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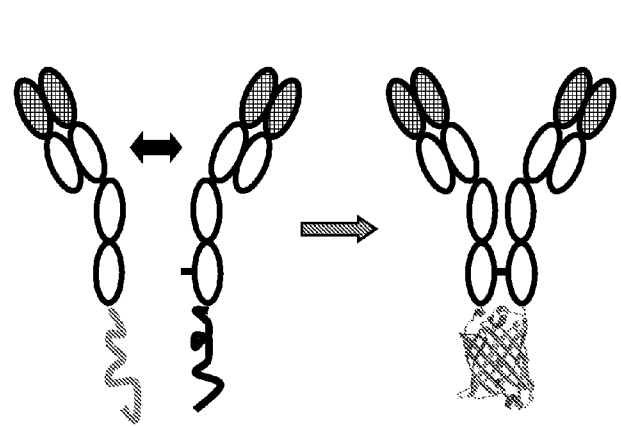
(54) Title: FLUORESCENT ANTIBODY FUSION PROTEIN, ITS PRODUCTION AND USE

(57) Abstract: Herein is reported a fusion protein comprising (a) a citrine moiety, which is either a single fluorescent citrine moiety or two corresponding fragments of a citrine moiety that upon combination form a fluorescent citrine moiety, and (b) an antibody fragment comprising at least a light chain variable domain and a heavy chain variable domain, wherein (i) the single fluorescent citrine moiety, or (ii) each of the corresponding fragments of the citrine moiety is linked to one terminus of the antibody fragment via a peptide bond independently of each other either directly or via a connector, and wherein each terminus of the antibody fragment is at most linked to one single functional citrine moiety or a fractional citrine moiety. Herein is also reported the use of the fusion protein for the fluorescence staining of cells, for high throughput fluorescence staining, for monitoring intracellular acidification, and for intracellular tracking.

Fig. 1



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Fluorescent antibody fusion protein, its production and use

Herein are reported an antibody conjugate comprising a yellow fluorescent protein which can be produced in mammalian cells, its use in fluorescence cell labeling as well as its use for molecule tracking within cells.

Background of the Invention

Recombinant antibodies have become widely used in research as well as in the treatment of diseases and in many diagnostically relevant applications. If fluorescent antibodies are required, usually antibodies are chemically conjugated to fluorophores. However, this approach has numerous limitations: it is usually not exactly determinable where the fluorophore is attached to the molecule, which can ultimately interfere with antigen binding, the stoichiometry of fluorophore to antibody can generally not be determined exactly and GFP derived proteins are more resistant to photobleaching than for example fluorescein.

Antibodies, which are hetero-tetrameric proteins, are stabilized and held together by disulfide bonds that are formed in the reducing environment of the ER. GFP related fluorescent proteins on the other hand, are designed to be folded in the cytosol of eukaryotic jellyfish. In general, these proteins can be produced in bacterial and mammalian cells, but the yield usually is in the low microgram range. An expression screen in E. coli indentified the YFPQ69M mutant called Citrine that showed an improved expression in intracellular compartments such as the ER, increased halide resistance and reduced but still pronounced pH dependent fluorescence.

In US 6,140,132 fluorescent protein sensors for measuring the pH of a biological sample are reported. FACS-optimized mutants of the green fluorescent protein (GFP) are reported in US 5,804,387. In US 7,166,424 fragments of fluorescent proteins for protein fragment complementation assays are reported. Real time nucleic acid detection in vivo using protein complementation is reported in US 2009/0029370. In US 7,585,636 protein subcellular localization assays using split fluorescent proteins are reported. Self-assembling split-fluorescent protein systems are reported in US 2005/0221343. US 2009/0035783 reports fluorescent proteins and uses thereof. Fluorescent proteins and chromoproteins from non-aequorea hydrozoa species and methods for using same are reported in US 2007/0298412. In US 2007/0105196 modified green fluorescent proteins and

methods for using same are reported. Novel fluorescent protein from aequorea coerulscens and methods for using the same are reported in US 2006/0167225.

In US 2009/203035 fluorescent proteins with increased stability are reported. Monoclonal amyloid beta (abeta)-specific antibodies and uses thereof are reported in WO 2009/033743. In WO 2006/099019 methods and composition related to in vivo imaging of gene expression are reported. Harnessing network to improve drug discovery is reported in WO 2006/058014. In WO 2006/096815 novel hTMC promoter and vectors for the tumor-selective and high-efficient expression of cancer therapeutic genes are reported. Myosin-like gene expressed in human heart and muscle is reported in US 2002/0048800.

Summary of the Invention

The use of a fluorescent antibody fusion protein as reported herein is suitable, e.g. compared to the detection of an antigen by a primary and/or secondary antibody. Especially when used in high throughput systems the fluorescence labeling of an antibody reduces the time required for staining.

Herein is reported as an aspect a fusion protein comprising

(a) a citrine moiety, which is either a fluorescent citrine moiety or two complementary fragments of a fluorescent citrine moiety that upon combination form a fluorescent citrine moiety,

(b) an antibody fragment comprising at least a light chain variable domain and a heavy chain variable domain,

wherein

(i) the fluorescent citrine moiety, or

(ii) each of the complementary fragments of a fluorescent citrine moiety

is linked to a terminus of the antibody fragment via a peptide bond independently of each other either directly, i.e. without a connector, or via a connector amino acid sequence, and

wherein

each terminus of the antibody fragment is at most linked to one fluorescent citrine moiety or to one fragment of a fluorescent citrine moiety.

In one embodiment the fluorescent citrine moiety or each of the complementary fragments of a fluorescent citrine moiety is linked to a C-terminus of the antibody fragment. In a further embodiment the fluorescent citrine moiety or each of the complementary fragments of a fluorescent citrine moiety is linked to a C-terminus of the antibody fragment via a peptide bond and/or via a connector that has the amino acid sequence of SEQ ID NO: 18. In one embodiment the fusion protein comprises at least one citrine moiety. In a further embodiment the fusion protein comprises one to four citrine moieties. In one embodiment the fusion protein comprises either two fluorescent citrine moieties or two complementary fragments of a fluorescent citrine moiety. In one embodiment the connector has an amino acid sequence selected from 'G', 'N', 'GST', SEQ ID NO: 18, 19, 20, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54. In another embodiment the connector has the amino acid sequence of SEQ ID NO: 18. In a further embodiment the antibody fragment is consisting of two full length light chains and two full length heavy chains. In one embodiment the citrine moiety has the amino acid sequence of SEQ ID NO: 21. In another embodiment the corresponding fragments of a fluorescent citrine moiety have an amino acid sequence of SEQ ID NO: 22 and 23.

In one embodiment the fusion protein comprises

(a) two fluorescent citrine moieties,

(b) a complete antibody consisting of two full length light chains and two full length heavy chains,

wherein the N-terminus of each of the citrine moieties

(i) is linked to the C-terminus of an antibody light chain via a peptide bond, and

(ii) is linked independently of each other either directly, i.e. without connector, or via a connector, and

wherein each C-terminus of the antibody light chains is linked to only one citrine moiety.

In another embodiment the fusion protein comprises

(a) one fluorescent citrine moiety,

(b) a complete antibody consisting of two full length light chains and two full length heavy chains,

wherein the N-terminus of the citrine moiety is linked to one C-terminus of an antibody chain via a peptide bond either directly, i.e. without connector, or via a connector.

In a further embodiment the fusion protein comprises

(a) two complementary fragments of a fluorescent citrine moiety,

(b) a complete antibody consisting of two full length light chains and two full length heavy chains,

wherein the N-terminus of each of the complementary fragments of the fluorescent citrine moiety

(i) is linked to the C-terminus of an antibody heavy chain via a peptide bond,

(ii) is linked independently of each other either directly, i.e. without connector, or via a connector, and

wherein each C-terminus of the antibody heavy chains is linked to one complementary fragment of a fluorescent citrine moiety.

In also an embodiment the fusion protein comprises

(a) two fluorescent citrine moieties,

(b) a complete antibody consisting of two full length light chains and two full length heavy chains,

(c) two single chain antibodies,

wherein the binding sites of the complete antibody bind to a first epitope or antigen and the binding sites of the two single chain antibodies bind to a second epitope or antigen different from the first one,

wherein the N-terminus of each of the citrine moieties

(i) is linked to the C-terminus of one antibody heavy chain via a peptide bond,

(ii) is linked independently of each other either directly, i.e. without connector, or via a connector, and

wherein each C-terminus of the antibody heavy chains is linked to one citrine moiety, and

wherein the N-terminus of each of the single chain antibodies is linked to the C-terminus of an antibody light chain and each C-terminus of the antibody light chains is linked to one single chain antibody.

In one embodiment the fusion protein comprises

(a) one fluorescent citrine moiety,

(b) two disulfide stabilized single chain antibodies,

wherein the binding sites of the sigle chain antibodies bind to the same or two different epitopes or antigens,

wherein one single chain antibody is linked to the N-terminus of the citrine moiety via a connector and the second single chain antibody is linked via the same or a different connector to the C-terminus of the citrine moiety.

Herein is reported as an aspect the use of a fusion protein as reported herein for the fluorescence staining of cells. Also is reported herein as an aspect the use of a fusion protein as reported herein for high throughput fluorescence labeling. Further is reported herein as an aspect the use of a fusion protein as reported herein for monitoring intracellular acidification. Also is reported herein as an aspect the use of a fusion protein as reported herein for intracellular tracking.

Herein is further reported as an aspect a method for staining cells comprising the following steps

(a) providing a fusion protein as reported herein with a binding site binding to a cell surface receptor, which upon binding can be internalized into the cell,
(b) incubating a population of cells with the fusion protein,
(c) dividing the incubated cell population in two sub-populations, whereof the first is fixed immediately and the second is further incubated prior to fixing and thereby staining cells.

Herein is also reported as an aspect a method for monitoring intracellular acidification comprising the following steps

(a) providing a fusion protein as reported herein with a binding site binding to a cell surface receptor, which upon binding can be internalized into the cell,
(b) incubating a population of cells with the fusion protein,
(c) determining the fluorescence intensity of the citrine moiety in samples removed from the incubated population of cells at defined time intervals and thereby monitoring intracellular acidification.

Herein is reported as an aspect a method for intracellular tracking comprising the flowing steps

(a) providing a fusion protein as reported herein with a binding site binding to a cell surface receptor, which upon binding can be internalized into the cell,
(b) incubating a population of cells with the fusion protein,
(c) determining the fluorescence localization of the citrine moiety in samples removed from the incubated population of cells at defined time intervals and thereby intracellular tacking the citrine moiety.

Herein is reported further a method for detecting the localization of a polypeptide in a cell comprising the following steps

(a) expressing in the cell or providing to the cell either (i) the polypeptide or (ii) a fusion protein comprising a) the polypeptide and b) a tag or a fragment of a fluorescent citrine moiety,
(b) (i) expressing in the cell or providing to the cell the corresponding fragment of the fluorescent citrine moiety, or
(ii) providing to the cell a fusion protein comprising
(a) the corresponding fragment of the fluorescent citrine moiety, and
(b) an antibody fragment comprising at least a light chain variable domain and a heavy chain variable domain,

wherein each fragment of the fluorescent citrine moiety is linked to one C-terminus of the antibody fragment either directly or via a connector, and each C-terminus of the antibody fragment is linked to one fragment of the fluorescent citrine moiety, and

wherein the antibody fragment is binding to a cell surface receptor, which upon binding can be internalized into the cell,

and
(c) detecting fluorescence in the cell, and thereby detecting the localization of the polypeptide in a cell.

A further aspect as reported herein is a composition comprising a fluorescent antibody fusion protein as reported herein in monomeric and aggregated form,

wherein the fraction of aggregated fluorescent antibody fusion protein is 5 % or less when determined by size exclusion chromatography.

Another aspect as reported herein is a method for producing a fluorescent antibody fusion protein in monomeric form comprising the following steps

a) cultivating a cell comprising a nucleic acid encoding the fluorescent antibody fusion protein and recovering the fusion protein from the cells or the cultivation medium,
b) applying the fluorescent antibody fusion polypeptide obtained in step a) to a size exclusion chromatography column, and
c) recovering the fluorescent antibody fusion protein from the size exclusion chromatography column and thereby producing a fluorescent antibody fusion protein in monomeric form.

Detailed Description of the Invention

Genetically encoded fluorescent antibodies can be used in many applications in biotechnology, proteomics, microscopy, cell biology and molecular diagnostics.

The use of a fluorescent antibody fusion protein as reported herein is especially suitable, e.g., compared to the detection of an antigen by a primary and/or secondary antibody. Although the elimination of the requirement of a secondary detection reaction provides for a weaker signal, it is less prone to a false positive result. Additionally this provides for less noise in the detection system and a better signal to noise ratio. Secondly, especially when used in high throughput systems, the use of a fluorescence labeled antibody reduces the time required for staining.

Herein is reported the expression of fluorescent antibody fusion proteins in HEK293 cells. Also reported is the purification of the fluorescent antibody fusion protein using protein A based purification techniques with yields similar to conventional antibody fusion proteins. The fluorescent antibody fusion proteins bind its target comparable to the non-fluorescent parent antibodies. The fluorescence properties including the pH dependency of citrine fluorescence were not altered by fusion to the antibody, especially to antibodies of the class IgG. These fluorescent antibody fusion proteins are not only tools with multiple application in diagnostics and biotechnology, but also serve as a photostable, pH sensitive tool to study cellular surface receptors. For example, due to the pH-dependency of the Citrine fluorescence IgG-Citrine fusion proteins are a valuable

tool to track antibody binding/internalization, followed by endosome acidification, or further routing to intracellular compartments in live cells.

It has been found that the fluorescent antibody fusion proteins as reported herein can be obtained with a high monomeric content if the fluorescent moiety is linked via a peptide bond to the C-terminus of an antibody fragment or an antibody chain. Furthermore, the fluorescent antibody fusion protein can be obtained in substantially homogeneous monomeric form by a chromatograpic purification when the fluorescent moiety is not directly linked to the C-terminus of the antibody fragment or antibody chain but is linked via a connector to the C-terminus. Especially suited is a connecter that has the amino acid sequence of SEQ ID NO: 18. It has further been found that this effect is pronounced when a fluorescent citrine moiety is linked to the C-terminus of an antibody light chain and when the complementary fragments of a fluorescent citrine moiety are linked to the C-terminus of an antibody heavy chain.

