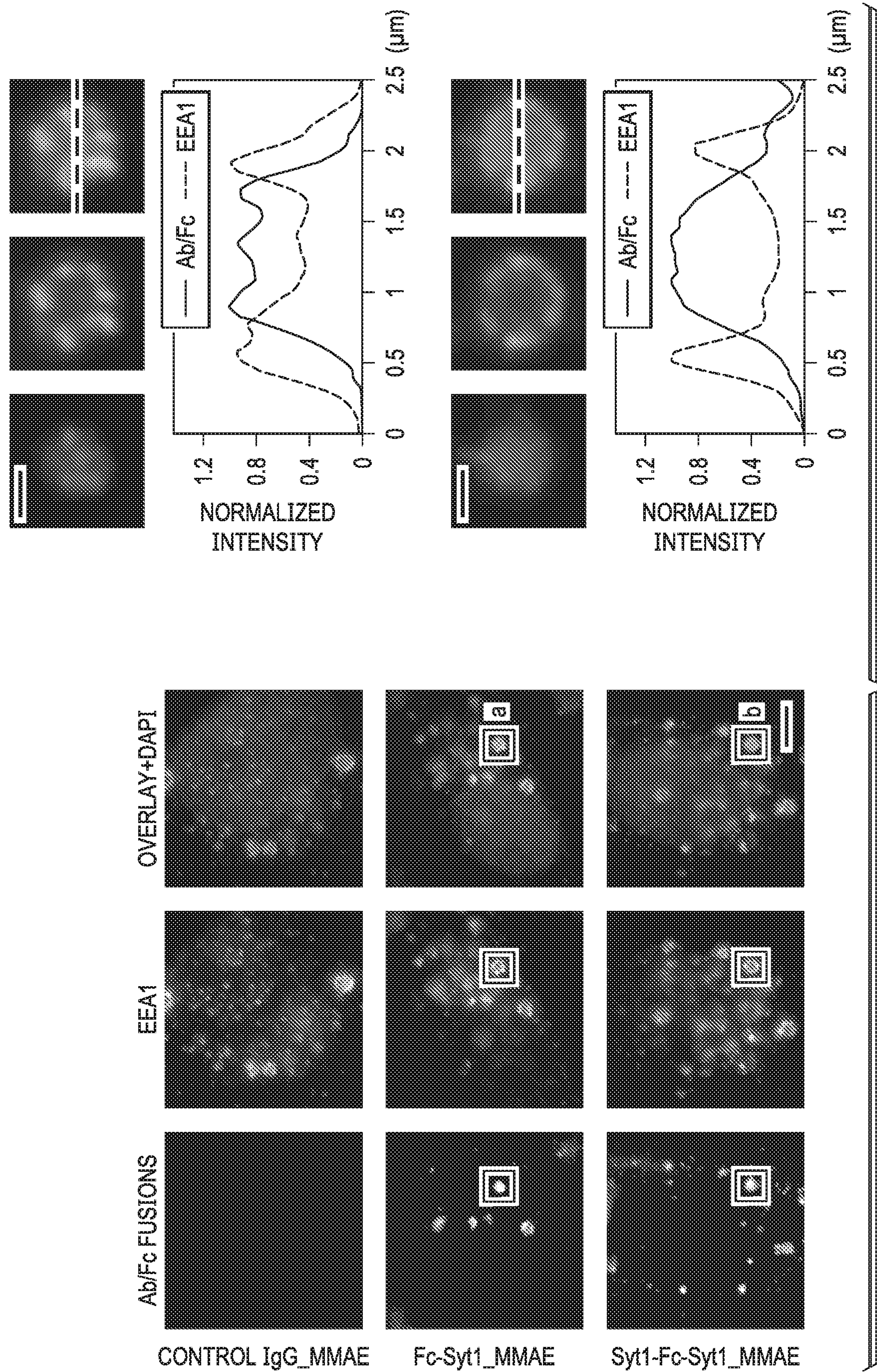


FIG. 12B

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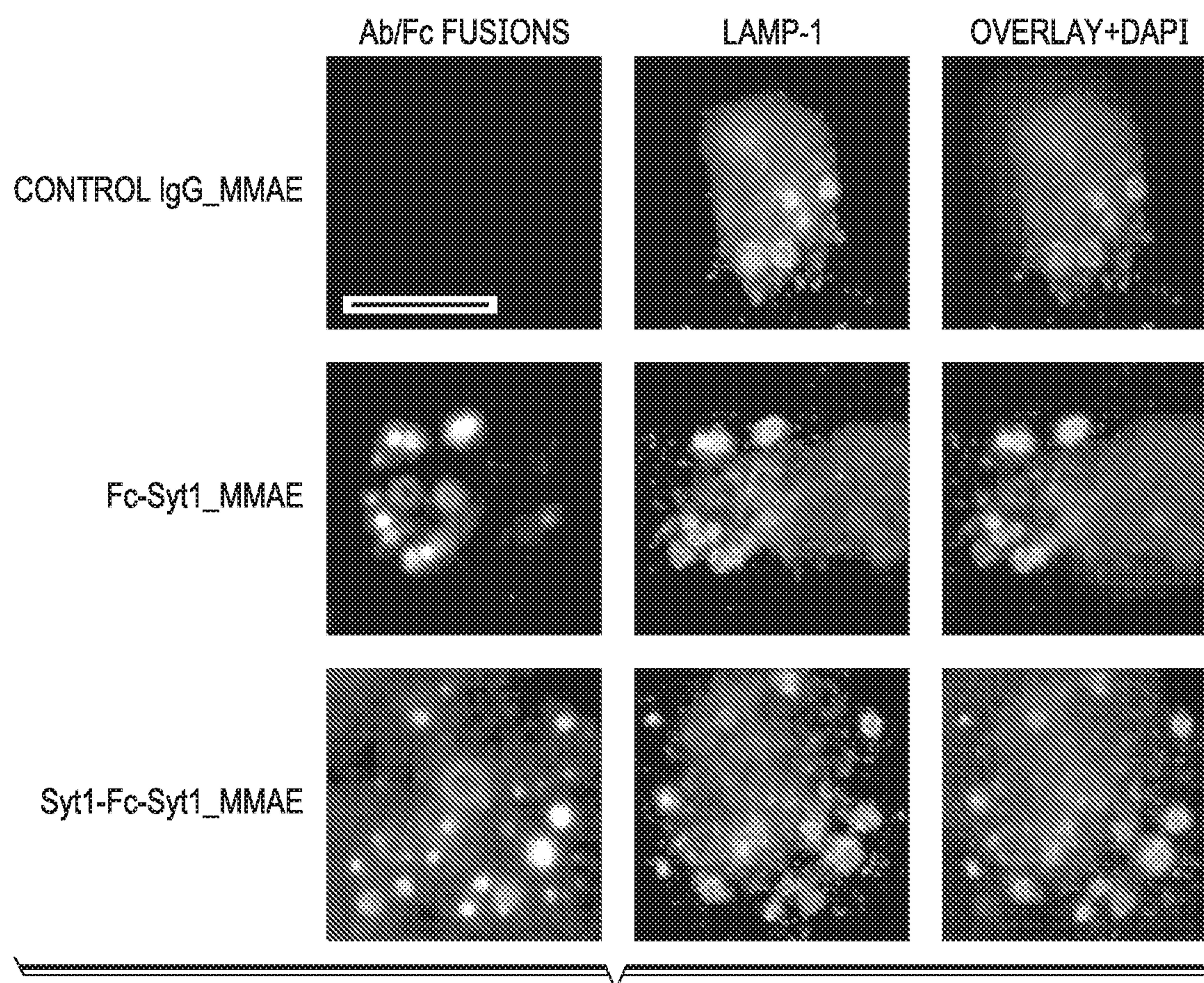


