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(54) Title: NON-COVALENT DISPLAY SYSTEM USING FIMGT/DSF

(57) Abstract: This invention is related to a novel non-covalent display system for use of immobilization of proteins on the surface of cells. This system is a powerful tool for library selection and protein engineering. The system relies on the capture of secreted proteins on the cell surface by using the very stable interaction of the pair FimGT/DsF. The field also encompasses the use of this method to generate cells which produce a desired level of secreted protein or secreted protein of a particular characteristic(s). In particular, the method allows rapid isolation of high expression recombinant antibody producing cell lines, or may be applied directly to rapid isolation of antibodies with specific characteristics. This method is applicable for any cell which secretes protein.



NON-COVALENT DISPLAY SYSTEM USING FIMGT/DSF

FIELD OF THE INVENTION

This invention is related to a novel non-covalent display system for use of immobilization of proteins on the surface of cells. This system is a powerful tool for library selection and protein engineering. The system relies on the capture of secreted proteins on the cell surface by using the very stable interaction of the pair FimGT/DsF. The field also encompasses the use of this method to generate cells which produce a desired level of secreted protein or secreted protein of a particular characteristic(s). In particular, the method allows rapid isolation of high expression recombinant antibody producing cell lines, or may be applied directly to rapid isolation of antibodies with specific characteristics. This method is applicable for any cell which secretes protein.

BACKGROUND OF THE INVENTION

In vitro surface display technologies are powerful tools that allow selection of proteins with desired properties from large libraries. Previously developed display platforms include ribosome, mRNA, phage, bacteria, yeast and mammalian display. Display platforms physically tether protein phenotype with the encoding genotype.

Phage display is the most widespread technique for constructing and screening antibody libraries, whereby the protein of interest is expressed as a polypeptide fusion to a bacteriophage coat protein and subsequently screened by binding to immobilized ligand. Fusions are made most commonly to a minor coat protein, called the gene III protein (pIII), which is present in three to five copies at the tip of the phage. Antibodies possessing desirable binding properties are selected by binding to immobilized antigen in a process called "panning." Phages bearing nonspecific antibodies are removed by washing. The bound phages are eluted and amplified by infection of E. coli. This approach has been applied to generate antibodies against many antigens. But the requirement for physical immobilization of an antigen to a solid surface produces many artificial difficulties.

Therefore several cell surface display methods have been developed which do not require immobilization of an antigen. Yeast in particular are good host cells for surface display and library selection because they contain an internal quality control apparatus that allows them to fold and process complex proteins while retaining and degrading poorly behaved ones. Traditional forms of yeast display involve the covalent fusion of the protein of interest (POI) to a cell wall protein directing the recombinant fusion protein to the outer cell surface. The POI is

fused to the C- or N-terminal part of the respective anchor protein dependent on its orientation related to the cell wall. Several different anchor proteins have been identified that facilitate the display of foreign proteins on yeast cells (van der Vaart et al. (1997) Appl. Environ. Microbiol., 63: 615–620; Sato et al. (2002) Appl. Microbiol. Biotechnol., 60: 469–474), but most prominent
5 is the Aga2p dependent surface-display (Boder and Wittrup (1997) Nat. Biotechnol., 15: 553–557; Boder et al., (2000) Proc. Natl. Acad. Sci. USA, 97: 10701–10705).

U.S. Patent Nos. 6,300,065 and 6,699,658 describe a yeast surface display system for screening combinatorial antibody libraries. The system relies on transforming yeast with vectors that express an antibody fragment as POI fused to a yeast cell surface anchoring
10 protein, using mutagenesis to produce a variegated population of mutants of the antibody fragment and then screening and selecting those cells that produce the antibody fragment with the desired enhanced phenotypic properties. U.S. Patent No. 7,132,273 discloses various yeast cell wall anchor proteins and a surface expression system that uses them to immobilize foreign enzymes or polypeptides on the cell wall.