The term "fluorescent antibody fusion protein in aggregated form" and grammatical equivalents thereof denotes an fluorescent antibody fusion protein which is associated, either covalently or non-covalently, with at least one additional antibody molecule, and which is eluted in a single peak from a size exclusion chromatography column. The term "fluorescent antibody fusion protein in monomeric form" and grammatical equivalents thereof denotes that at least 95 % of a fluorescent antibody fusion protein are present in monomeric form, i.e. in substantially monomeric form. The term "in monomeric and in aggregated form" denotes a mixture of fluorescent antibody fusion proteins not associated with other antibody molecules and of fluorescent antibody fusion proteins associated with other antibody molecules. In this mixture neither of the monomeric form nor the aggregated form is present exclusively. The term "fluorescent antibody fusion protein in monomeric form" and grammatical equivalents thereof denotes a fluorescent antibody fusion protein that is not associated with a second antibody molecule, i.e. neither covalently nor non-covalently bound to another antibody molecule.

As antibodies are hetero-tetrameric proteins, multiple points to fuse citrine to an antibody can be used. The fluorescent protein can be fused to the N- or C-terminus of either antibody chain, thus it can be fused to the heavy or light chain. Techniques for producing bispecific antibodies can be used e.g. to target only one

of the dimeric heavy or light chains. Therefore, different formats can be generated with either one or two fused citrine moieties (see Figure 1).

The term "antibody" denotes a protein consisting of one or more polypeptide(s) substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the different constant region genes as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies. A "complete antibody" denotes a molecule that comprises two light chain polypeptides (light chains) and two heavy chain polypeptides (heavy chains). Each of the heavy and light chain polypeptides contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light chain polypeptides comprises a constant region (generally the carboxyl terminal portion). The variable domain of an immunoglobulin's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR). A "full length light chain" comprises a light chain variable domain (V_L) and a light chain constant domain (C_L). A "full length heavy chain" comprises a heavy chain variable domain (V_H), the heavy chain constant domains 1 to 3 (C_H1, C_H2, C_H3) and a hinge region.

Applying optimized connector sequences, one or more citrine molecules can be fused to different positions of the antibody without interfering with folding, secretion or function of the fusion protein. These proteins can be transiently expressed and purified to similar yields as unmodified antibodies using standard techniques. IgG-citrine fusions fully retain binding specificity and affinity of the antibody part and can be applied to assays that require fluorescent labeled IgG.

For example, in one exemplary fluorescent antibody fusion protein a citrine part can be at each C-terminus of the light chains using a connector. This fusion protein comprises an antibody part and two fluorescent protein moieties. The connector can be selected for example from no linker, the $(G_4S)_2$-connector (SEQ ID NO: 18), the $(G_4S)_3$-connector (SEQ ID NO: 19), or the GIHRPVAT-connector (SEQ ID NO: 20), whose sequence is derived from the commonly used eGFP-N2 expression vector (obtainable from BD Biosciences Clontech Cat. No 6081-1) (see Table 1). Further connectors are listed in Table 2. It has been found that using a connector of SEQ ID NO: 18 provides for a molecule that can be obtained in substantially monomeric form.

Table 1: Summary of fluorescent antibody fusion proteins.

targeting entity	**kind of citrine moiety**	**number of citrine moieties**	**fused to**	**connector**	**amount of purified protein per liter of cultivation supernatant (after SEC)**
anti-digoxygenin antibody	none	0	none	none	25 mg**
anti-IGF-1 R antibody	none	0	none	none	20 mg**
bispecific anti-IGF-1 R and digoxygenin antibody	none	0	none	none	15 mg**
anti-digoxygenin antibody	citrine	2	C-terminus of light chain	$(G_4S)_2$	23 mg
anti-digoxygenin antibody (knobs into holes)	citrine	1	C-terminus of heavy chain	$(G_4S)_2$	13 mg
anti-digoxygenin antibody (knobs into holes)	split-citrine	1	C-terminus of heavy chain / C-terminus of heavy chain	$(G_4S)_2$	3 mg
anti-IGF-1 R antibody	citrine	2	C-terminus of light chain	$(G_4S)_2$	5 mg
bispecific anti-IGF-1 R and digoxygenin antibody	citrine	2	C-terminus of light chain	$(G_4S_)_2$	4 mg

targeting entity	kind of citrine moiety	number of citrine moieties	fused to	connector	amount of purified protein per liter of cultivation supernatant (after SEC)
bispecific anti-IGF-1 R and digoxygenin antibody	citrine	1	scFv-citrine-scFv	$(G_4S)_2$ -X-$(G_4S)_2$	2 mg
anti-digoxygenin antibody	citrine	2	C-terminus of light chain	GIHRPVAT	6.7 mg
anti-digoxygenin antibody	citrine	2	C-terminus of light chain	none	4.5 mg
anti-digoxygenin antibody	eGFP	2	C-terminus of light chain	$(G_4S)_2$	3 mg
anti-IGF-1 R antibody	eGFP	2	C-terminus of light chain	$(G_4S)_2$	2.6 mg
anti-digoxygenin antibody (knobs into holes)	split-eGFP	1	C-terminus of heavy chain / C-terminus of heavy chain	$(G_4S)_2$	----

**: average value of multiple preparations.

Table 2: Connectors.

Connector	SEQ ID NO:
G	-
N	-
GST	-
LSLSGG	24
LSLSPGG	25
LSLSPGK	26
LSPNRGEC	27
GIHRPVAT	20
$(GQ_4)_3$	28

Connector	SEQ ID NO:
$(GQ_4)_3G$	29
$(GQ_4)_3GNN$	30
$(G_2S)_3$	31
$(G_2S)_4$	32
$(G_2S)_5$	33
$(G_3S)_3$	34
$(G_3S)_4$	35
$(G_3S)_5$	36
$(G_3S)_5GGG$	37
$(G_4S)_2$	18
$(G_4S)_2G$	38
$(G_4S)_2GG$	39
$(G_4S)_2GGG$	40
$(G_4S)_2GN$	41
$(G_4S)_3$	19
$(G_4S)_3G$	42
$(G_4S)_3T$	43
$(G_4S)_3GG$	44
$(G_4S)_3GGT$	45
$(G_4S)_3GGN$	46
$(G_4S)_3GAS$	47
$(G_4S)_4$	48
$(G_4S)_5$	49
$(G_4S)_5G$	50
$(G_4S)_5GG$	51
$(G_4S)_5GAS$	52
$G(S)_{15}G$	53
$G(S)_{15}GAS$	54

One exemplary fluorescent antibody fusion protein comprises only one fluorescent protein moiety. Therefore the C-terminus of only one chain is fused to a fluorescent citrine moiety (see Figure 1). To produce this fusion protein the “knobs into holes” technique can be used. With this technique two different heavy chains can be combined in one antibody. Therefore, one heavy chain can be with “hole” mutations and one heavy chain can be with the “knob” mutation and one of them is fused via a $(G_4S)_2$-connector to a single fluorescent citrine moiety. The “knob into holes” technology is reported in e.g. WO 96/027011; Ridgway, J.B., et al., Protein Eng. 9 (1996) 617-621; Merchant, A.M., et al., Nat. Biotechnol. 16 (1998) 677-681; Atwell, S., et al. J. Mol. Biol. 270 (1997) 26-35.

Another exemplary fluorescent antibody fusion protein comprises the fluorescent citrine moiety as two complementary fragments wherein each fragment is

conjugated to the C-terminus of a different antibody heavy chain. To produce this fusion protein the "knobs into holes" technique can be used. Thus, one heavy chain can be with a "knob" mutation and comprises the N-terminal fragment of the fluorescent citrine moiety connected via a $(G_4S)_2$-connector. The complementary fragment of the citrine moiety can be attached to the heavy chain with "hole" mutations via a $(G_4S)_2$-connector and comprises the C-terminal fragment of the fluorescent citrine moiety connected via a $(G_4S)_2$-connector (Figure 1). When the antibody is folded in the endoplasmatic reticulum (ER) both heavy chains come in close contact and the corresponding fragments of the citrine moiety are brought together and form the fluorescent moiety, which has the same biophysical properties as a single chain fluorescent citrine moiety. They can be connected for example by a $(G_4S)_2$-connector.

The term "fluorescent citrine moiety" denotes a fluorescent protein consisting of a single contiguous amino acid chain that has an excitation maximum at about 516 nm and an emission maximum at about 529 nm. The term "about" denotes in this context a variance of +/- 2 nm. In one embodiment the fluorescent citrine moiety has the amino acid sequence of SEQ ID NO: 21. The term "fragment of a fluorescent citrine moiety" denotes a fragment of a fluorescent citrine moiety that when combined with the corresponding other fragment provides for a moiety with about the same excitation maximum and about the same emission maximum as a fluorescent citrine moiety but which in turn is consisting of two amino acid chains. In one embodiment the corresponding fragments of a fluorescent citrine moiety have an amino acid sequence of SEQ ID NO: 22 and 23.

A further exemplary fluorescent antibody fusion protein comprises a bi-specific antibody. In this format one or two binding sites of the first specificity that bind to a first epitope or antigen can be formed by the pairs of heavy and light variable domains of a full length antibody. The additional one or two binding sites of the second specificity binding to a second epitope or antigen can be formed by two, optionally disulfide stabilized, scFvs whereby each of the C-termini of the heavy chains of the full length antibody can be conjugated to a single, i.e. only one, scFvs, e.g. connected via a $(G_4S)_2$-connector (see Figure 1).

Also an exemplary fluorescent antibody fusion protein comprises two disulfide stabilized scFvs binding to the same or two different epitopes or antigens, wherein one scFv can be conjugated to the N-terminus of the citrine moiety via a $(G_4S)_2$-connector and the second scFv can be conjugated via the same or a different

connector to the C-terminus of the citrine moiety. For purification purposes this format can further comprise an additional hexahistidine-tag at the C-terminus.

Some of the exemplary fluorescent antibody conjugates as outlined above have been obtained wherein the citrine moiety had been replaced by the eGFP moiety (see Table 1).

Production and purification

The fluorescent antibody fusion proteins as reported herein can be transiently expressed in human embryonic kidney cells, such as HEK 293 cells. The fusion proteins can be secreted into the cell culture media like not conjugated or fused antibodies. The fluorescent antibody fusion proteins can be purified by a protein A affinity chromatography and/or a size exclusion chromatography. Hexahistidine-tag comprising fusion proteins alternatively can be purified by a Ni-chelate affinity chromatography. In Figure 2a an exemplary elution profile of a protein A chromatography of a fusion protein comprising one citrine moiety at each C-terminus of the light chains is shown. In Figure 2b an analytical SEC elution profile of the same fusion protein as above after SEC purification is shown.

The purification yields obtained are shown in Table 1. Purified protein recovered from one liter of cell culture supernatant after transient expressions (without optimization of expression conditions) were e.g. 25 mg/l for the non-fused parent antibody and 23 mg/l for a fusion protein comprising fluorescent citrine moieties conjugated to the light chains. The yield of fusion proteins comprising complementary fragments of a fluorescent citrine moiety is sufficient for e.g. in vitro analytics or microscopy.

When the connector for linking the citrine moiety to the antibody was omitted and the citrine moiety was directly fused to the antibody, a significant reduction of the yield can be observed (see Table 1). Furthermore, the purified recombinant protein can contain a higher percentage of aggregates and can be prone to precipitation (see Table 3).

Table 3: Aggregate content of different fluorescent antibody fusion proteins.

targeting entity	kind of citrine moiety	number of citrine moieties	fused to	connector	aggregate content after SEC purification	aggregate content prior SEC purification
anti-digoxygenin antibody	citrine	2	C-terminus of light chain	$(G_4S)_2$	1.3 %	25.5 %
anti-digoxygenin antibody	citrine	2	C-terminus of light chain	GIHRPVAT	50 %	60 %
anti-digoxygenin antibody	citrine	2	C-terminus of light chain	none	32 %	55 %
anti-digoxygenin antibody	citrine	1	C-terminus of heavy chain	$(G_4S)_2$	n.d.	34 %
anti-digoxygenin antibody	split-citrine	1	C-terminus of heavy chain	$(G_4S)_2$	4.5 %	57 %
bispecific anti-IGF-1 R and digoxygenin antibody	citrine	2	C-terminus of light chain	$(G_4S)_2$	3 %	59 %
anti-digoxygenin antibody	eGFP	2	C-terminus of light chain	$(G_4S)_2$	10 %	63 %
anti-IGF-1 R antibody	eGFP	2	C-terminus of light chain	$(G_4S)_2$	0.8 %	25 %

Supernatants and cell lysates of cells expressing fusion proteins comprising citrine or eGFP show comparable amounts of total protein mass (as analyzed by western blot, Figure 5). Fusion proteins comprising eGFP show a reduction of yield of properly folded active protein after purification. Furthermore, eGFP cannot be used in the approach that has two complementary fragments of the fluorescent protein on both C-termini of the heavy chain as no protein could be purified despite the fact that expression could be observed (as analyzed by western blot, Figure 5).

Antigen binding

The fluorescent antibody fusion proteins can be captured onto the surface of a chip using e.g. an antibody specific for the human Fc region. Thereafter the binding to Myoglobine carrying one antigen moiety can be analyzed (see Figure 3) and K_D

values can be determined (see Table 4). The fluorescent antibody fusion proteins as reported herein have affinities comparable to not conjugated parent antibody. This is independent of the localization of the citrine moiety in the fusion protein.

Table 4: Antigen binding properties of different antibody formats.

Molecule	Kind of citrine moiety	k_a (1/Ms)	k_d (1/s)	K_D (M)
bispecific anti-IGF-1 R and digoxygenin antibody	no citrine	$3.4*10^5$	0.013	$3.9*10^{-08}$
bispecific anti-IGF-1 R and digoxygenin antibody	2 citrine moieties on light chain	$3.3*10^5$	0.014	$4.1*10^{-08}$
anti-digoxygenin antibody	no citrine	$6.1*10^5$	0.011	$1.8*10^{-08}$
anti-digoxygenin antibody	2 citrine moieties on light chain	$5.9*10^5$	0.01	$1.7*10^{-08}$
anti-digoxygenin antibody	1 citrine moiety on heavy chain	$6.7*10^5$	0.009	$1.4*10^{-08}$
anti-digoxygenin antibody	citrine moiety fragments on heavy chains	$7.7*10^5$	0.009	$1.1*10^{-08}$

pH dependency of fluorescence

To analyze whether the fusion to antibodies alters the pH dependent fluorescence of the citrine moiety an ELISA based assay can be used.

For this assay 50 nM of the different fluorescent antibody fusion proteins can be incubated in buffer adjusted to the respective pH value. Thereafter the sample can be excited with light of 516 nm and the fluorescence can be determined at 529 nm. Fluorescence can be measured and normalized to 1 for the highest value for all fusion proteins independently in order to generate a value independent from the number of citrine moieties in the fusion protein.