FIG. 13B

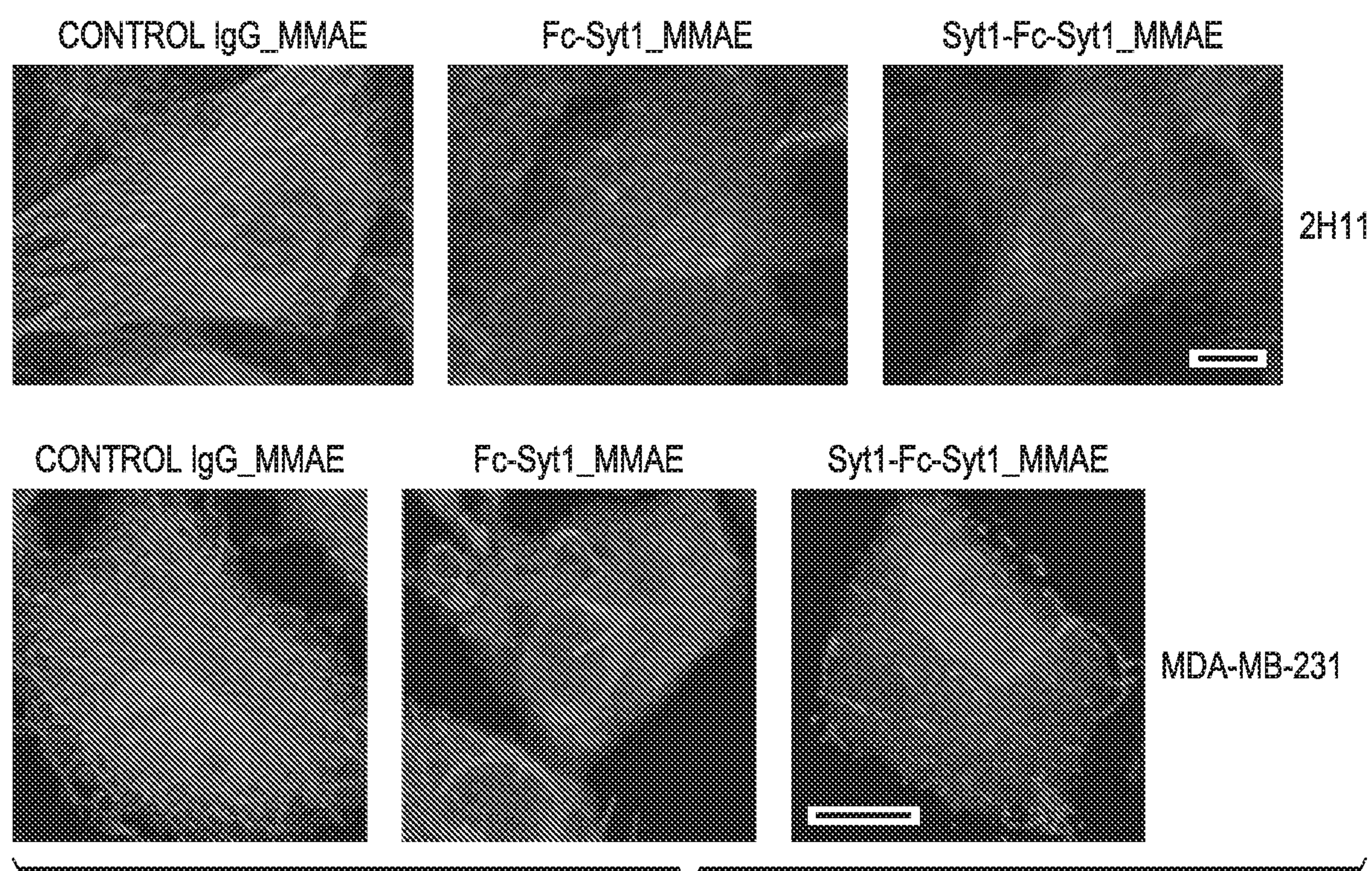


FIG. 13C

FIG. 14A

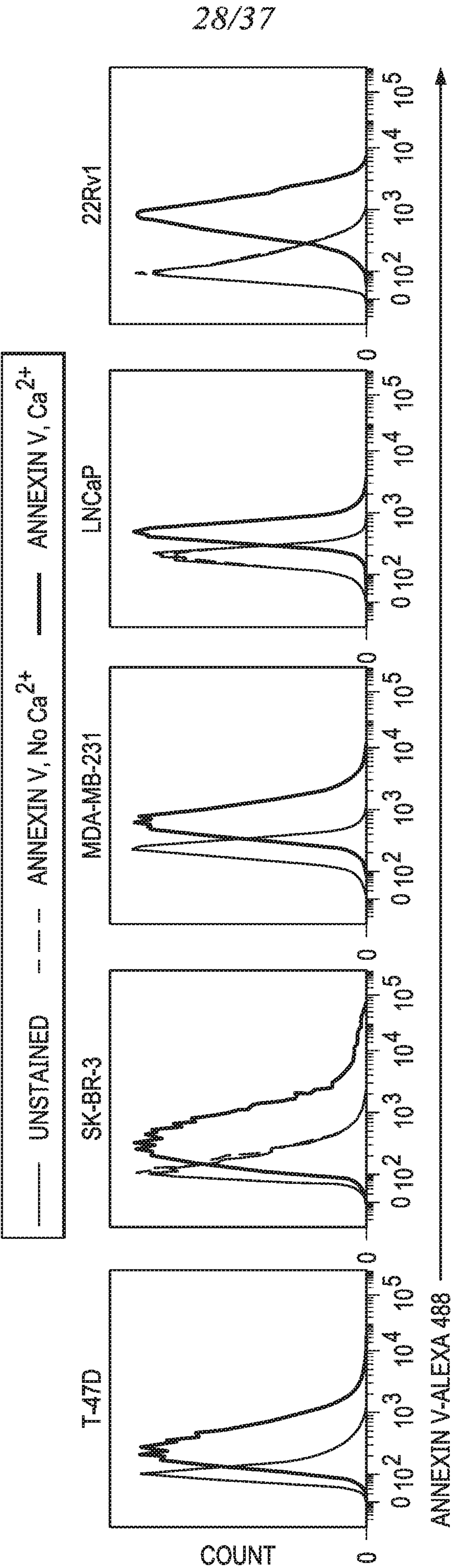


FIG. 14B