Several alternative yeast display systems have been disclosed. WO 02/057423 A2 describes a method of detecting and isolating a eukaryotic cell that produces a secreted protein of interest (POI) which is captured on the cell surface. A cell surface capture molecule and / or the POI are transfected into the cell. As a potential antibody binding protein an Fc receptor, an anti-immunoglobulin antibody, an anti-immunoglobulin ScFv, Protein A, Protein G, or functional
20 fragments thereof are disclosed. WO 2010/069913 A1 describes a display system including (i) a cell surface molecule, (ii) a display molecule comprising a modified polypeptide, and (iii) an adapter molecule comprising two binding sites. The interaction between the binding molecule and the adapter molecule is manifested via covalent interaction of disulfide bridges of the PDZ domain of InaD (n-terminal part of the modified display molecule) and the PDZ domain of NorpA as part of the adapter molecule. WO 2010/005863 A1 describes a switchable display
25 system of yeast host cells that express an immunoglobulin of interest, and a capture moiety involving a cell surface anchoring protein fused to protein A. The expression of the immunoglobulin of interest and the capture moiety are under the control of differently inducible promoters. WO 2012/074948 A2 describes a bait/pray system comprising as bait a heavy Fc immunoglobulin domain fused to a surface anchor polypeptide and as pray full length
30 antibodies and monovalent antibody fragments comprising an Fc moiety. WO 2013/169609 A1 describes a bait/pray antibody display system comprising a bait including a light immunoglobulin chain or functional fragment thereof fused to a surface anchor polypeptide or functional fragment thereof. WO 2009/111183 A1 describes an adapter based lower eukaryotic
35 display systems. Adapter-mol 2 is fused to the POI and adapter-mol1 is fused to an outer

surface anchoring molecule. As adapter molecules interacting peptides are disclosed especially coiled-coiled-peptides.

For the cell surface display of full-length antibodies on yeast cells an alternative approach was developed that relies on secretion of antibodies followed by capture to the surface by binding
5 to a capturing agent which is chemically coupled to the cell wall. Chemical coupling of the capturing moiety has the advantage that the system can be easily converted to soluble production formats which means that the system is switchable from a displayed POI into a soluble form of the POI. A system based on in vivo biotinylation of the POI which comprises a small biotinylation peptide and binding after secretion to a surface immobilized avidin was
10 disclosed by Rakestraw et al. (WO 2008/118476 A2; Rakestraw et al. (2011) Protein Eng. Des. Sel., 24: 525–530). An alternative system for capturing secreted antibodies uses chemical coupling of an Fc-binding ZZ domain to the cell wall (Rhiel et al. (2014) PLoS One. 9(12):e114887). The above mentioned non-covalent yeast surface display systems disclosed by Rakestraw et al. and Rhiel et al. have the disadvantage that during the selection phase of
15 antibodies biotinylated antigens and / or Fc-domain fused antigens cannot be used. Yeast-based cell secretion and capture technologies are switchable, but secreted POIs are not captured until after they have completely left the cell, leading to a potential loss of the genotype-phenotype linkage. This means there is a high impact of the capturing moiety to maintain the genotype-phenotype linkage. To overcome all this shortcomings a non-covalent
20 surface display system was developed which relies on the FimGT/DsF interaction pair.

The interaction pair FimGT/DsF was published by Giese et al. (Giese et al. (2012) Angew. Chem. Int. Ed. Engl. 51(18):4474-4478) as a new affinity purification system for protein complexes and large-scale investigations of protein–protein interaction networks. FimGT (SEQ ID NO: 12) is derived from type 1 pili FimG of E. coli comprising 132 amino acid residues
25 lacking first 12 N-terminal amino acid residues compared to the endogens protein and has a molecular weight of 13.7 kDa. DsF (SEQ ID NO: 13) is a peptide (15aa) corresponding to the binding site of the donor strand of the neighboring subunit FimF of FimG in the pilus formation. The interaction between FimGT and DsF could be characterized as the kinetically most stable protein–ligand complex known to date (K_D value of 10^{-20}). This interaction pair was used in the
30 context of protein purification as a new single-step affinity purification system.