The data (see e.g. Figure 4) shows that independent of the localization of the citrine moiety in the fusion protein the fluorescence of the fusion protein is pH-dependent. The fluorescence of the citrine moiety is highest at pH values above pH 7.5. The fluorescence diminishes by lowering the pH value and follows a sigmoid curve with a fluorescence of 50 % of the maximum value at a pH value of about pH 5.5. The complemented citrine composed of two corresponding fragments attached separately to the two heavy chain C-termini had the same properties (see Figure 4).

Fluorescent FABs

Antibodies can be cleaved by the proteases pepsin or papain to obtain smaller modules called FAB or FAB_2, respectively. Thus, fluorescent antibody fusion proteins comprising citrine at one terminus of each light chain can be used to obtain fluorescent FAB or FAB_2 molecules.

In order to obtain fluorescent antibody fragment fusion proteins comprising one citrine moiety at each of the C-termini of the light chains of the fusion protein can be digested with papain. The resulting protein mixture can be purified via a protein A affinity chromatography step to remove the Fc part.

Staining of fixed cells

Upon binding to a target receptor the bound antibody can not only block further ligand binding, but can also induce internalization of the receptor-antibody complex via the endocytotic pathway.

For example, after applying an anti-IGF-1 receptor antibody comprising fluorescent antibody fusion protein to target IGF-1 receptor positive cells the sample can be either fixed immediately or can be incubated further in growth medium. After the further incubation the cells can be fixed.

The fluorescent signal of citrine moieties obtained with immediately fixed cells that can be observed in the FITC channel is distributed equally over the cell surface (see Figure 6). The signals that can be obtained with a secondary antibody against the kappa light chain shows that both fluorescence signals are found to be co-localized.

The majority of the fluorescent signal of the citrine moiety obtained with further incubated cells can be found in intracellular vesicles (see Figure 6). Only a minor fraction of florescence intensity can be found on the cell surface. The signals that can be obtained with a secondary antibody against the kappa light chain shows that

both fluorescence signals are co-localized. However, a few membrane bound structures with dominant green fluorescence can be observed (see arrow in Figure 6).

The fluorescent antibody fusion protein comprising citrine are not prone to rapid proteolysis once internalized into cells and exposed to the cellular interior for prolonged time.

Imaging living cells

A fluorescent monospecific anti-IGF-1 receptor antibody fusion protein and a fluorescent bispecific anti-IGF-1 receptor and anti-digoxygenin antibody fusion protein comprising citrine moieties can be applied to IGF-1 receptor positive cells. From the signal that can be obtained with a secondary antibody against the Fc-region it can be seen that binding of both the anti-IGF-1 receptor antibody fused to one citrine moiety at each of the C-termini of the light chains as well as the anti-IGF-1 receptor and digoxygenin bispecific antibody fusion protein comprising one citrine moiety at each of the C-termini of the light chain can be determined (see Figure 7a). As can be seen from Figure 7b neither the cells nor the secondary antibodies gives rise to a significant signal, whereas both the anti-IGF-1 receptor antibody fused to citrine as well as the anti-IGF-1 receptor and anti-digoxygenin bispecific antibody fused to citrine gives rise to a signal in the FITC channel.

A bispecific monovalent fusion protein comprising citrine between an anti-IGF-1 receptor scFv and an anti-digoxygenin scFv can be constructed. It can be shown (see Figure 7c) that the module binds to the target cells. In the same experiment a fluorescent labeled digoxygenin can be used. Only a minor binding of the fluorescent labeled digoxygenin to the cells can be detected (see Figure 7c). The binding can be enhanced strongly in the presence of the monovalent bispecific module (see Figure 7c).

Monitoring intracellular acidification

Upon binding to a cell surface receptor, such as the IGF-1 receptor, an antibody can induce internalization of the receptor into intracellular vesicles. The vesicles can be transported along the endocytotic pathway and can be acidified, i.e. from a neutral pH value at their internalization via a pH value of about pH 6 to pH 5.5 in endosomal compartments to a pH value of about pH 5 in lysosomal compartments.

To prevent the acidification after internalization weak bases, which accumulate in acidic compartments and lead to an increase of the luminal pH value, can be used.

For example, one population of cells can be treated with an NH_4Cl solution prior to the antibody incubation, whereas a different population of cells can be left untreated. Both populations can be treated thereafter with the fluorescent anti-cell surface receptor antibody fusion protein comprising citrine for one hour on ice to allow binding of the antibody but preventing internalization. After the incubation, a fraction of both populations can be analyzed by FACS for antibody binding (for an example see Figure 8a). Independent of the pre-treatment with an NH_4Cl solution both populations show a similar antibody binding to the cell surface monitored by citrine fluorescence. After incubation at 37 °C the surface-bound antibodies are co-internalized with the receptors they are bound to. Thereafter cells without NH_4Cl solution pre-treatment show a significant reduction in fluorescence as compared to pre-treated cells (see Figure 8b). This shows that the fluorescence of fluorescent antibody fusion proteins comprising citrine can be reduced by acidification.

The fusion proteins as reported herein can provide for a framework of a fluorescent antibody fusion protein wherein solely with the exchange of the V regions fluorescent antibodies with a specificity of interest can be obtained.

Thus, the combination of citrine with secreted receptor ligands or extracellular portions of receptors can be used in research and diagnostics. For example, a citrine tagged receptor can be used to analyze binding of a cyan labeled ligand by FRET analysis. If the complementary fragments of a citrine moiety are attached to the N-termini of a light and a heavy chain, it can be possible to distinguish association of cognate pairs of light and heavy chains. Another application can be that the association and dissociation of antibodies of the IgG4 isotype can be detected via corresponding citrine fragments attached to the constant regions. Furthermore, a module with three fluorescent subunits can be generated by simple combination of the elements as reported herein. The reduction of the fluorescent properties of citrine when exposed to reduced pH can be used as a marker for tracking of the intracellular trafficking of endocytosed receptors. Thus, fluorescent antibody fusion proteins as reported herein can be used to screen for factors that interfere with the acidification of endosomal compartments, or can be used to identify factors that interfere with the integrity of endosomal membranes.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Sequence Listing

SEQ ID NO: 1	anti-digoxygenin antibody light chain with a $(G_4S)_2$ connected citrine
SEQ ID NO: 2	anti-digoxygenin antibody heavy chain "knob" variant with a $(G_4S)_2$ connected citrine
SEQ ID NO: 3	anti-digoxygenin antibody heavy chain "hole" variant
SEQ ID NO: 4	anti-digoxygenin antibody heavy chain "knob" variant with a $(G_4S)_2$ connected citrine fragment comprising amino acids 1-158 of citrine
SEQ ID NO: 5	anti-digoxygenin antibody heavy chain "hole" variant with a $(G_4S)_2$ connected citrine fragment comprising amino acids 159-239 of citrine
SEQ ID NO: 6	anti-IGF-1 R antibody light chain with a $(G_4S)_2$ connected citrine
SEQ ID NO: 7	anti-IGF-1 R disulfide stabilized scFv with a $(G_4S)_2$ connected citrine with a $(G_4S)_2$ connected anti-digoxygenin disulfide stabilized scFv
SEQ ID NO: 8	anti-digoxygenin antibody light chain with a GIHRPVAT connected citrine
SEQ ID NO: 9	anti-digoxygenin antibody light chain with no linker connected citrine
SEQ ID NO: 10	anti-digoxygenin antibody light chain with a $(G_4S)_2$ connected eGFP
SEQ ID NO: 11	anti-IGF-1 R antibody light chain with a $(G_4S)_2$ connected eGFP
SEQ ID NO: 12	anti-digoxygenin antibody heavy chain "knob" variant with a $(G_4S)_2$ connected eGFP fragment comprising amino acids 1-158 of eGFP
SEQ ID NO: 13	anti-digoxygenin antibody heavy chain "hole" variant with a $(G_4S)_2$ connected eGFP fragment comprising amino acids 159-239 of eGFP
SEQ ID NO: 14	anti-digoxygenin antibody light chain

SEQ ID NO: 15	anti-digoxygenin antibody heavy chain
SEQ ID NO: 16	anti-IGF-1 R antibody heavy chain
SEQ ID NO: 17	anti-IGF-1 R antibody heavy chain with a $(G_4S)_3$ connected anti-digoxygenin disulfide stabilized scFv
SEQ ID NO: 18	$(G_4S)_2$ connector
SEQ ID NO: 19	$(G_4S)_3$ connector
SEQ ID NO: 20	GIHRPVAT connector
SEQ ID NO: 21	Citrine moiety amino acid sequence
SEQ ID NO: 22	Fractional citrine moiety amino acid sequence
SEQ ID NO: 23	Fractional citrine moiety amino acid sequence
SEQ ID NO: 24 to 54	Connectors

Description of the Figures

Figure 1 Different formats of fluorescent antibody fusion proteins:
a) a citrine moiety fused to both light chains,
b) a citrine moiety fused to both light chains of a bispecific antibody,
c) a citrine moiety fused to one heavy chain using knobs into holes,
d) a citrine moiety assembly via two corresponding fragments using knobs into holes,
e) a citrine moiety between two single chain antibodies.

Figure 2 Expression and purification of Citrine fusion proteins: (a) Isolation of the fusion protein from cell culture supernatant by protein A affinity chromatography; (b) fusion protein containing fractions from the affinity chromatography can be identified by fluorescence in ambient light; (c) size exclusion chromatography confirms that purified fusion proteins are of monomeric status, preparations do not contain protein aggregates.

Figure 3 Fluorescent antibody fusion proteins retain full binding specificity and affinity of their respective parent antibody.

Figure 4 All fusion proteins fully retain the pH-dependence of the citrine fluorescence.

Figure 5 Western blot of supernatants and cell lysates of cells expressing fusion proteins comprising citrine or eGFP.

Figure 6 Fusion proteins to detect cell binding and internalization: Confocal microscopy demonstrates that binding and incubation

on ice (upper panel) displays citrine fluorescence on cell surfaces due to antibody-mediated attachment to the cell surface; subsequent incubation at 37 °C (lower panel) shows antigen mediated internalization of citrine. Antibody-signals and citrine signals are co-localized (right panels) which indicates that the entities of the fusion protein do not separate within cells during the course of this experiment. The internalization of the anti-IGF-1 receptor antibody citrine fusion protein was comparable to an antibody against the IGF-1 receptor lacking the citrine moiety. When stained with a secondary antibody, the internalization patterns observed were comparable. No binding was observed to antigen negative cell lines. On these cell lines, no internalization was observed as well.

Figure 7 IgG-citrine fusion proteins to detect binding to live cells: FACS analyses on live cells shows specific citrine fluorescence on antigen positive cells due to antibody-mediated targeting of the citrine moiety. The binding of the anti-IGF-1 receptor antibody citrine fusion protein was comparable to an antibody against the IGF-1 receptor lacking the citrine moiety when stained with a secondary antibody. However, this antibody did not give rise to a significant signal in the FITC channel. No binding was observed to antigen negative cell lines. On these cell lines, no citrine signal was observed in the FITC channel as well.

Figure 8 IgG-citrine fusions to track acidification in living cells: FACS analyses on live cells shows binding of an anti-IGF-1 receptor antibody citrine fusion protein to IGF1R positive target cells (a). After one hour, citrine fluorescence of an internalized anti-IGF-1 receptor antibody citrine fusion protein is diminished due to decrease of pH (b). This loss can be counteracted by one hour preincubation of the target cells with the weak base NH_4Cl. Under these conditions endosomal acidification is inhibited. Binding of anti-IGF-1 receptor antibody citrine fusion protein is not inhibited (a) but the loss of citrine fluorescence is diminished (b).

Example 1

Encoding sequence of fluorescent antibody fusion protein comprising citrine

DNA stretches of interest were synthesized (Geneart, Regensburg, Germany). Subsequently, the synthesized DNA stretches were subcloned into appropriate expression vectors and amplified in E.coli XL1Gold (Invitrogen).

The coding sequence of an exemplary anti-IGF-1 receptor antibody is reported in WO 2004/087756 and WO 2007/045465 and WO 2007/115814. The coding sequence of an exemplary anti-digoxygenin antibody is reported in EP 09164612.5.

Example 2

Expression and purification of fluorescent antibody fusion proteins comprising citrine

The fluorescent antibody fusion proteins comprising citrine were expressed by transient transfection of human embryonic kidney 293 cells using the FreeStyle™ 293 Expression System according to the manufacturer's instruction (Invitrogen, USA). Light and heavy chain encoding nucleic acids of the corresponding antibodies were constructed in expression vectors carrying pro- and eukaryotic selection markers. These plasmids were amplified in E.coli, purified, and subsequently applied to transient transfection. The suspension FreeStyle™ 293 cells were cultivated in FreeStyle™ 293 Expression 25 medium at 37 °C/8 % CO_2. Therefore the cells were seeded in fresh medium at a density of 1-2 x 10^6 viable cells/ml on the day of transfection. The DNA-293fectin™ complexes were prepared in Opti-MEM medium (Invitrogen, USA) using 333 µl of 293fectin™ (Invitrogen, Germany) and 250 µg of heavy and light chain plasmid DNA in a 1:1 molar ratio for a final transfection volume of 250 ml. The fluorescent antibody fusion protein containing cell culture supernatant was clarified 7 days after transfection by centrifugation at 14,000 g for 30 minutes and filtrated through a sterile filter (0.22 µm). Supernatants were stored at -20 °C until purification.

The recombinant protein was purified from the supernatant by a two step affinity chromatography using protein A-Sepharose™ (GE Healthcare, Uppsala, Sweden) and Superdex™200 size exclusion chromatography. Briefly, the clarified culture supernatant was applied on a HiTrap 15 ProteinA HP (5 ml) column equilibrated with PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Unbound protein was removed by washing with equilibration buffer. The fusion protein was recovered with 0.1 M citrate buffer, pH 2.8, and the fusion

protein containing fractions were neutralized with 0.1 ml 1 M Tris, pH 8.5. The fusion protein comprising fractions were pooled, concentrated with an Amicon Ultra centrifugal filter device (MWCO: 30,000 Da, Millipore) to a final volume of 3 ml. The concentrated solution was loaded on a Superdex^{TM}200 HiLoad 120 ml 16/60 gel filtration column (GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM histidine, 140 mM NaCl, pH 6.0. The protein concentration was determined by measuring the optical density (OD) at 280 nm with the OD at 320 nm as the background correction, using a molar extinction coefficient of the proteins calculated on basis of the amino acid sequence. Fusion protein comprising fractions were pooled, snap-frozen and stored at -80 °C. The homogeneity of the recombinant proteins was confirmed by SDS-PAGE in the presence and absence of a reducing agent (5 mM 1,4-dithiotreitol) and staining with Coomassie brilliant blue. The NuPAGE® Pre-Cast gel system (Invitrogen, USA) was used according to the manufacturer's instruction (4-20 % Tris-Glycine gels).