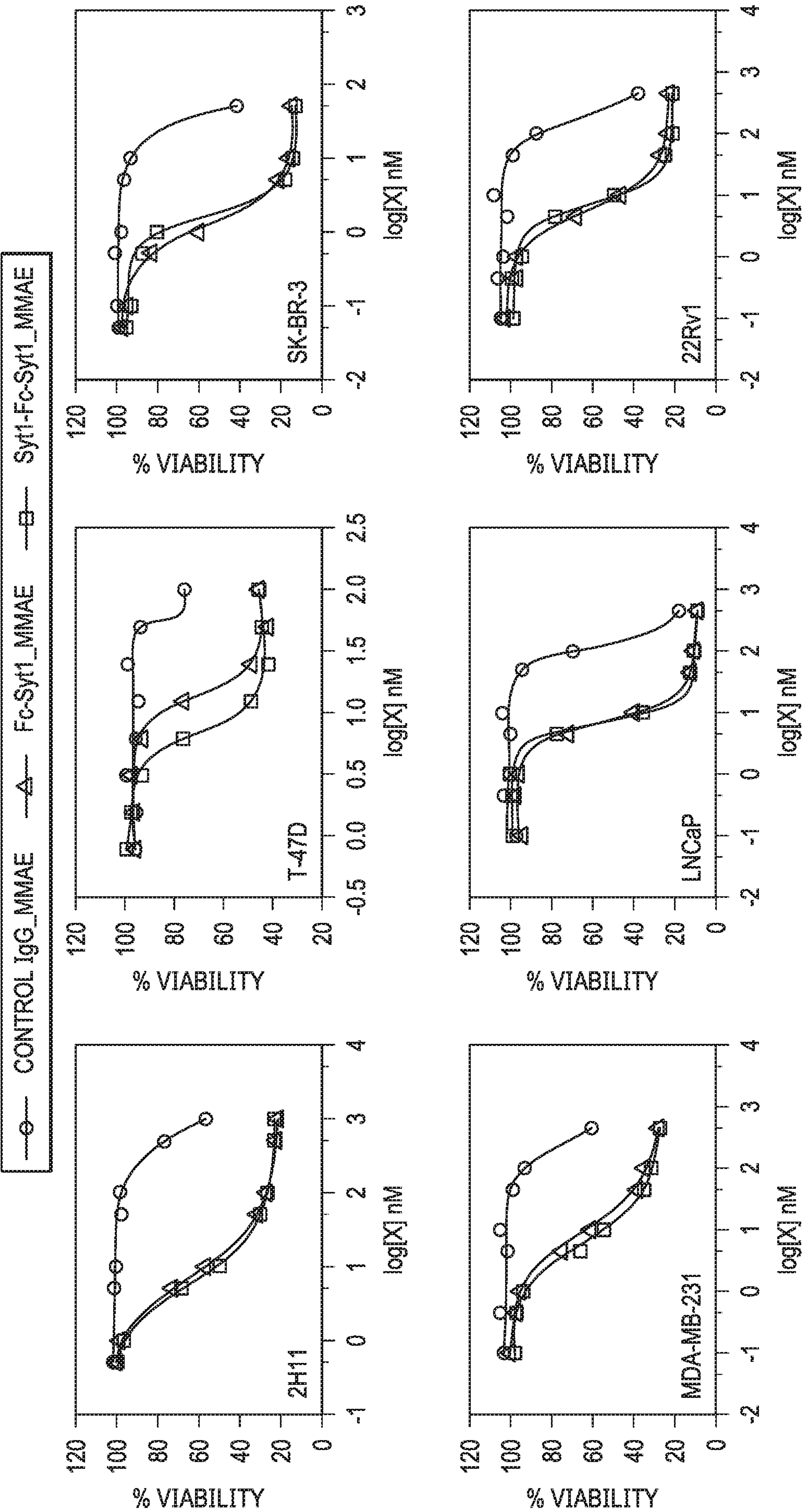
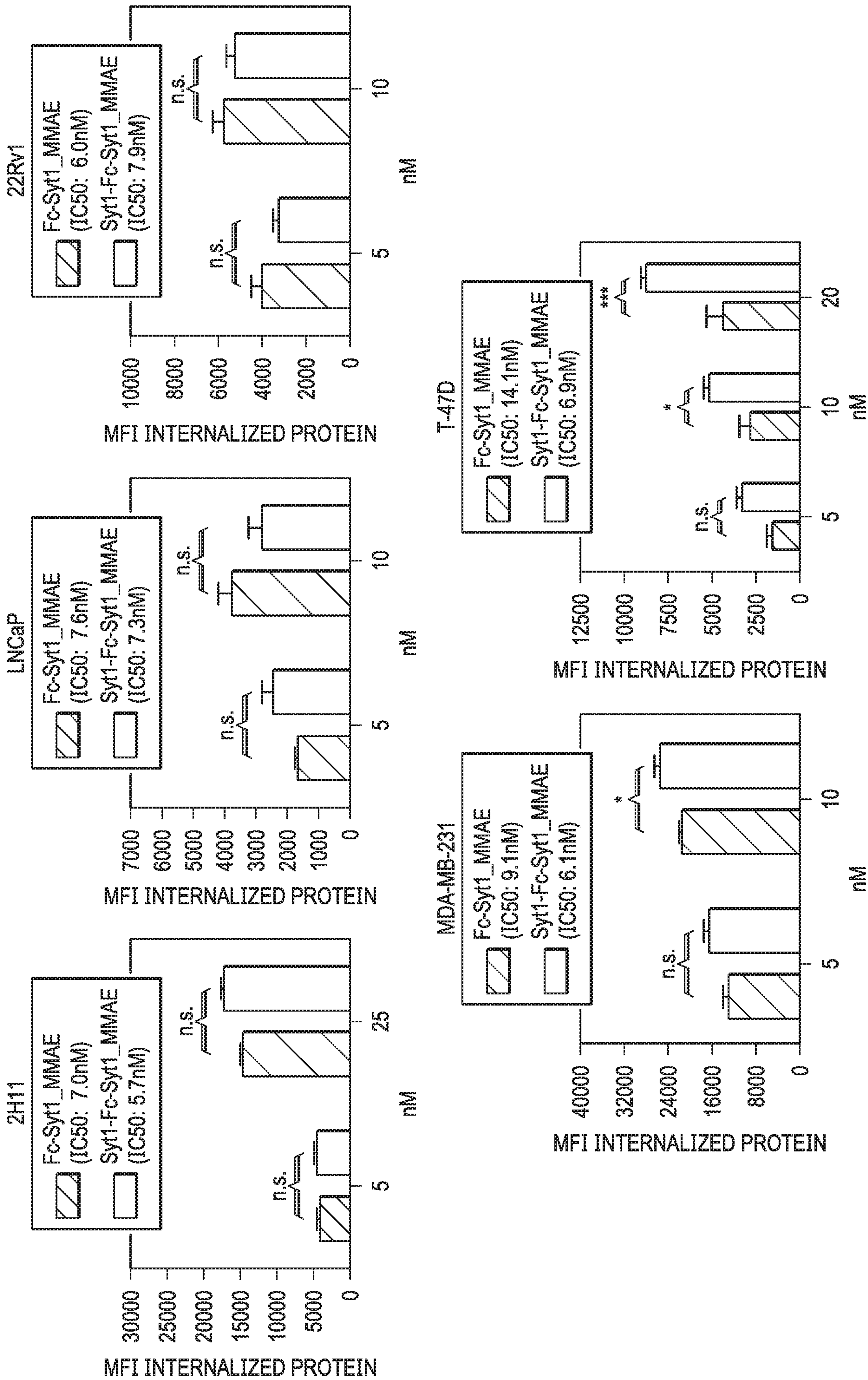
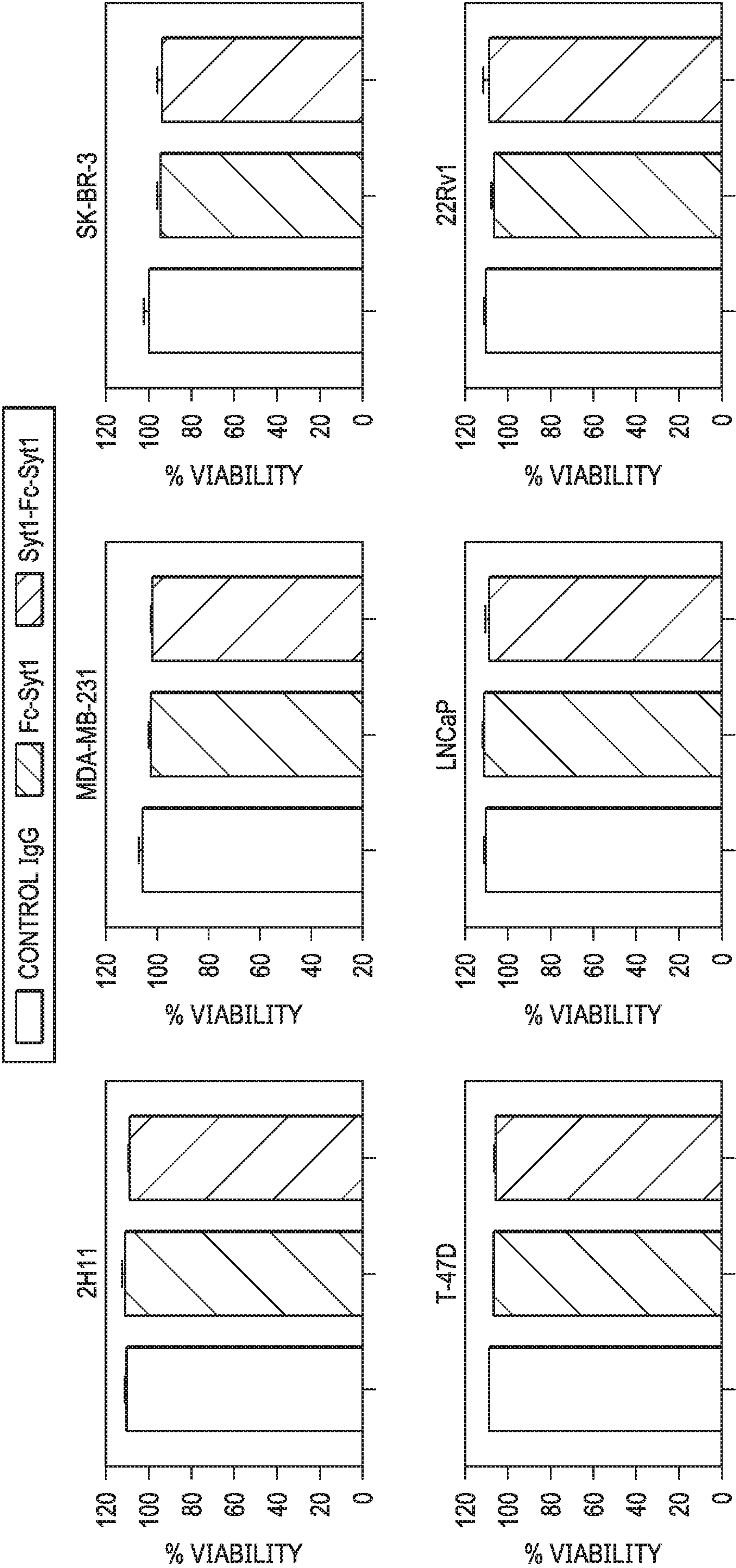