In order to circumvent the exclusion of biotinylated and / or Fc-domain fused antigens in the screening process later on, this present invention propose the establishment of a switchable display system, based on the coating/re-capturing approach using a different, non-streptavidin or ZZ domain based coating agent.

In this present invention a new coating/immobilization method is described using FimGT/DsF as coating/recapturing interaction pair. The interaction of these two partners might enable a strong genotype phenotype coupling necessary for screening process in the context of library screening against antigens by yeast display.

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DESCRIPTION OF THE FIGURES

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Figure 1: Direct coating of yeast cells via DsF maleimide. (A): Yeast cell are coated with DsF via its functional group maleimide which interact covalently with sulfur groups on the surface of the yeast cell. IgGs fused to the counterpart FimGT will be soluble produced upon induction, and captured on the cell surface via the interaction of FimGT and DsF. (B) Structure of the coating agent DsF fused to the active group maleimide.

Figure 2: Overview of the expression cassette of pETF67: Hc and Lc were fused to a leader peptide app8 under the control of the bicystronic promoter Gal1/Gal10. The Lc was fused to a myc tag, the Hc is fused to 3xHA and a FLAG-tag followed by the anchor peptide FimGT

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Figure 3: Verification of the interaction of FimGT and DsF in a three layer approach (A) first layer consists of NHS-biotin, the second layer of Streptavidin-APC and the third layer of DsF-biotin. All three layers were applied in separate steps on the yeast strain yscETF42 (vwk18gal+ pETF67)

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Figure 4: Analysis of NHS-biotin/streptavidin-APC/DsF-biotin coated yeast cells (yscETF42) on IgG display. A,B,C representing yscETF42 cells coated with NHS-biotin and streptavidin-APC without DsF-biotin as third layer. D,E,F representing yscETF42 cells coated with NHS-biotin and streptavidin-APC with DsF-biotin as third layer (A+D) Dotplot FACS analysis detecting efficient labeling via APC conjugated streptavidin and captured IgG-FimGT fusion via anti-human-PE antibody. (B+E) Histogram FACS analysis detecting efficient labeling via APC conjugated streptavidin. (C+F) Histogram FACS analysis detecting IgG display on the surface by anti-human-PE antibody.

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Figure 5: Potential variation of Dsf/FimGT mediated full IgG display. 1. FimGT as a catching moiety will be displayed via alpha-agglutinin under the control of an inducible promoter and 2. A full IgG molecule fused to the counterpart DsF will also be expressed by the host yeast cell and caught by displayed FimGT molecule on the cell surface.

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Figure 6: FACS analysis of yscETF13 and yscETF40. FACS analyses depict a sufficient display level (60 %) of FimGT via alpha-agglutinin. Displayed FimGT was detected via a myc-tag in combination with an anti-rabbit-APC conjugated secondary antibody. Caught DsF-biotin peptides were labeled with streptavidin-PE. 50 % of the analyzed cells showed an efficient

display level of FimGT (APC positive) and the ability to catch DsF-biotin (PE positive). A,B,C representing yscETF13 cells carrying the empty plasmid pEsc-leu. D,E,F representing yscETF42 cells displaying FimGT (A+D) Dotplot FACS analysis detecting efficient FimGT display via myc-tag in combination with an anti-rabbit-APC conjugated secondary antibody. (B+E) Histogram FACS analysis detecting efficient display of FimGT via a myc-tag in combination with an anti-rabbit-APC conjugated secondary antibody. (C+F) Histogram FACS analysis detecting biotinylated DsF caught by displayed FimGT via PE conjugated streptavidin.

Figure 7: Summary of all tested conditions for the identification of the optimal coating condition of yeast cells with DsF-maleimide-biotin (comparison of mean values of all single cells analyzed).