Example 3

Surface plasmon resonance

To analyze the binding properties of the recombinant fluorescent antibody fusion proteins surface plasmon resonance (SPR) technology using a BIAcore T100 or BIAcore 3000 instrument was employed (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Therefore a capturing anti-human IgG antibody was immobilized on the surface of a CM5 biosensor chip using amine coupling chemistry according to then manufacturer's instructions. Flow cells were activated with a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.1 M 3-(N,N-dimethylamino) propyl-N-ethylcarbodiimide at a flow rate of 5 µl/min. The anti-human IgG antibody was injected in 10 mM sodium acetate buffer, pH 5.0, at 2 µg/ml resulting in a surface density of approximately 500 RU. A reference control flow cell was treated in the same way with vehicle buffers instead of the capturing antibody. Surfaces were blocked with an injection of 1 M ethanolamine/HCl pH 8.5. To evaluate the anti-digoxygenin antibodies binding to mono-digoxygenylated Myoglobine was analyzed. In order to determine binding affinities digoxygenylated Myoglobine was injected at increasing concentrations. The regeneration of the flow cell was carried out by injecting a 0.85 % (w/v) H_3PO_4 for 60 sec. at 5 µl/min. and then injecting 5 mM NaOH for 60 sec. at 5 µl/min. The samples to be analyzed were diluted in HBS-P buffer (10 mM HEPES buffer, pH 7.4, comprising 150 mM NaCl, 0.005 % Surfactant P20) and injected at a flow rate of 5 µl/min. The contact time was 3 min. for the antibodies at a concentration between 1 nM and 5 nM. The

dissociation time was 5 min. for each molecule at a flow rate of 30 µl/min. All determinations were performed at 25 °C. Signals were detected at a rate of one signal per second.

Example 4

PH dependent fluorescence

To measure the pH dependent fluorescence of the fluorescent antibody fusion proteins the plate fluorescence reader Safire2 (Tecan Systems, Austria) was used. Buffers of the pH values of pH 2.0, pH 4.0, pH 5.0, pH 5.5, pH 6.0, pH 7.4 and pH 9.0 were generated by adjusting PBS using either HCl or NaOH. A pH row of increasing values was generated in a multi-well plate and the different fusion proteins were added to a final concentration of 50 nM in a total volume of 150 µl. Subsequently, the multi-well plate was shaken for 30 sec. at 750 rpm. Fluorescence was excited at 516 nm for fusion proteins comprising a citrine moiety and emission was determined at 529 nm. Both emission and excitation bandwidth were 5 nm. Each fluorescence read was performed 10 times with an integration time of 40 µs.

Example 5

FAB cleavage

FAB fragments can be obtained by cleavage with papain (Roche, Cat # 11546644) in 20 mM histidine, 140 mM NaCl, pH 6.0. The solution can be incubated for 1.5 hours at 37 °C. The sample can be immediately analyzed by SEC (GFC300) and SDS-PAGE in the presence and absence of a reducing agent (5 mM 1,4-dithiotreitol) and staining with Coomassie brilliant blue. The NuPAGE® Pre-Cast gel system (Invitrogen, USA) can be used according to the manufacturer's instruction (4-20 % Tris-Glycine gels).

Example 6

Microscopy

For microscopy I24 cells were grown on glass coverslips to a density of about 50-70 %. Afterwards they were treated with the fluorescent antibody fusion protein in a concentration of 5 nM for two hours on ice. Thereafter the cells were washed in cold PBS and one set was fixed immediately. A second set of cells was incubated at 37 °C for the respective durations. The incubated cells were fixed with paraformaldehyde. For staining, the fixed cells were washed, incubated with the blocking reagent GSDB and incubated with a rabbit anti-human kappa light chain

antibody (DAKO) at a concentration of 6.5 µg/ml for 1.5 to 2 hours in a humidity chamber. After another wash, the cells were incubated with a Cy3-labeled goat anti-rabbit antibody (Molecular Probes) in a concentration of 28.6 µg/ml for 1.5 hours in a humidity chamber. Then the cells were washed. Next, the DNA was labeled with DAPI (Roche Diagnostics GmbH, Mannheim, Germany) at a concentration of 10 µg/ml for 2-3 min., washed again and covered with mounting medium. The cells were analyzed with a Leica SP20 confocal microscope.

Example 7

FACS analysis

For FACS analysis, MCF7 cells were detached by incubation in Accutase for 15 min. After washing in FACS buffer (PBS containing 5 % FCS) the cells were seeded in a 96 well rounded bottom multi-well plate (Corning Inc. Cat# 3799, USA) to a final density of 3 x 105 cells/ml and used immediately. The cells were incubated in the presence of 3.43 nM of fluorescent antibody fusion protein or isotype control antibody (Jackson Immunoresearch Laboratories) for 30 min. on ice to allow binding but prevent internalization. For detection of bound antibodies, a secondary Cy5 labeled antibody (Jackson Immunoresearch Laboratories; 709- 15 176-1490; Cy5 labeled $F(ab')_2$ donkey anti-human IgG (H +L) antibody) was added to a final concentration of 3.43 nM. The cells were subsequently washed in FACS buffer to remove unbound antibody. After washing the cells incubated with the small scFv module were incubated with DIG-Cy5 in an equimolar concentration. After another washing step 10,000 cells were analyzed with the FACS canto II (BD Biosciences). Cy5 was analyzed in the Cy5 channel, citrine fluorescence was analyzed using the FITC channel.

Example 8

FACS pH analysis

To determine the pH dependency using FACS, one group of MCF7 cells were incubated in the presence of a 50 mM NH_4Cl solution for one hour before the start of the experiment. All subsequent steps for this group were carried out in the presence of a 50 mM NH_4Cl solution. The cells were detached by incubation in Accutase for 15 min. After washing in FACS buffer the cells were seeded in a 96 well rounded bottom multi-well plate (Corning Inc. Cat# 3799, USA) to a final density of 3 x 10^5 cells/ml and used immediately. The cells were incubated in the presence of 3.43 nM of fluorescent antibody fusion protein for 30 min. on ice to

allow binding but prevent internalization. The cells were subsequently washed in FACS buffer to remove unbound antibody. The cells were measured immediately and again after 2 hours with a FACS canto II (BD Biosciences). Citrine fluorescence was analyzed using the FITC channel.

Patent Claims

1. A fusion protein comprising
   (a) a citrine moiety, which is either a fluorescent citrine moiety or two complementary fragments of a fluorescent citrine moiety that upon combination form a fluorescent citrine moiety,
   (b) an antibody fragment comprising at least a light chain variable domain and a heavy chain variable domain,

   wherein
   (i) the fluorescent citrine moiety, or
   (ii) each of the complementary fragments of a fluorescent citrine moiety
   is linked to a single C-terminus of the antibody fragment via a peptide bond either directly or via a connector, and

   wherein each C-terminus of the antibody fragment is at most linked to one fluorescent citrine moiety or to one complementary fragment of a fluorescent citrine moiety.

2. The fusion protein according to claim 1, characterized in that the fluorescent citrine moiety or the complementary fragments of a fluorescent citrine moiety is linked to a single C-terminus of the antibody fragment via a peptide bond and via a connector that has the amino acid sequence of SEQ ID NO: 18.

3. The fusion protein according to any one of the preceding claims, characterized in that the fusion protein comprises either two fluorescent citrine moieties or two complementary fragments of a fluorescent citrine moiety.

4. The fusion protein according to any one of the preceding claims, characterized in the antibody fragment is consisting of two full length light chains and two full length heavy chains.

5. The fusion protein according to any one of the preceding claims, characterized in that the fusion protein comprises
   (a) two fluorescent citrine moieties,
   (b) a complete antibody consisting of two full length light chains and two full length heavy chains,

wherein each of the citrine moieties is linked to the C-terminus of an antibody light chain via a peptide bond independently of each other either directly or via a connector, and each C-terminus of the antibody light chains is linked to one citrine moiety.

6. The fusion protein according to any one claims 1 to 4, characterized in that the fusion protein comprises
   (a) one fluorescent citrine moiety,
   (b) a complete antibody consisting of two full length light chains and two full length heavy chains,

wherein the citrine moiety is linked to one C-terminus of an antibody chain via a peptide bond either directly or via a connector.

7. The fusion protein according to any one of claims 1 to 4, characterized in that the fusion protein comprises
   (a) two corresponding fragments of a fluorescent citrine moiety,
   (b) a complete antibody consisting of two full length light chains and two full length heavy chains,

wherein each of the fragments of the citrine moiety is linked to one C-terminus of an antibody heavy chain via a peptide bond independently of each other either directly or via a connector, and each C-terminus of the antibody heavy chains is linked to one fragment of the citrine moiety.

8. The fusion protein according to any one of claims 1 to 4, characterized in that the fusion protein comprises
   (a) two fluorescent citrine moieties,
   (b) a complete antibody consisting of two full length light chains and two full length heavy chains,
   (c) two single chain antibodies,

wherein the binding sites of the complete antibody bind to a first epitope or antigen and the binding sites of the two single chain antibodies bind to a second epitope or antigen different from the first one,

wherein each of the citrine moieties is linked to one C-terminus of an antibody heavy chain via a peptide bond independently of each other either

directly or via a connector, and each C-terminus of the antibody heavy chains is linked to one citrine moiety, and

wherein each of the single chain antibodies is linked to one C-terminus of an antibody light chain and each C-terminus of the antibody light chains is linked to one single chain antibody.

9. Use of a fusion protein according to any one of claims 1 to 8 for the fluorescence staining of cells.

10. Use of a fusion protein according to any one of claims 1 to 8 for high throughput fluorescence staining.

11. Method for staining cells comprising the following steps
   (a) providing a fusion protein according to any one of claims 1 to 8 with a binding site binding to a cell surface receptor, which upon binding can be internalized into the cell,
   (b) incubating a population of cells with the fusion protein,
   (c) dividing the incubated cell population in two sub-populations, whereof the first is fixed immediately and the second is further incubated prior to fixing.

12. Use of a fusion protein according to any one of claims 1 to 8 for monitoring of intracellular acidification.

13. Method for monitoring intracellular acidification comprising the following steps
   (a) providing a fusion protein according to any one of claims 1 to 8 with a binding site binding to a cell surface receptor, which upon binding can be internalized into the cell,
   (b) incubating a population of cells with the fusion protein,
   (c) determining the fluorescence intensity of the citrine moiety in samples removed from the incubated population of cells at defined time intervals and thereby monitoring intracellular acidification.

14. Use of a fusion protein according to any one of claims 1 to 8 for intracellular tracking.

15. Method for intracellular tracking comprising the flowing steps
    (a) providing a fusion protein according to any one of claims 1 to 8 with a binding site binding to a cell surface receptor, which upon binding can be internalized into the cell,
    (b) incubating a population of cells with the fusion protein,
    (c) determining the fluorescence localization of the citrine moiety in samples removed from the incubated population of cells at defined time intervals and thereby intracellular tacking the citrine moiety.

16. Method for detecting the localization of a polypeptide in a cell comprising the following steps
    (a) expressing in the cell or providing to the cell either (i) the polypeptide or (ii) a fusion protein comprising a) the polypeptide and b) a tag or a fragment of a citrine moiety,
    (b) (i) expressing in the cell or providing to the cell the corresponding other fragment of the citrine moiety, or
    (ii) providing to the cell a fusion protein comprising
        (a) the corresponding other fragment of the citrine moiety,
        (b) an antibody fragment comprising at least a light chain variable domain and a heavy chain variable domain,

    wherein the N-terminus of the corresponding other fragment of the citrine moiety is linked to one C-terminus of the antibody fragment either directly of via a connector, and

    wherein the antibody fragment is binding to a cell surface receptor, which upon binding can be internalized into the cell,

    and
    (c) detecting fluorescence in the cell, and thereby detecting the localization of the polypeptide in a cell.

17. A composition comprising a fluorescent antibody fusion protein according to any one of claims 1 to 8 in monomeric and aggregated form, characterized in that the fraction of aggregated fluorescent antibody fusion protein is 5 % or less when determined by size exclusion chromatography.