FIG. 14C



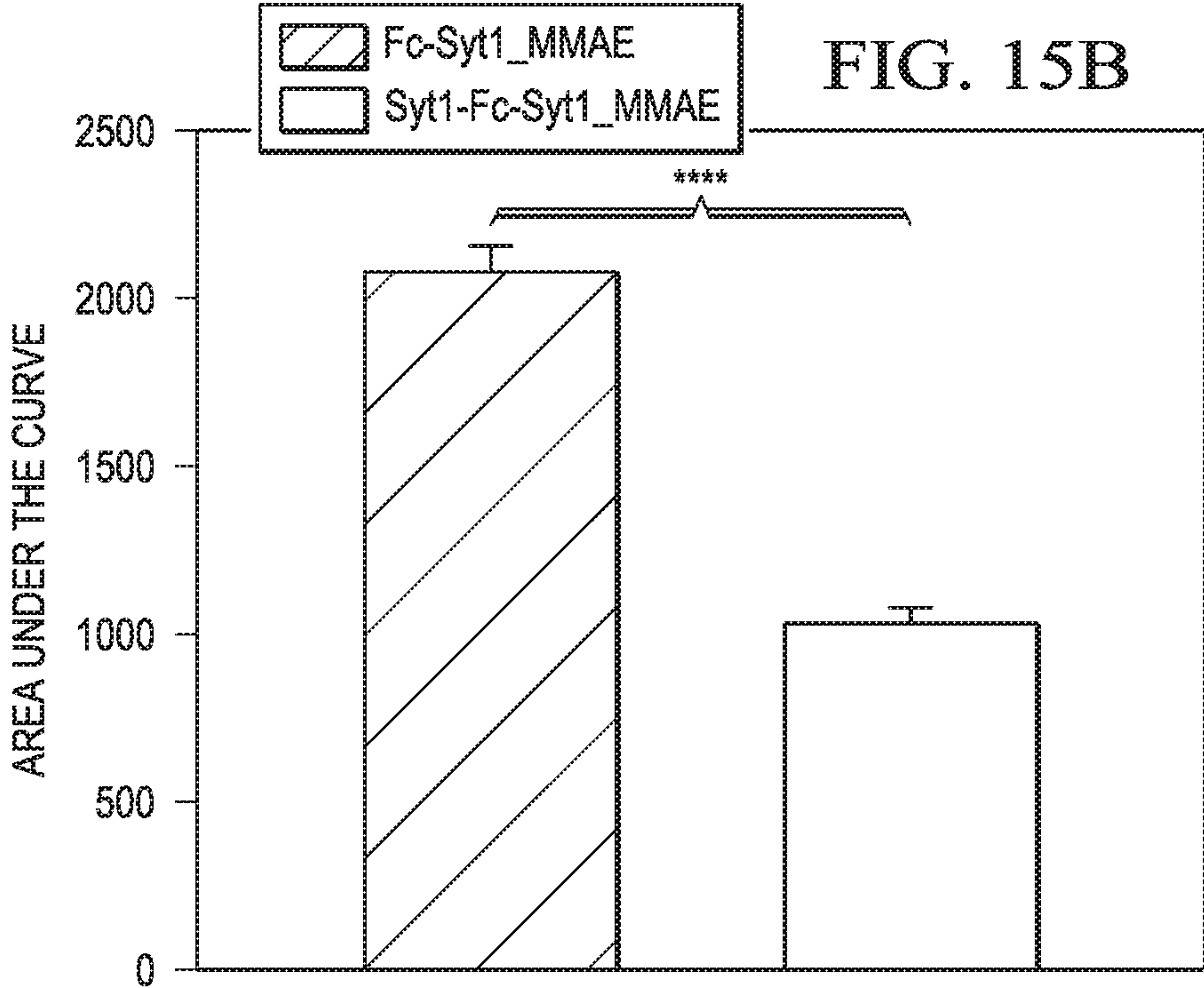
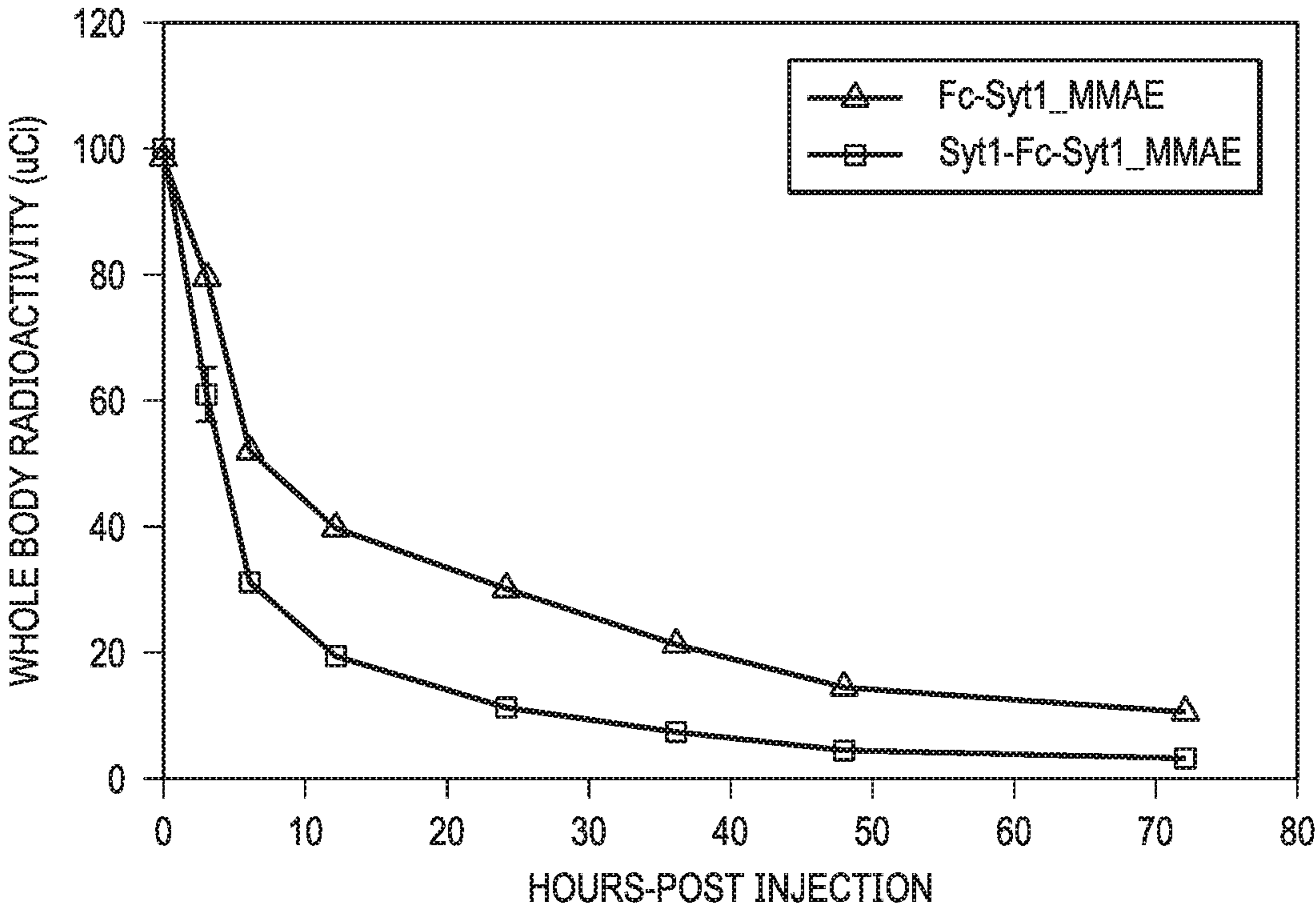
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FIG. 14D



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FIG. 15A



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FIG. 15C

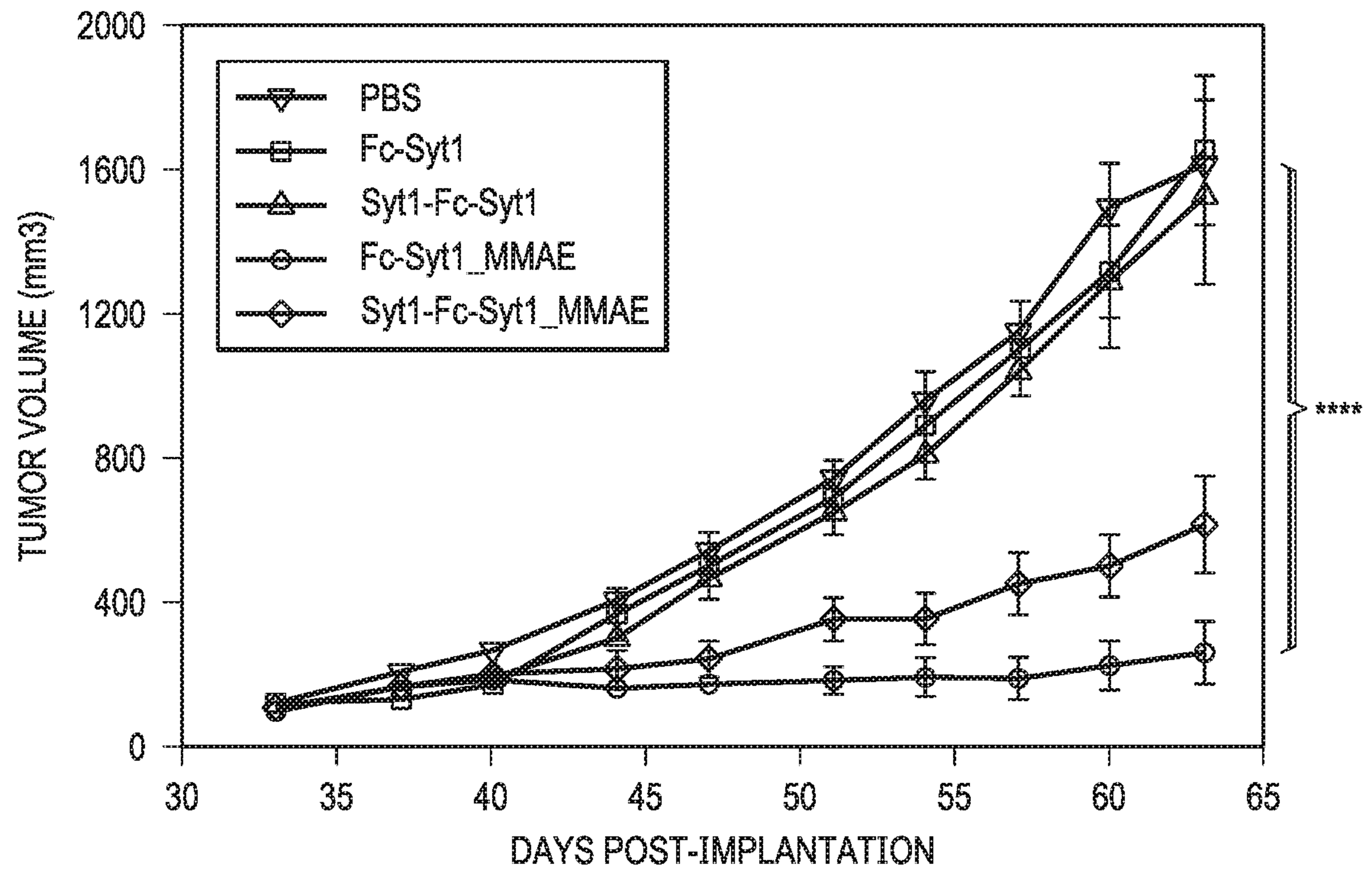
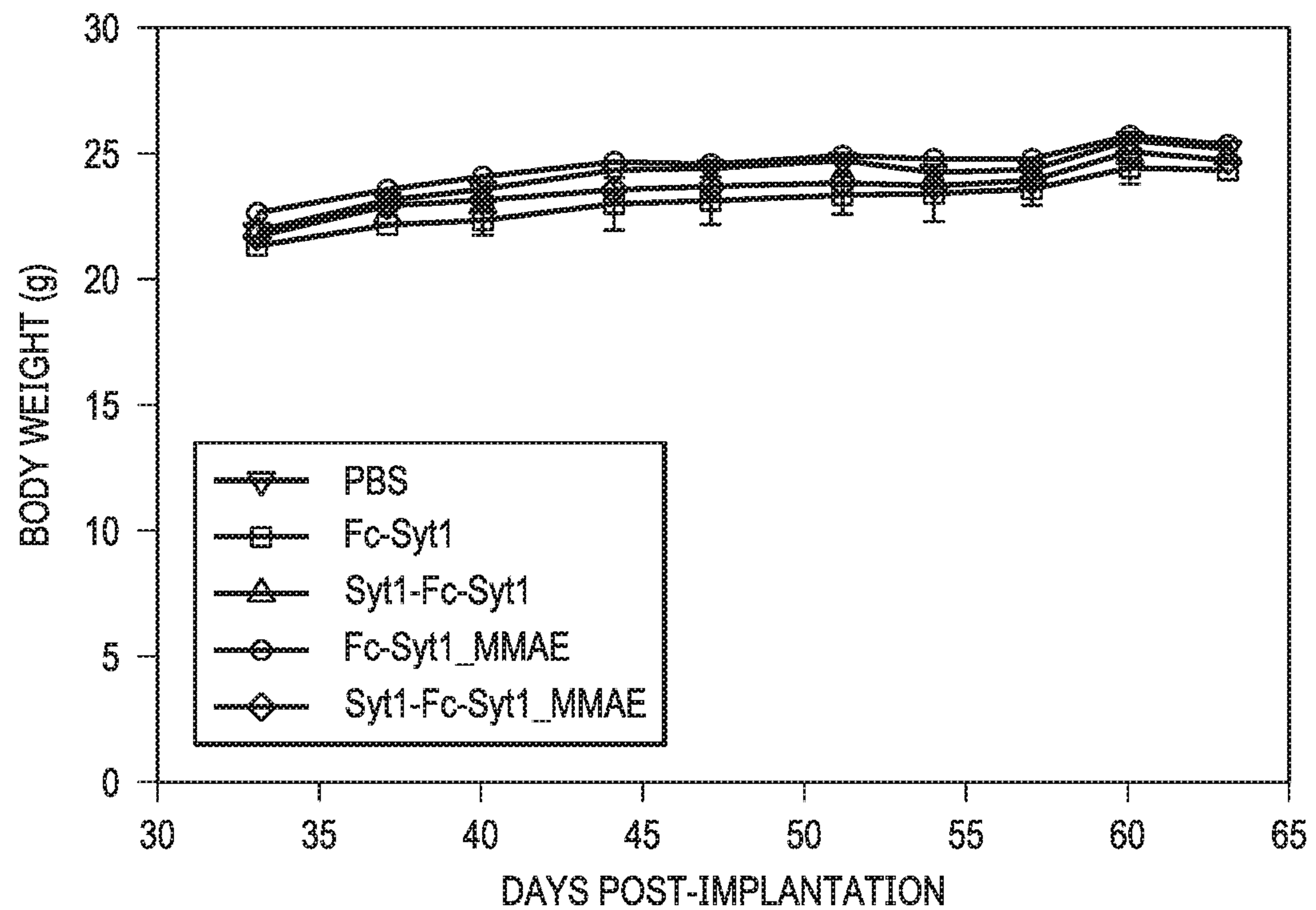


FIG. 15D



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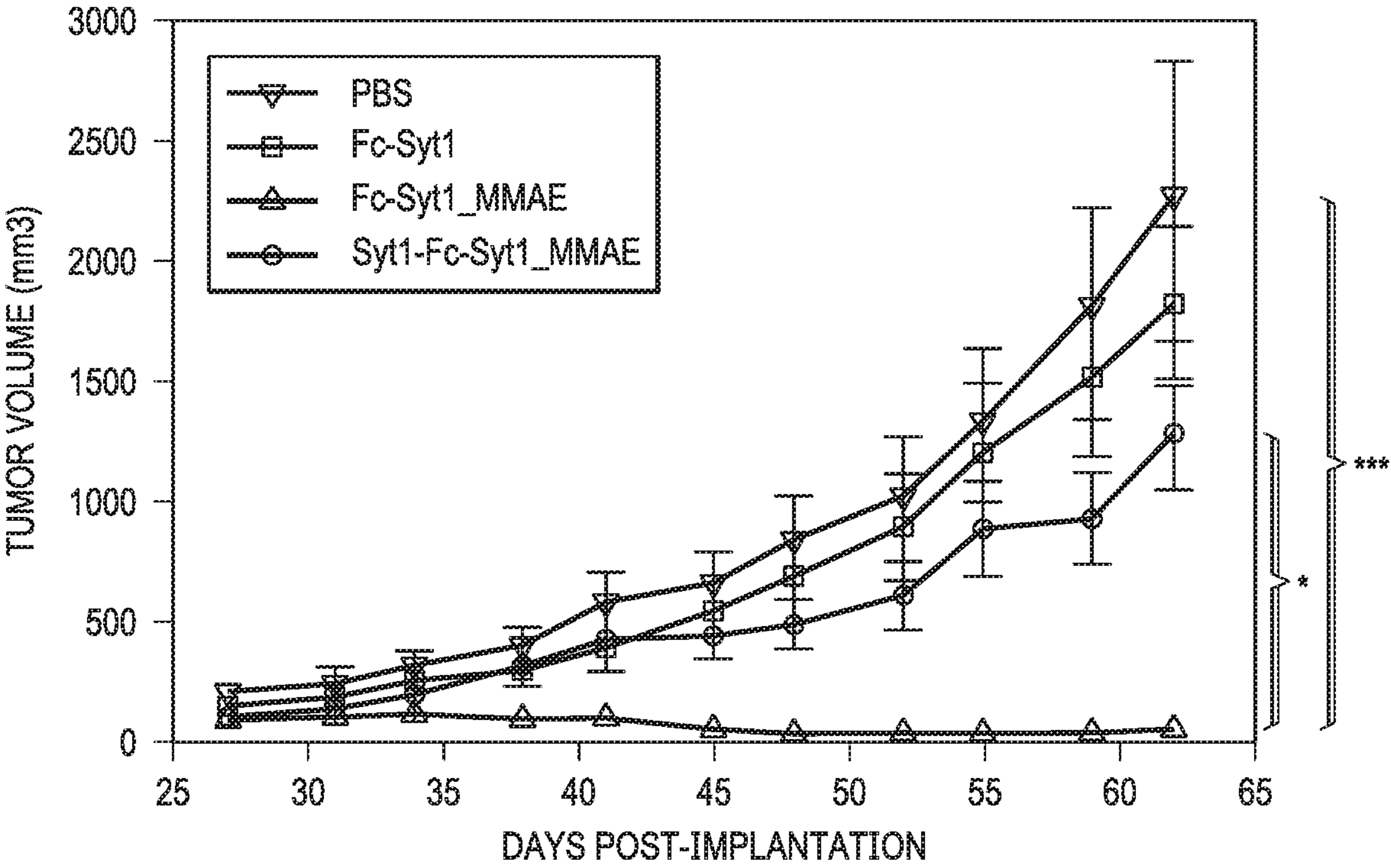