Fig. 1

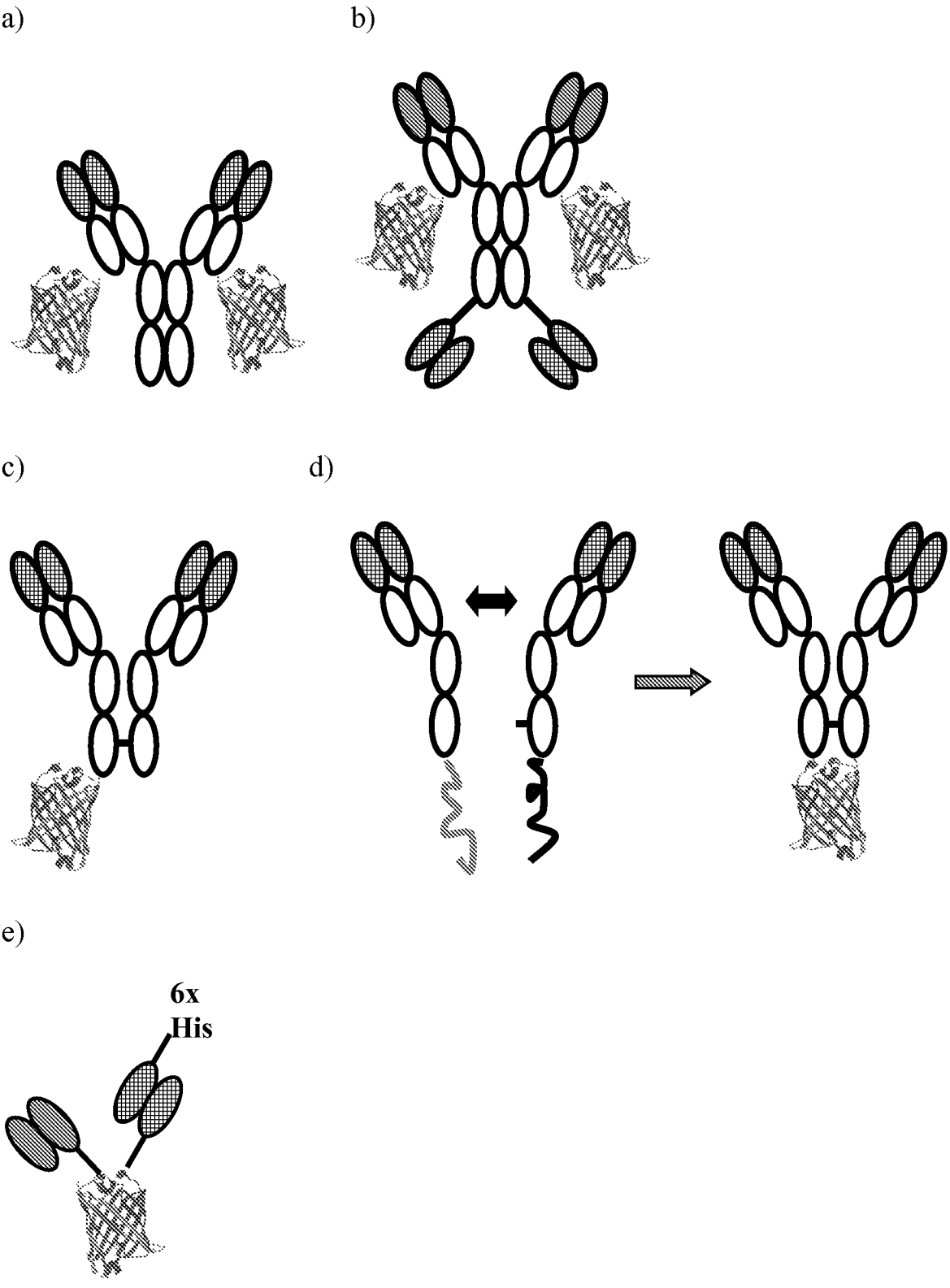
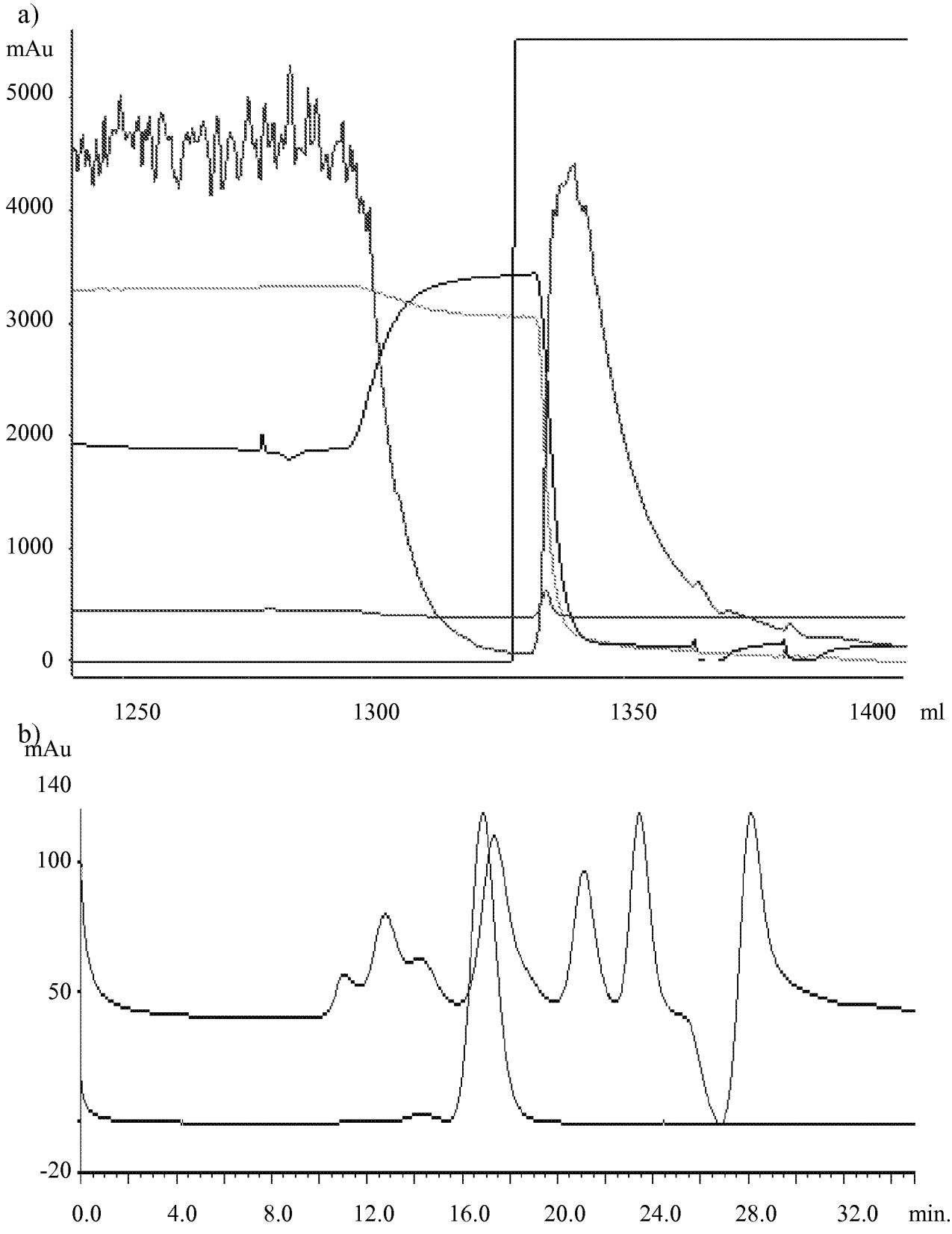


Fig. 2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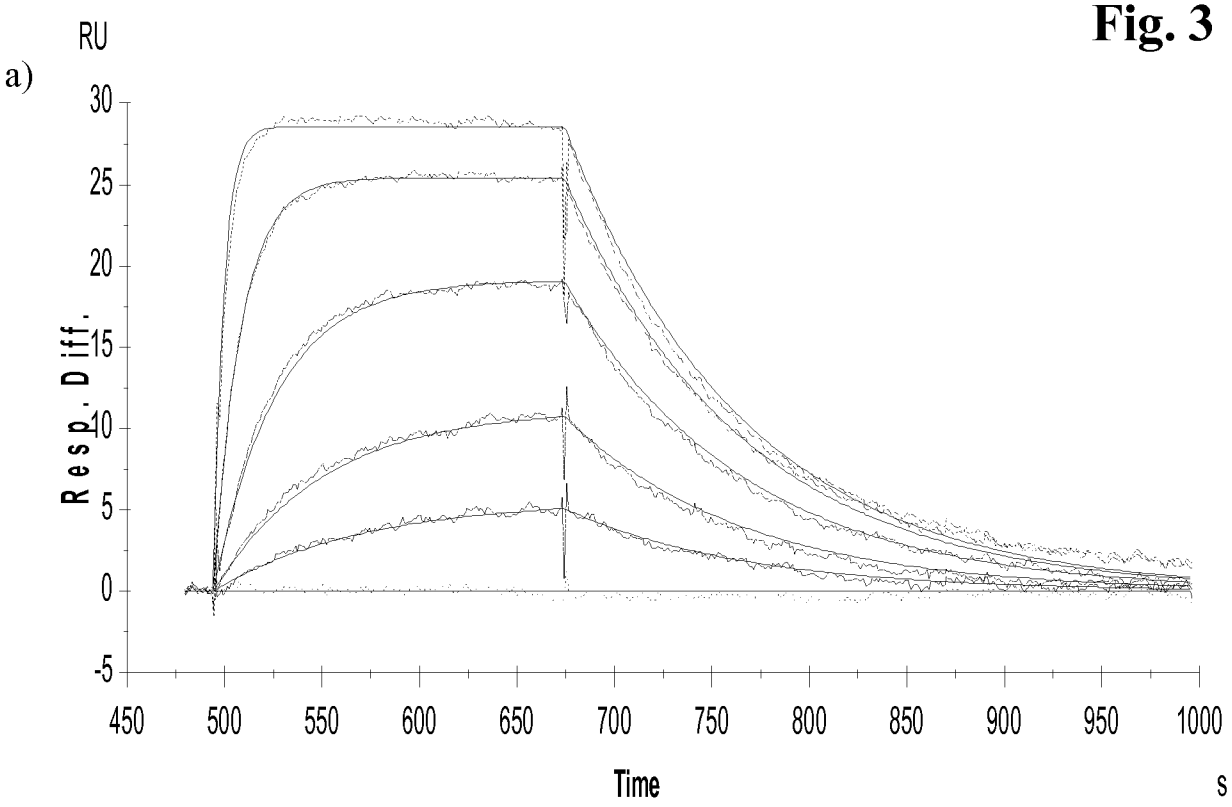
min.

Fig. 3

a)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b)

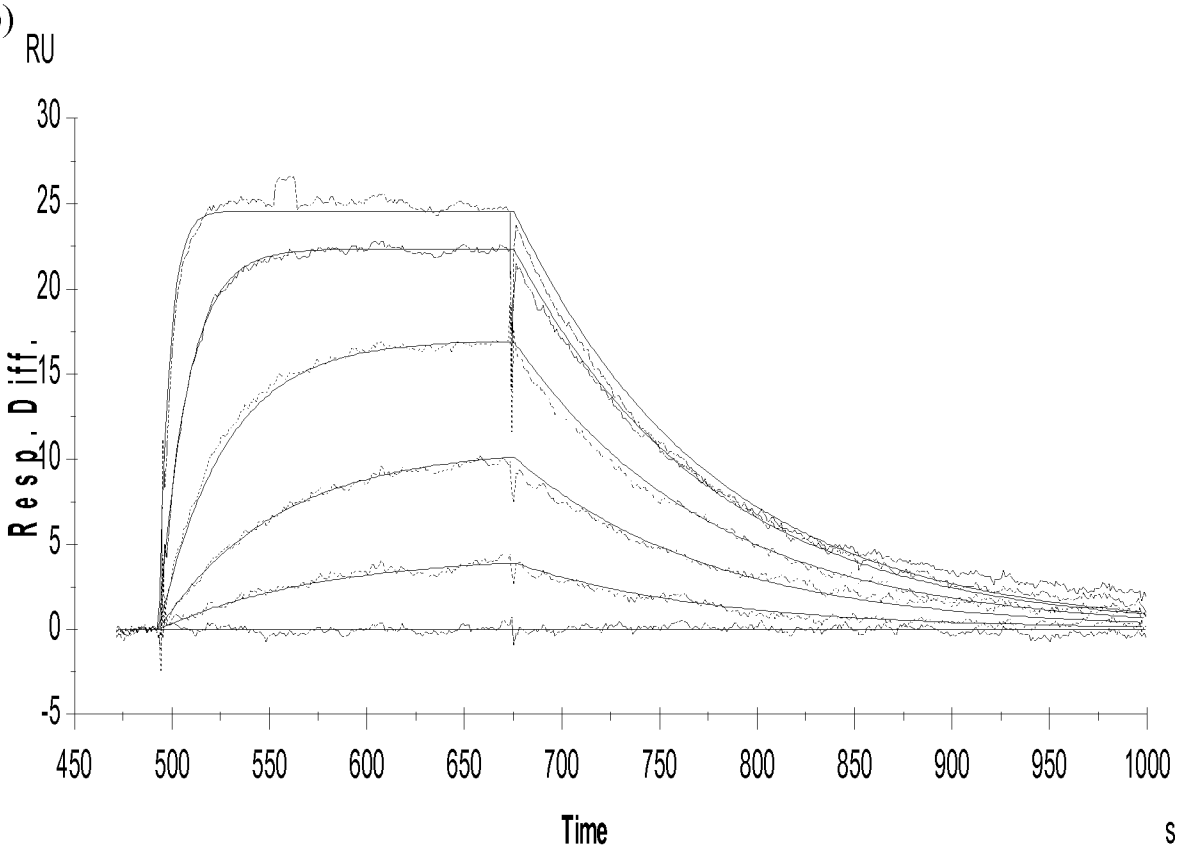
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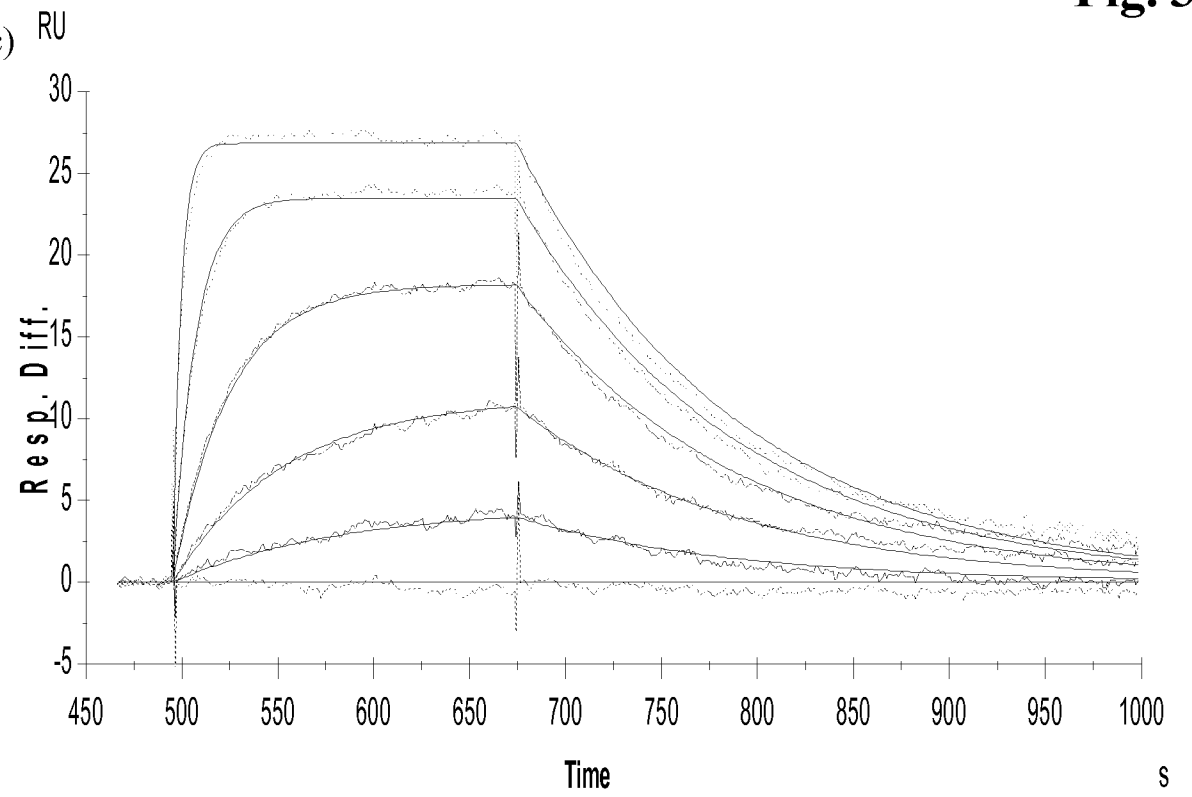
Fig. 3

c)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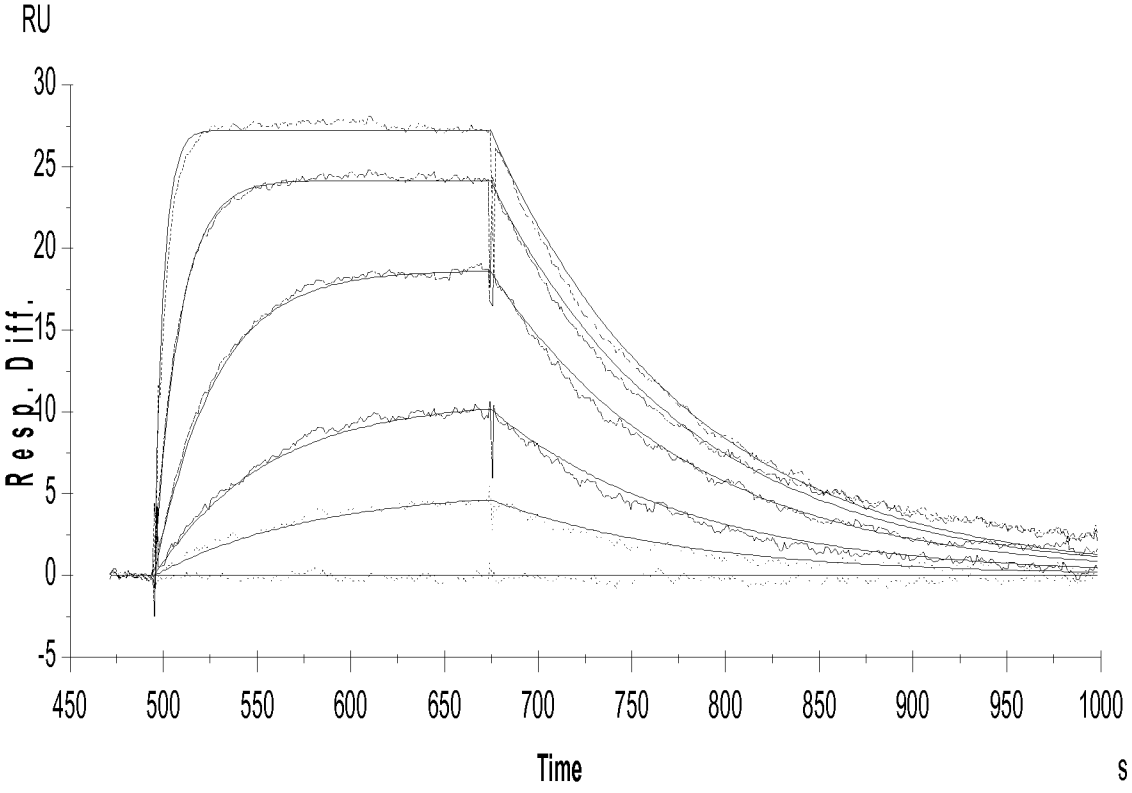
500
550
600
650
700
750
800
850
900
950
1000
Time
s

d)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25
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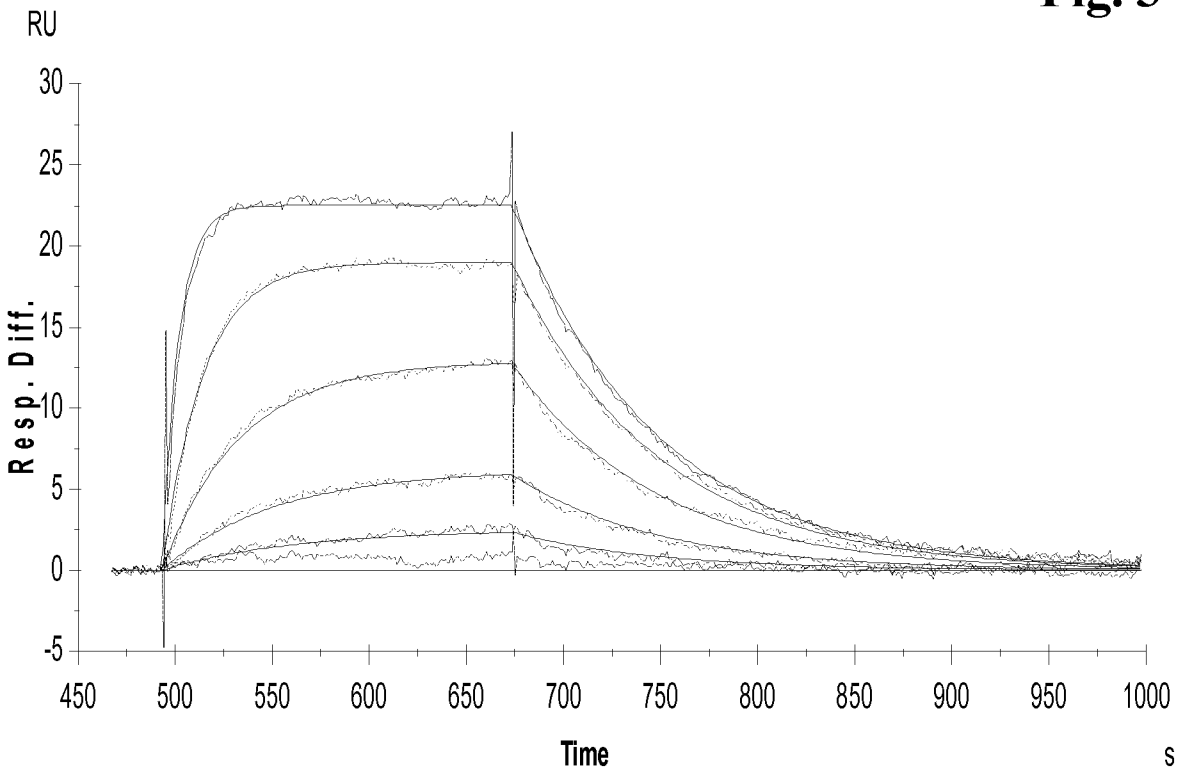
15
10
5
0
-5
Resp. Diff.
450
500
550
600
650
700
750
800
850
900
950
1000
Time
s

Fig. 3

e)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Resp. Diff.
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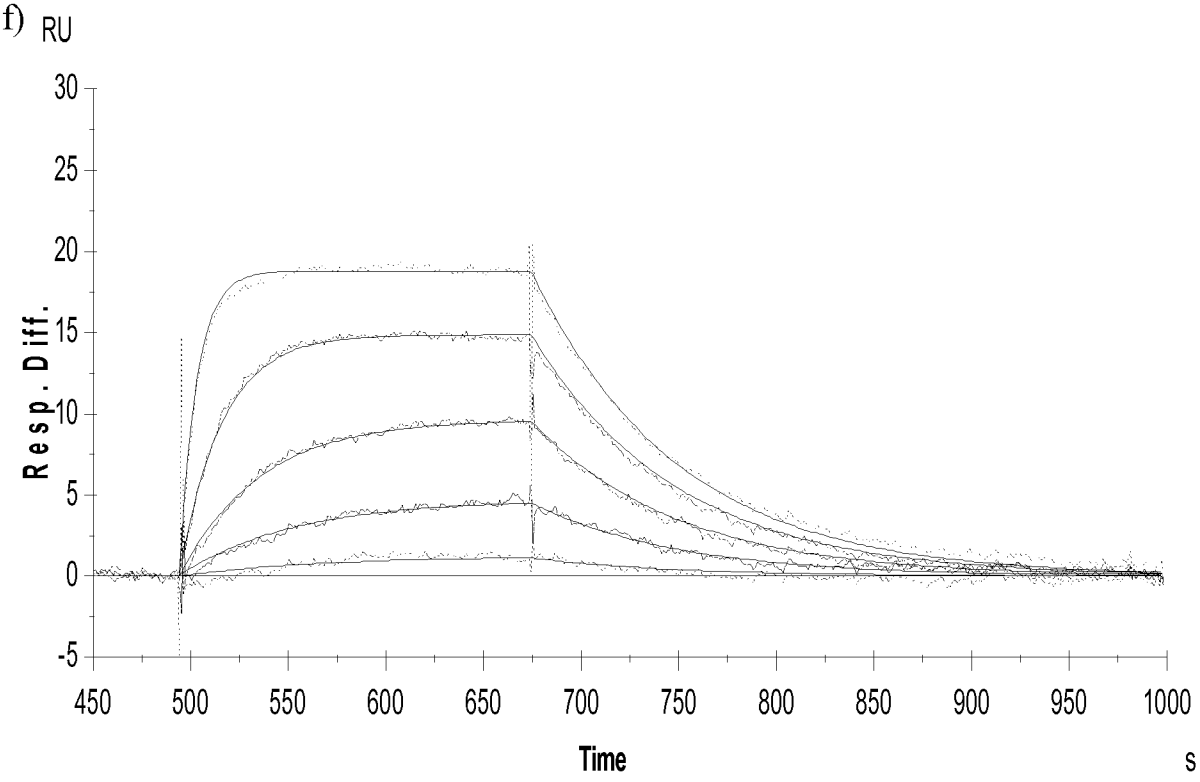
900
950
1000
Time
s

f)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Resp. Diff.
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500
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600
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700
750
800
850
900
950
1000
Time
s

Fig. 4

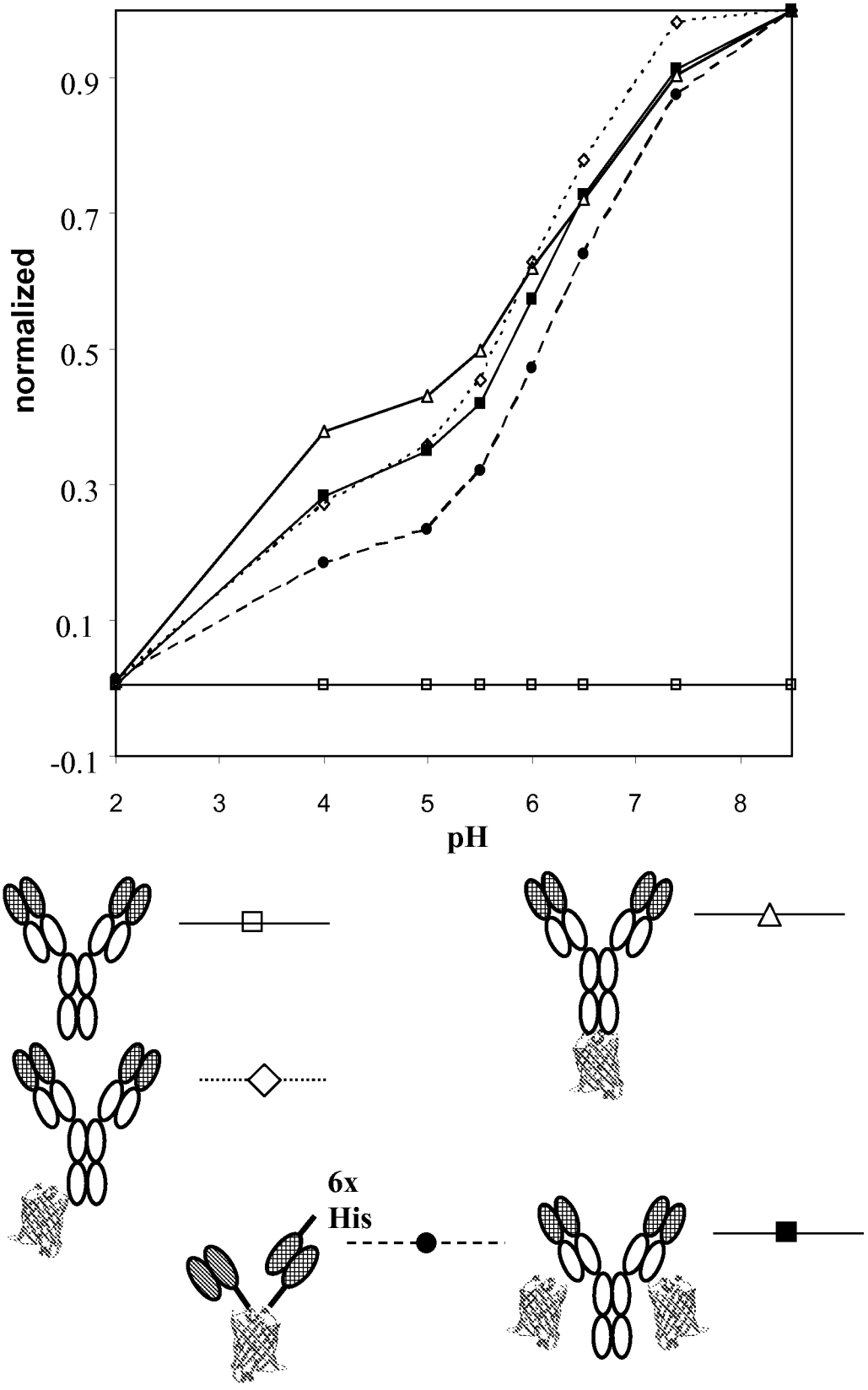


Fig. 5

a)