FIG. 15E

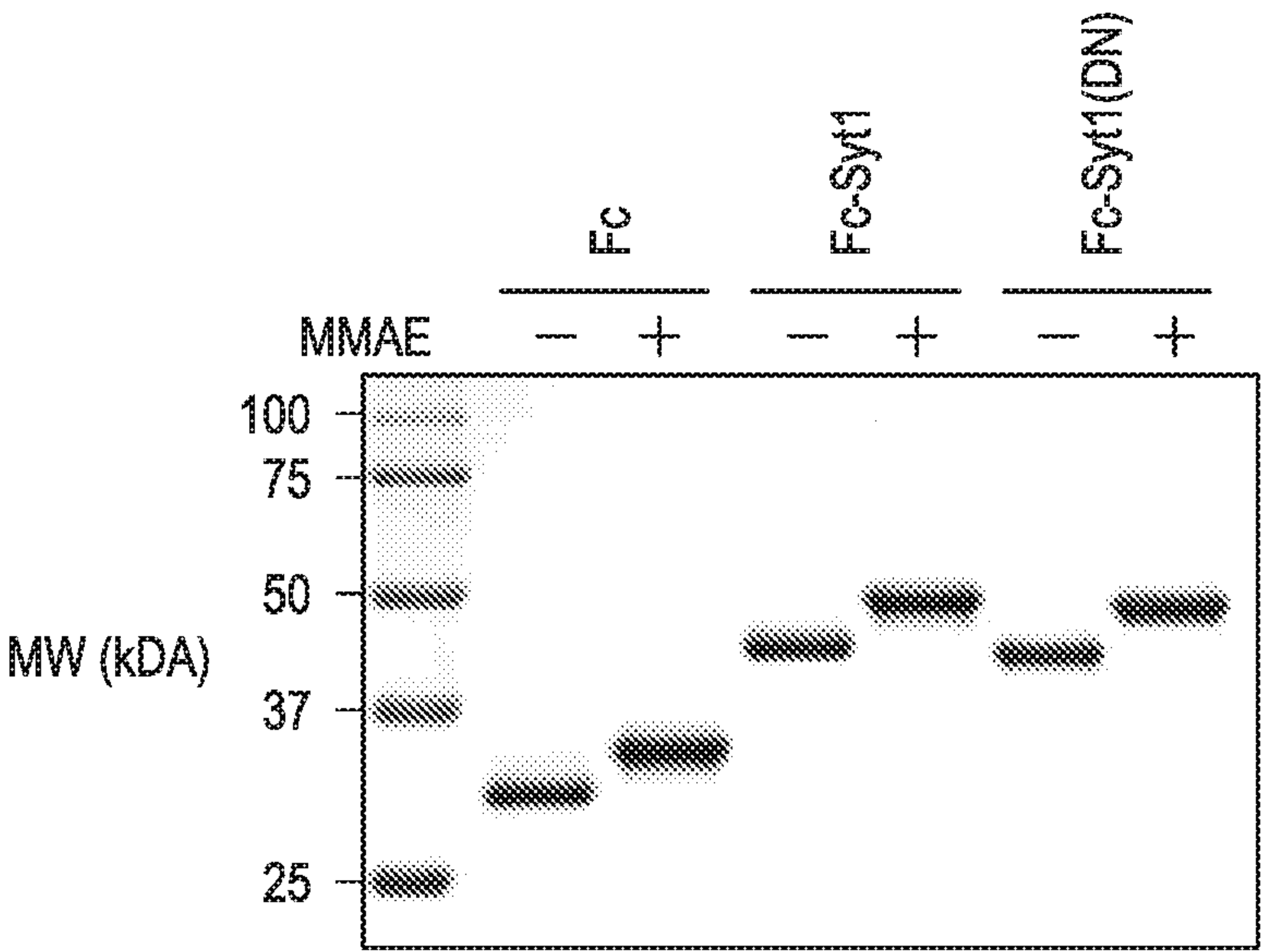


FIG. 16A

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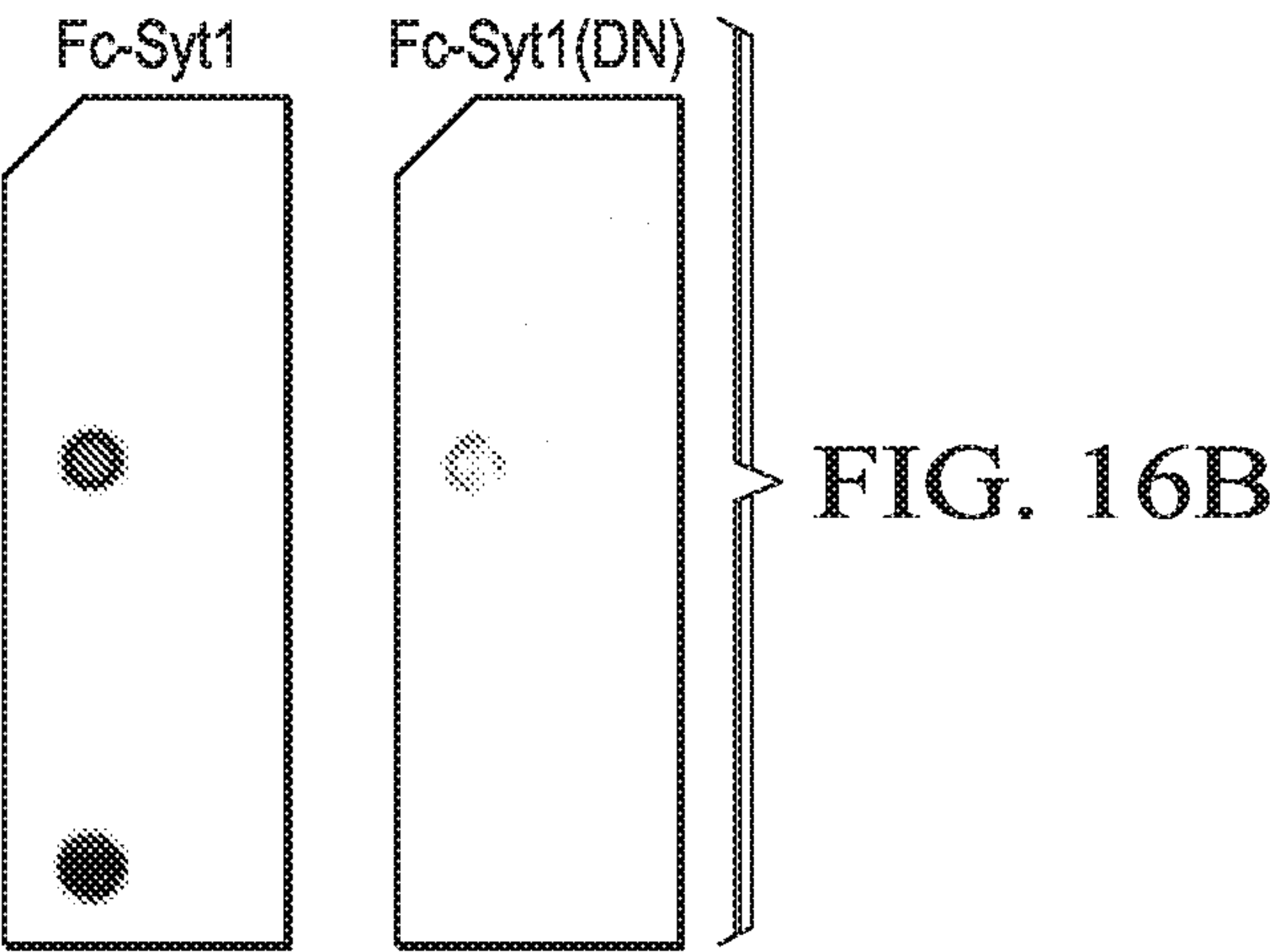


FIG. 16C

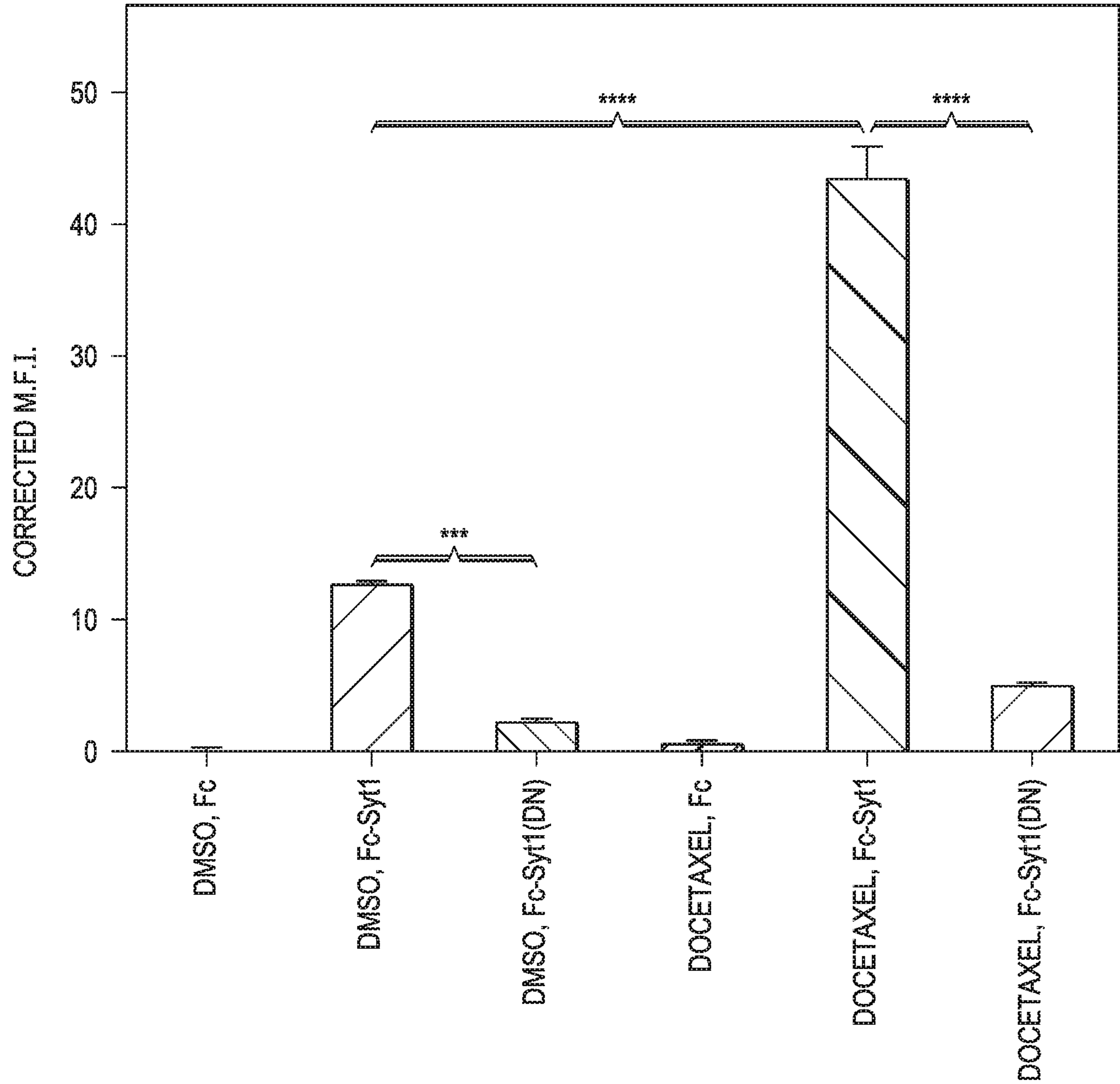
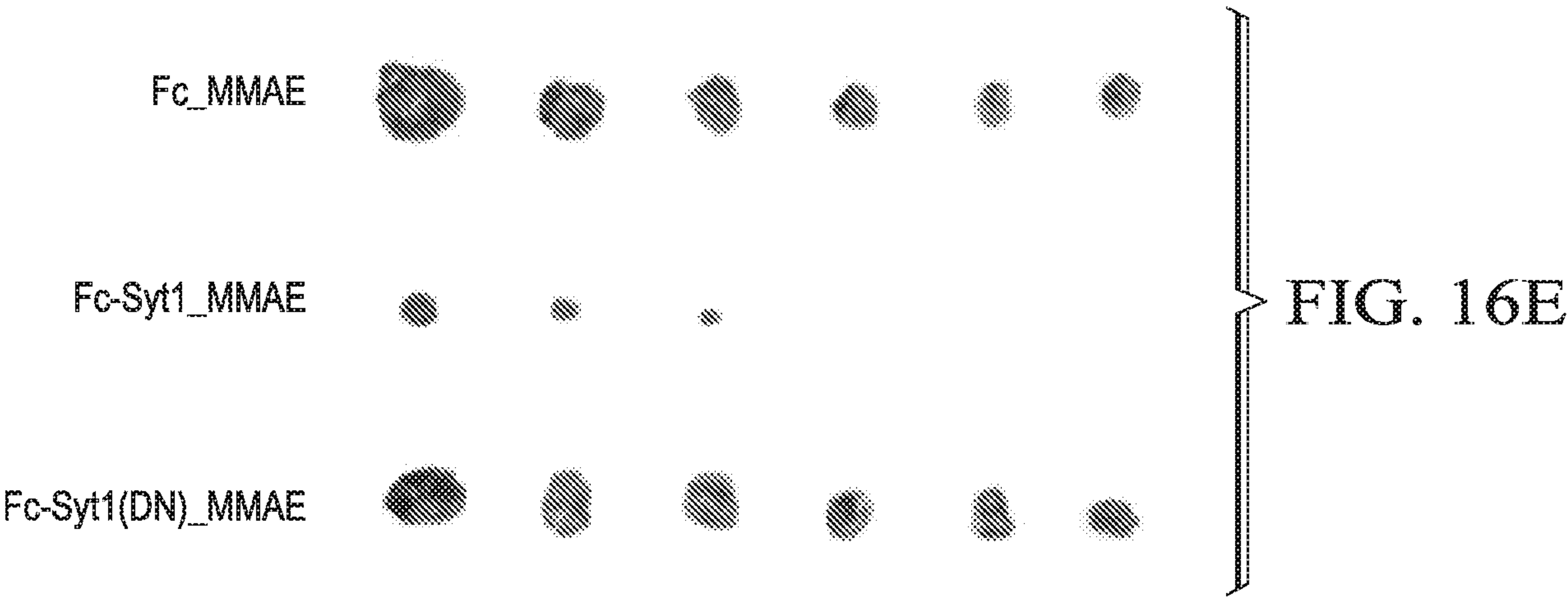
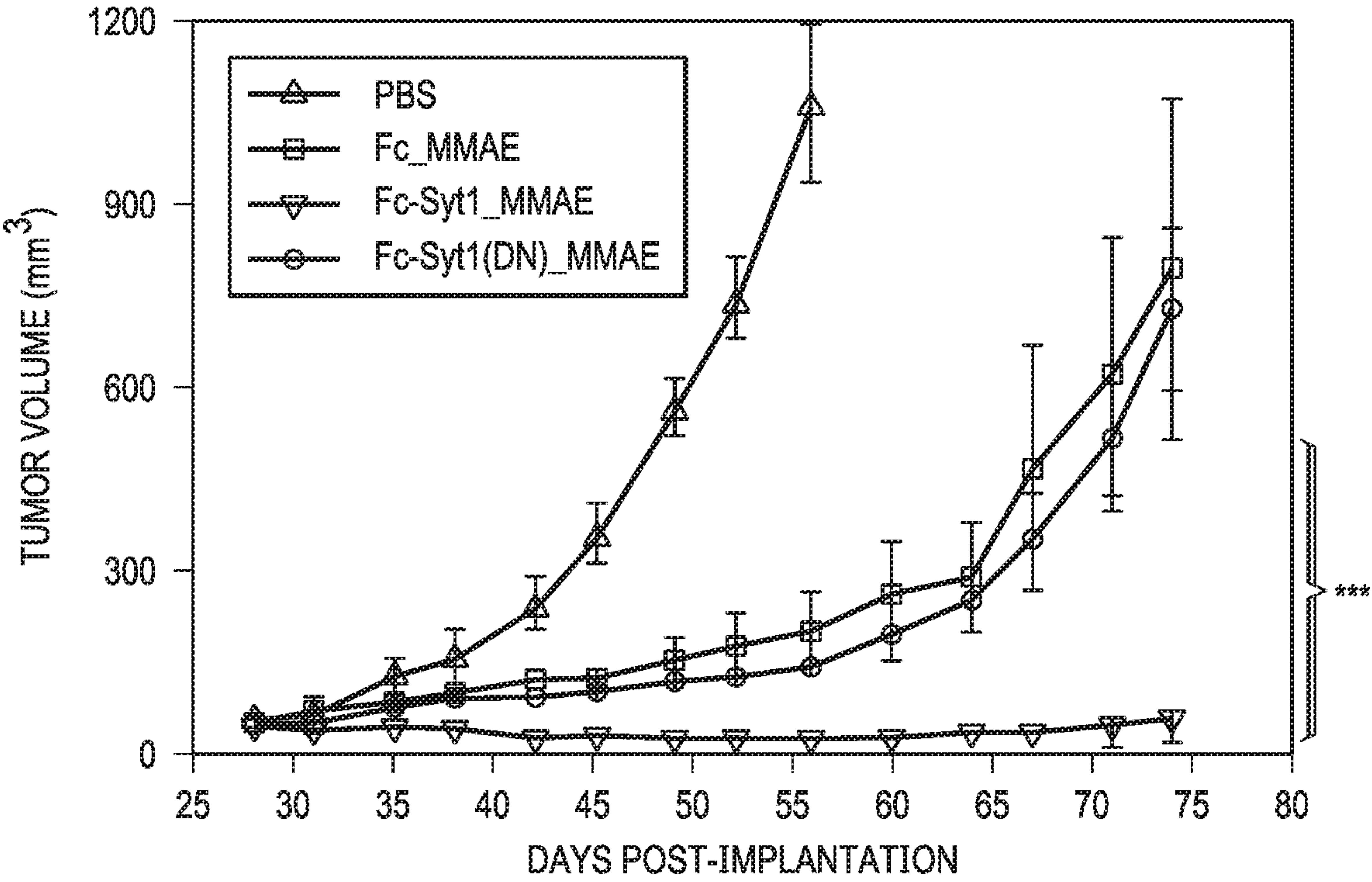


FIG. 16D



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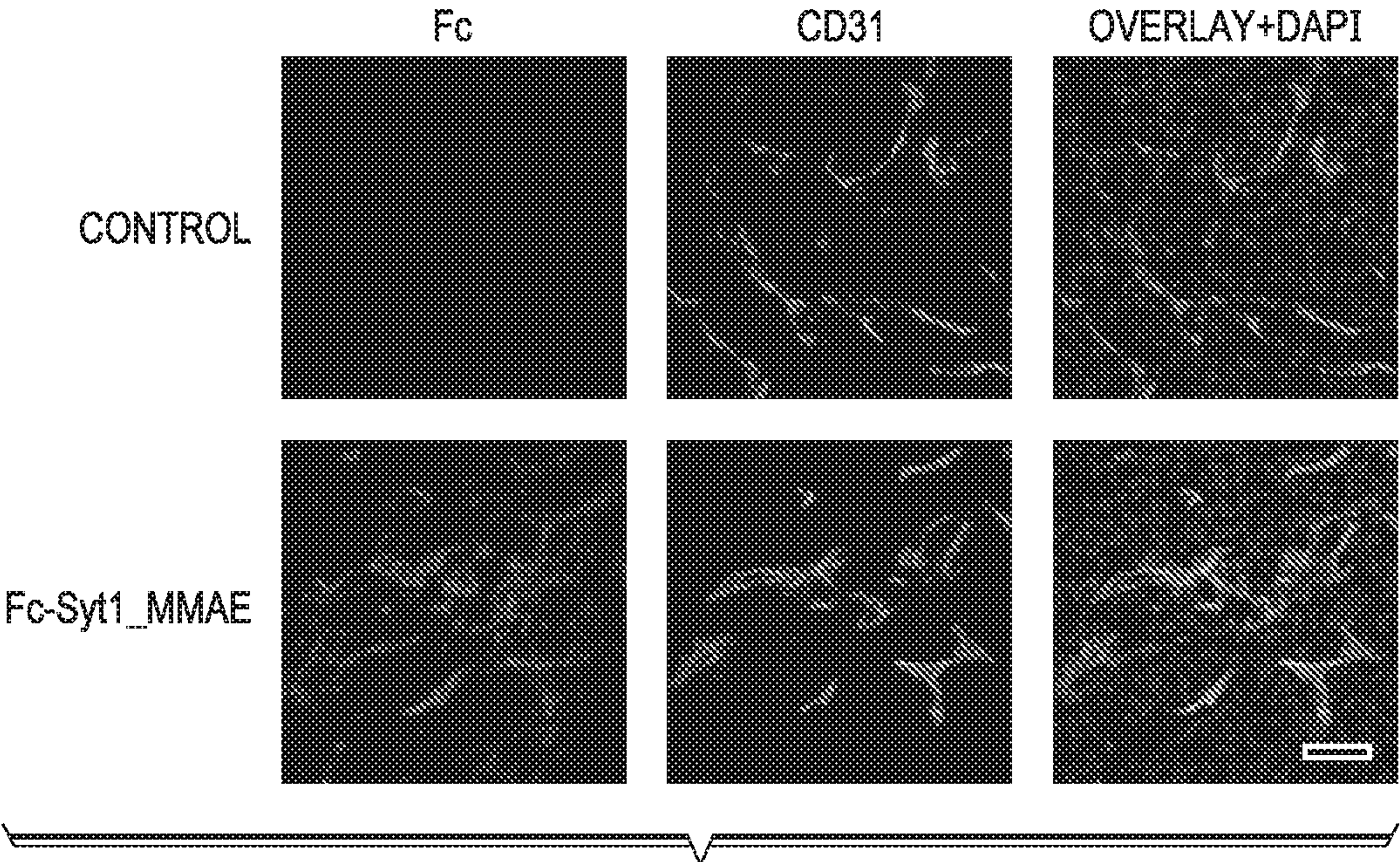