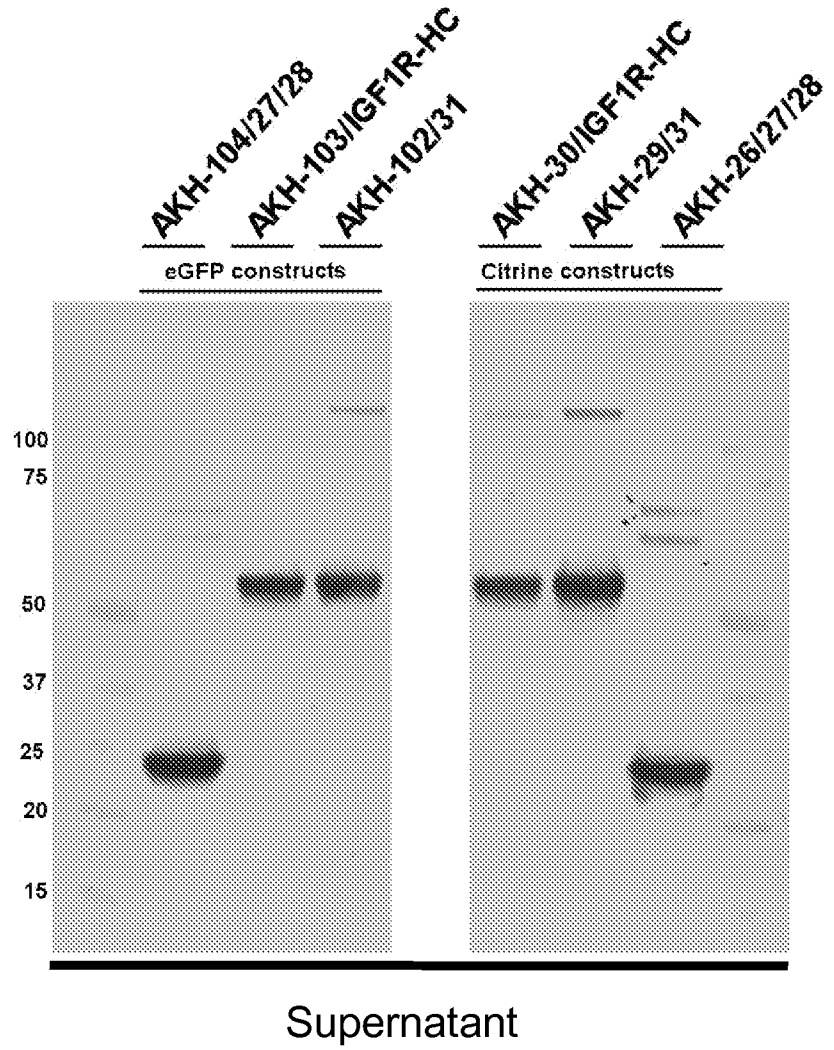
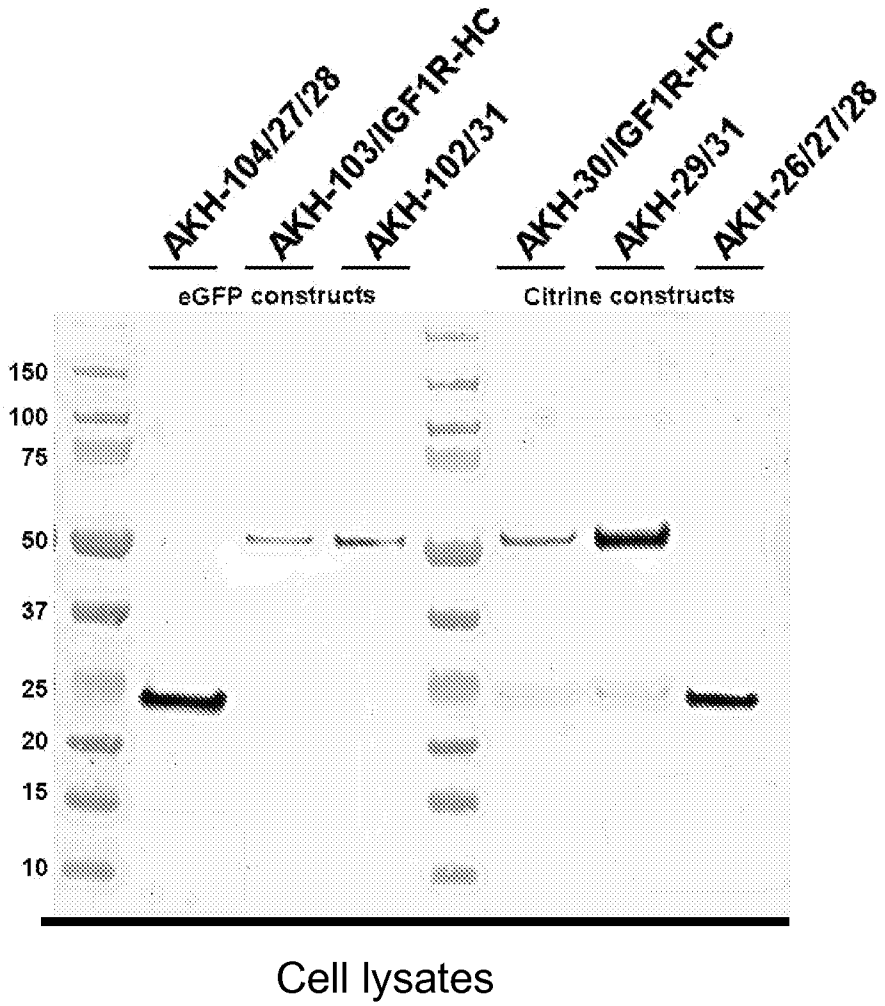

AKH-104/27/28
AKH-103/IGF1R-HC
AKH-102/31
AKH-30/IGF1R-HC
AKH-29/31
AKH-26/27/28
eGFP constructs
Citrine constructs
100
75
50
37
25
20
15
Supernatant

Fig. 5

b)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AKH-103/IGF1R-HC
AKH-102/31
AKH-30/IGF1R-HC
AKH-29/31
AKH-26/27/28
eGFP constructs
Citrine constructs
150
100
75
50
37
25
20
15
10
Cell lysates

Fig. 6

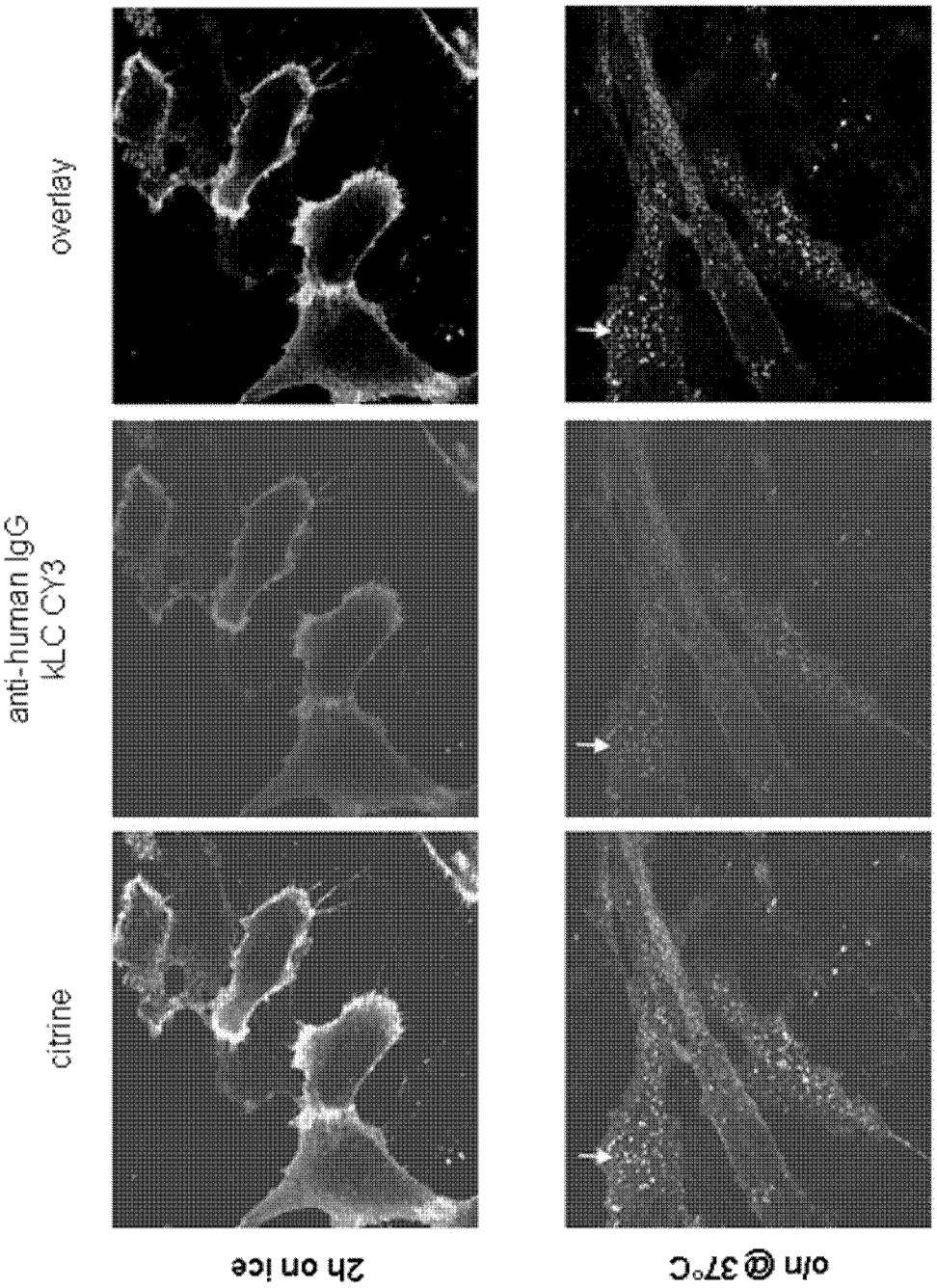
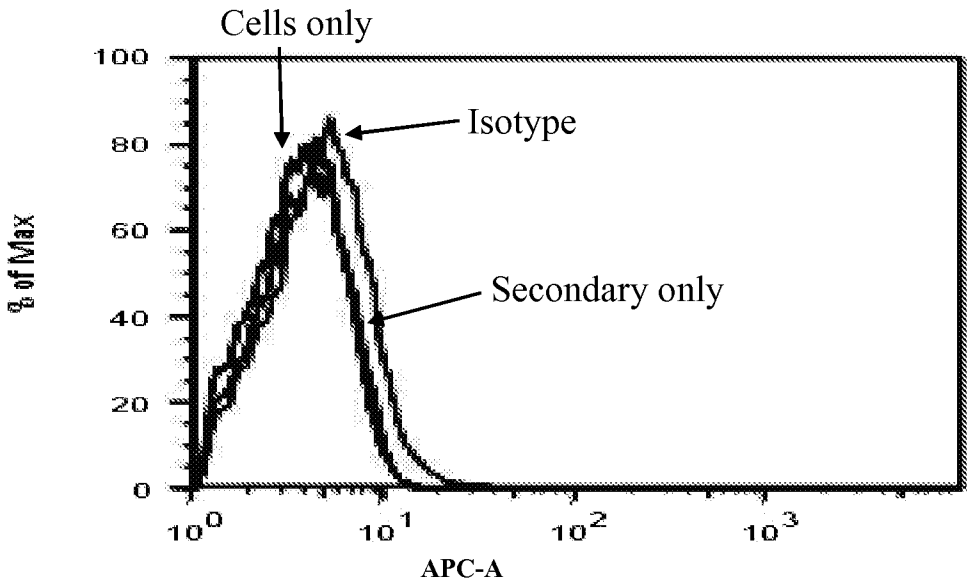
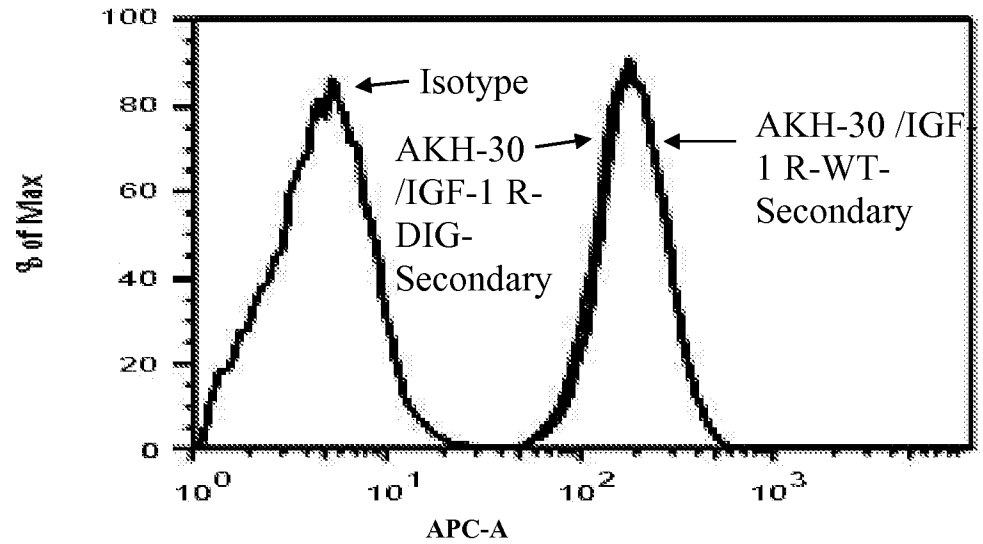


Fig. 7

a)



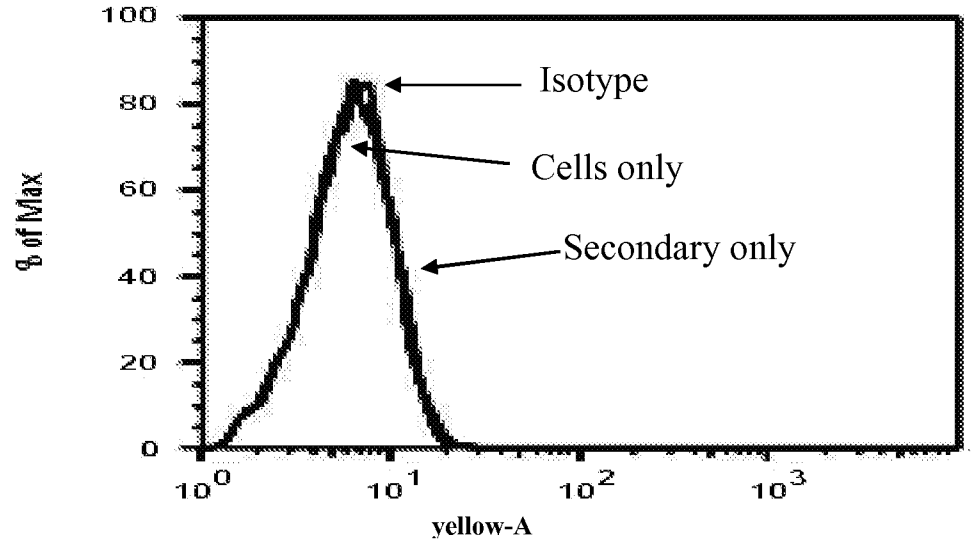
	WELL ID	Geom. Mean, APC-A	Count
	secondary only Canto II	3.74	8801
	Isotype Canto II	4.53	9417
	Cells only FACS Canto II	3.51	8425