FIG. 17A

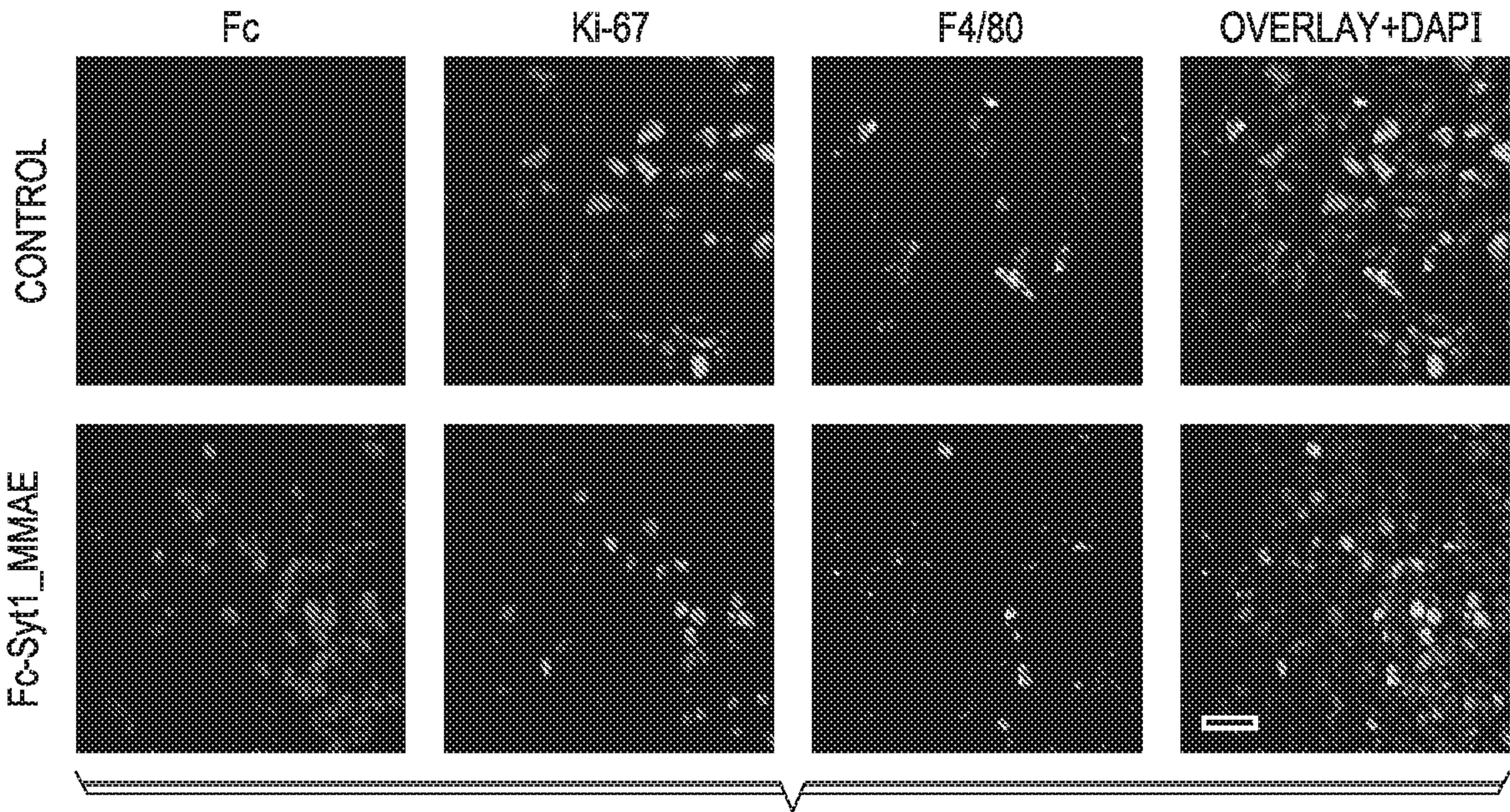


FIG. 17B

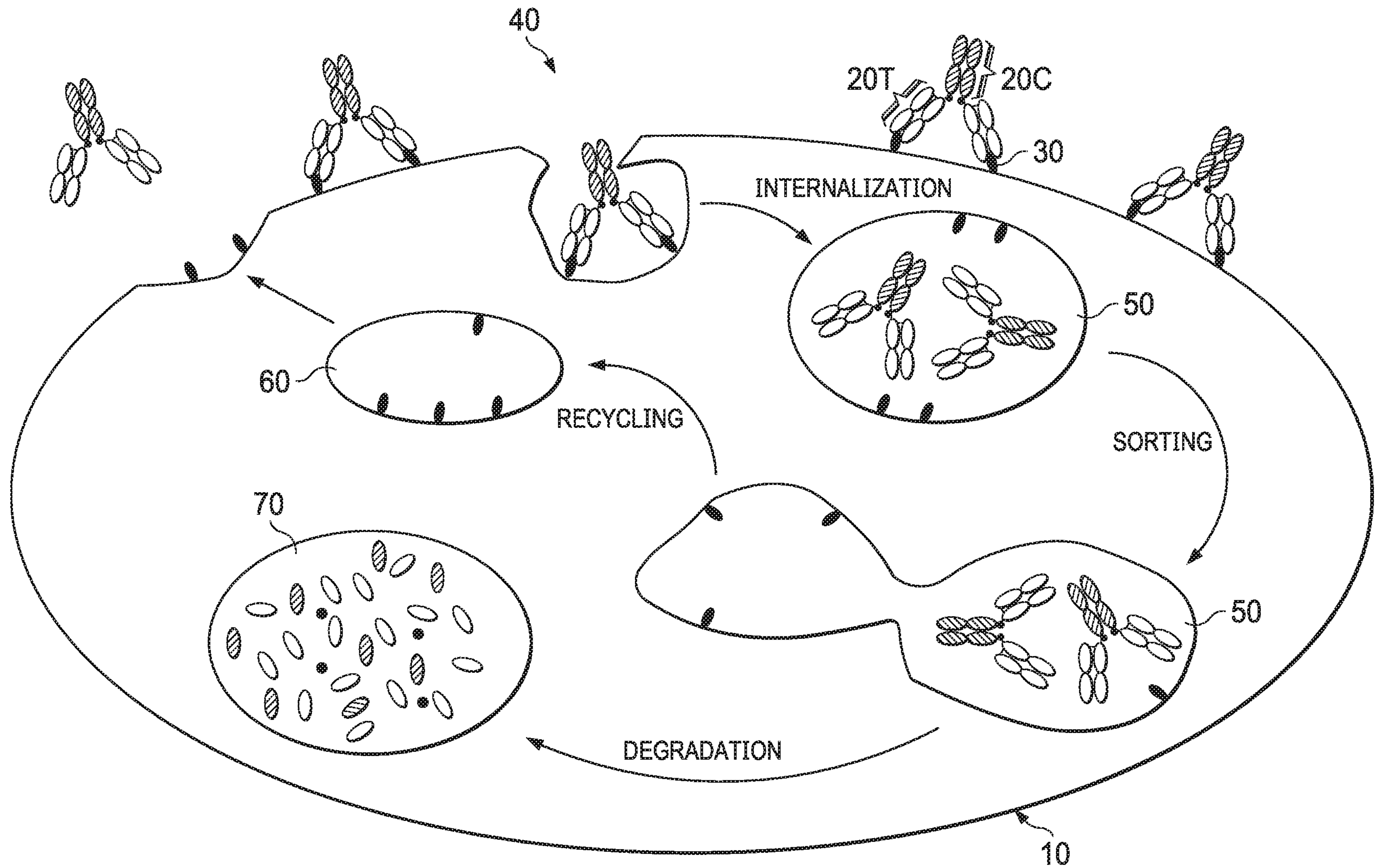


FIG. 1