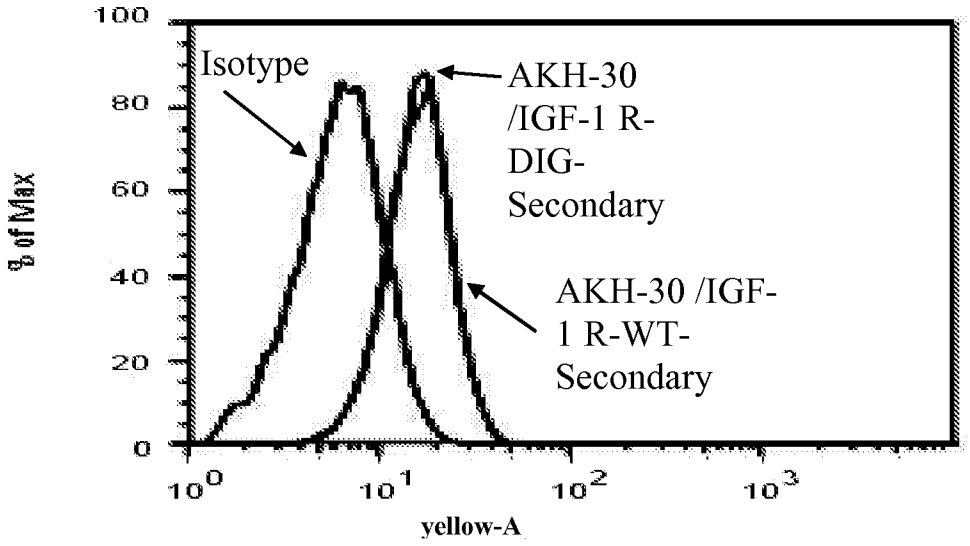
	WELL ID	Geom. Mean APC-A	Count
	AKH30-IGF-1 R-DIG + secondary Canto II	181.78	11146
	AKH30-IGF-1 R-wt + secondary Canto II	174.51	11283
	Isotype Canto II	4.53	9417

Fig. 7

b)



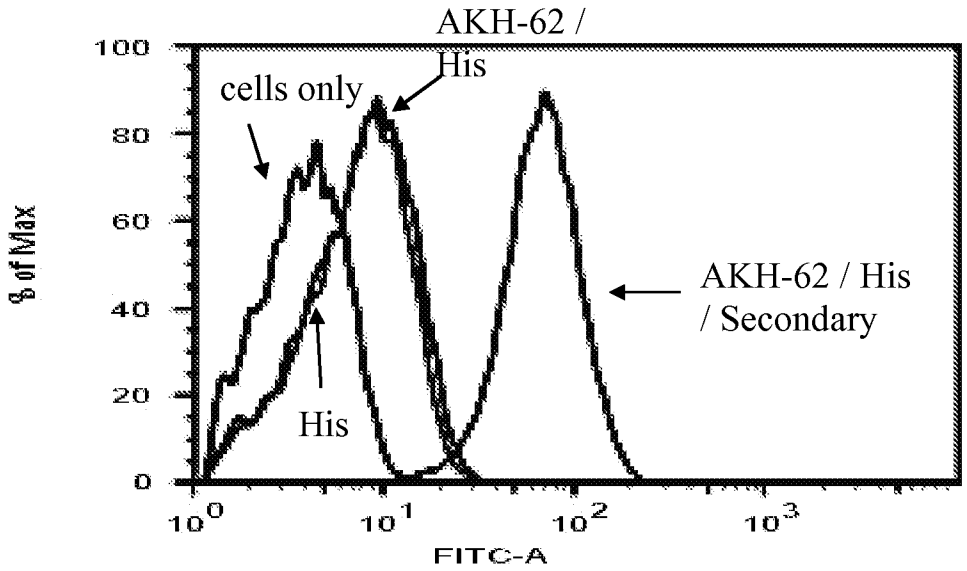
	WELL ID	Geom. Mean yello-A	Count
	secondary only array	5.81	5948
	Isotype array	5.94	5884
	Cells only array	5.76	6081



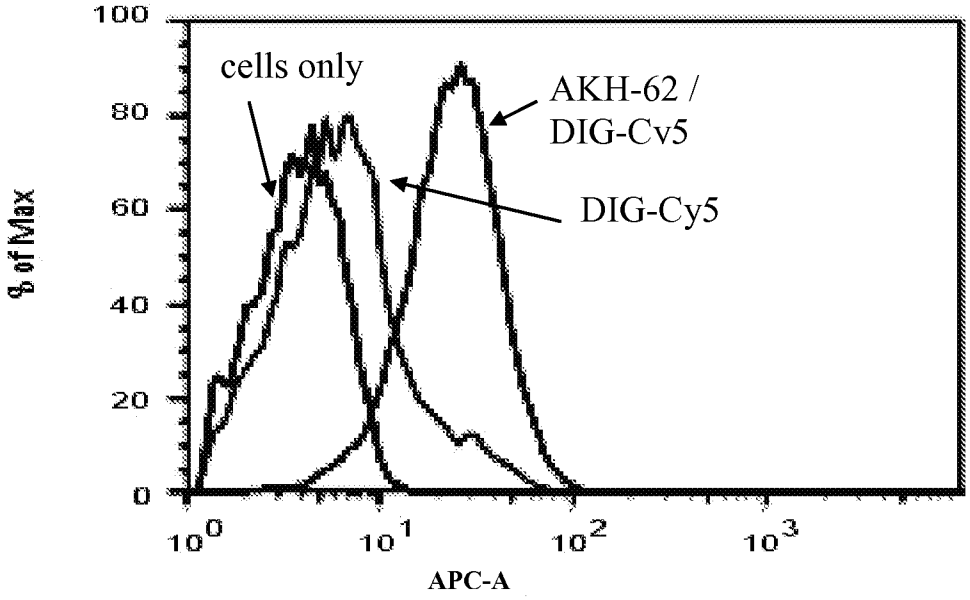
	WELL ID	Geom. Mean yello-A	Count
	AKH30-IGF-1 R-DIG array	15.42	6430
	AKH30-IGF-1 R-wt array	15.44	6247
	Isotype array	5.94	5884

Fig. 7

c)



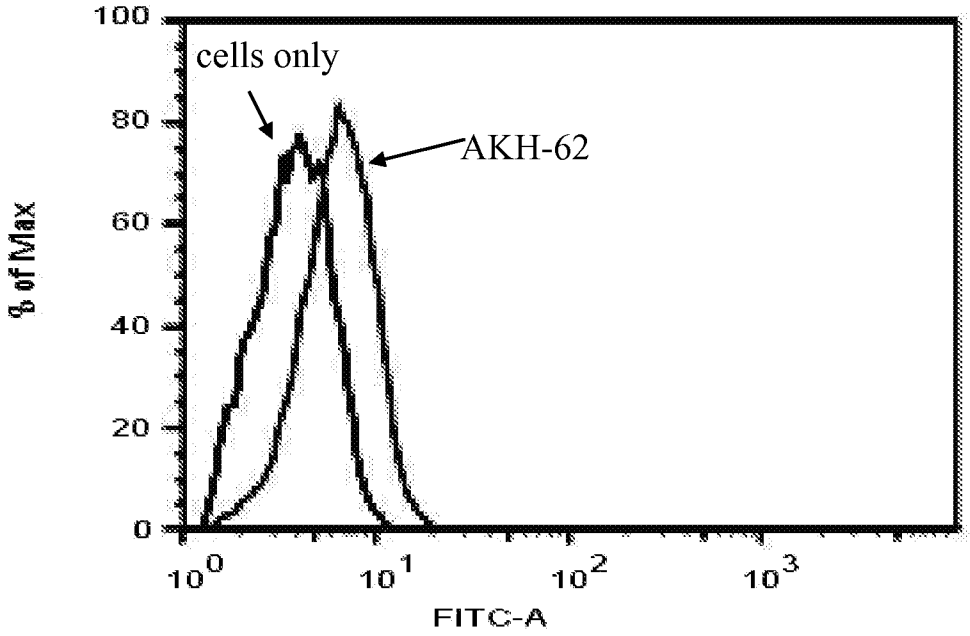
	WELL ID	Geom. Mean , FITC-A	Count
	AKH62 + anti-His + anti-mouse secondary	64.69	10914
	AKH62 + anti-mouse secondary	7.13	9138
	anti-His	6.98	9670
	cells only	3.64	8612



	WELL ID	Geom. Mean ,APC-A	Count
	AKH62 + DIG-Cy5	22.59	10863
	DIG-Cy5	6.08	9125
	cells only	3.64	8612

Fig. 7

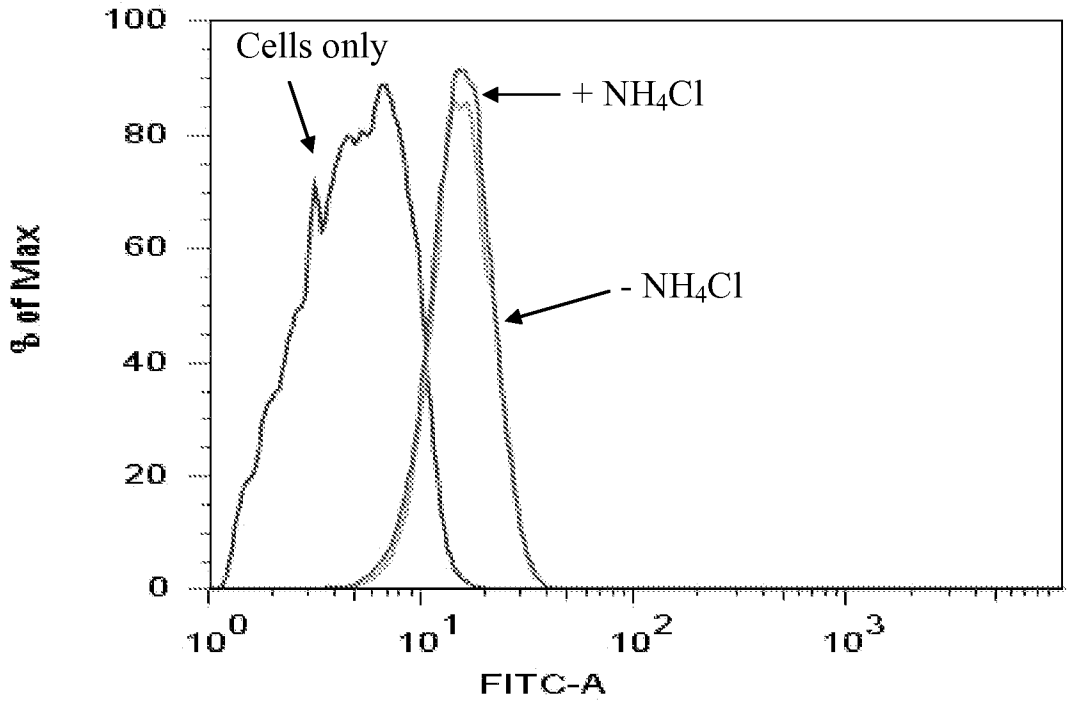
d)



	WELL ID	Geom. Mean , FITC-A	Count
	AKH62 only	6.13	10748
	cells only	3.66	9640

Fig. 8

a)

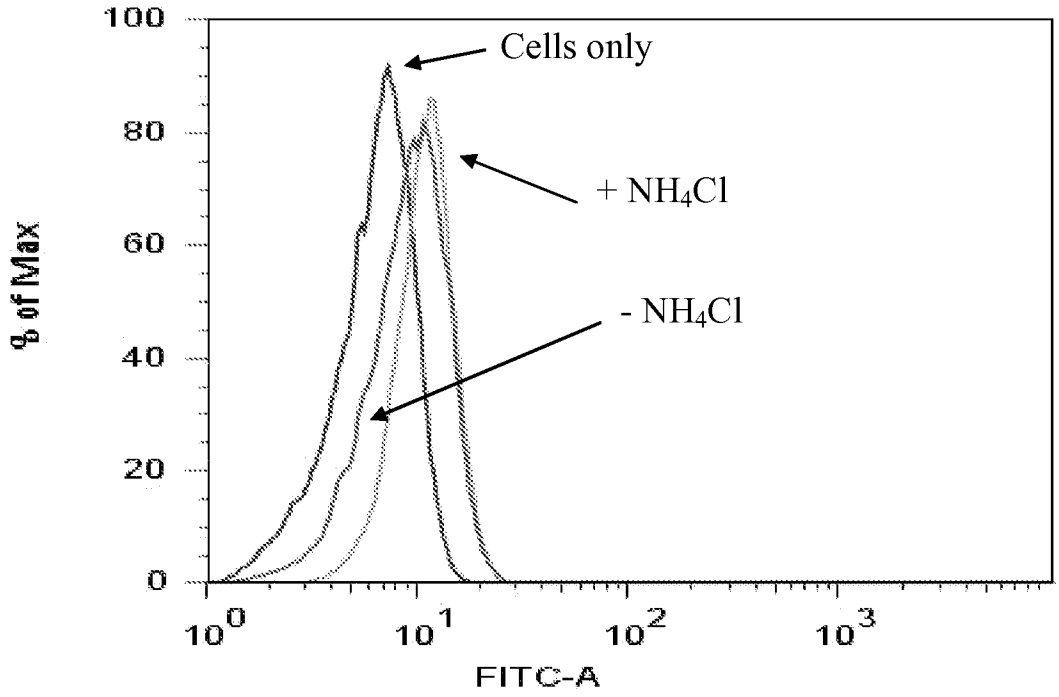


Viable/FITC-A subset

	WELL ID	Geom. Mean , FITC-A	Count
	MCF7 1 hour - IGF1R-citrine only	14,82	9204
	MCF7 1 hour - IGF1R-citrine NH4 1 hour before	14,94	6004
	MCF7 1 hour - Cells only	4,60	8432

Fig. 8

b)



Viable/FITC-A subset

	WELL ID	Geom. Mean , FITC-A	Count
	MCF7 3 hours - IGF1R-citrine only	8,62	9196
	MCF7 3 hours - IGF1R-citrine NH4 1 hour before	10,50	6023
	MCF7 3 hours - Cells only	5,93	9758

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/056770

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/533 G01N33/58
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C07K A61K

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

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

EPO-Internal, BIOSIS, EMBASE, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/203035 A1 (SHANER NATHAN C [US] ET AL) 13 August 2009 (2009-08-13) paragraph [0006] paragraph [0068] paragraph [0113] -----	1-17
Y	WO 2009/033743 A1 (UNIV ZURICH PROREKTORAT FORSCH [CH]; NITSCH ROGER [CH]) 19 March 2009 (2009-03-19) page 30, line 24 - line 26 -----	1-17
Y	WO 2006/099019 A2 (UNIV TEXAS [US]; KUNDRA VIKAS [US]; FANG BINGLIANG [US]; JI LINX X [US) 21 September 2006 (2006-09-21) paragraph [0024] paragraph [0100] ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 2011	15/07/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Routledge, Brian

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 2006/058014 A2 (ODYSSEY THERA INC [US]; MACDONALD MARNIE L [US]; WESTWICK JOHN K [US];) 1 June 2006 (2006-06-01) paragraph [0463] paragraph [0473] paragraph [0486]	1-17
Y	WO 2006/096815 A2 (UNIV TEXAS [US]; FANG BINGLIANG [US]; ROTH JACK A [US]; JI LINX [US]) 14 September 2006 (2006-09-14) paragraph [0116]	1-17
Y	US 2002/048800 A1 (GU YIZHONG [US] ET AL GU YIZHONG [US] ET AL) 25 April 2002 (2002-04-25) paragraph [0271] paragraph [0413] - paragraph [0416]	1-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/056770

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