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs. The following references, however, can provide one of skill in the art to which this invention pertains with a general definition of many of the terms used in this invention, and can be referenced and used so long as such definitions are consistent with the meaning commonly understood in the art. Such references include, but are not limited to, Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); Hale & Marham, The Harper Collins Dictionary of Biology (1991); and Lackie et al., The Dictionary of Cell & Molecular Biology (3d ed. 1999); and Cellular and Molecular Immunology, Eds. Abbas, Lichtman and Pober, 2nd Edition, W.B. Saunders Company. Any additional technical resource available to the person of ordinary skill in the art providing definitions of terms used herein having the meaning commonly understood in the art can be consulted. For the purposes of the present invention, the following terms are further defined. Additional terms are defined elsewhere in the description. As used herein and in the appended claims, the singular forms "a," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a gene" is a reference to one or more genes and includes equivalents thereof known to those skilled in the art, and so forth.

The terms "polypeptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid

polymer. Unless otherwise indicated, a particular polypeptide sequence also implicitly encompasses conservatively modified variants thereof.

Amino acids may be referred to herein by their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

5 Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"FimGT" is a polypeptide comprising the amino acid sequence of SEQ ID NO: 12, preferentially

"FimGT" is a polypeptide consisting of the amino acid sequence of SEQ ID NO: 12.

"DsF" is a polypeptide comprising the amino acid sequence of SEQ ID NO: 13, preferentially

"DsF" is a polypeptide consisting of the amino acid sequence of SEQ ID NO: 13.

10 "FimGT/DsF interaction pair" means a pair of polypeptides comprising the binding partners "FimGT" and "DsF", which means one polypeptide comprises "FimGT" and the other polypeptide comprises "DsF".

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules, preferably comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains

15 which are typically inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region.

The heavy chain constant region can comprise e.g. three domains CH1, CH2 and CH3. Each

light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain (CL). The

20 VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is typically composed of three CDRs and up to four FRs arranged from amino-terminus to carboxy-terminus e.g. in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

25 As used herein, the term "Complementarity Determining Regions" (CDRs; e.g., CDR1, CDR2, and CDR3) refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three CDR regions identified as CDR1, CDR2 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" as defined by

30 Kabat (e.g. about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (e.g. about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light

chain variable domain and 26- 32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain (Chothia and Lesk; J Mol Biol 196: 901-917 (1987)). In some instances, a complementarity determining region can include amino acids from both a CDR region defined according to Kabat and a hypervariable loop.

5 Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these maybe further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. A preferred class of immunoglobulins for use in the present invention is IgG.

10 The heavy-chain constant domains that correspond to the different classes of antibodies are called [alpha], [delta], [epsilon], [gamma], and [mu], respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. As used herein antibodies are conventionally known antibodies and functional fragments thereof.

A "functional fragment" or "antigen-binding antibody fragment" of an antibody/immunoglobulin
15 hereby is defined as a fragment of an antibody/immunoglobulin (e.g., a variable region of an IgG) that retains the antigen-binding region. An "antigen-binding region" of an antibody typically is found in one or more hyper variable region(s) of an antibody, e.g., the CDR1, -2, and/or -3 regions; however, the variable "framework" regions can also play an important role in antigen binding, such as by providing a scaffold for the CDRs. Preferably, the "antigen-binding
20 region" comprises at least amino acid residues 4 to 103 of the variable light (VL) chain and 5 to 109 of the variable heavy (VH) chain, more preferably amino acid residues 3 to 107 of VL and 4 to 111 of VH, and particularly preferred are the complete VL and VH chains (amino acid positions 1 to 109 of VL and 1 to 113 of VH; numbering according to WO 97/08320).

"Functional fragments", "antigen-binding antibody fragments", or "antibody fragments" of the
25 invention include but are not limited to Fab, Fab', Fab'-SH, F(ab')₂, and Fv fragments; diabodies; single domain antibodies (DABs), linear antibodies; single-chain antibody molecules (scFv); and multispecific, such as bi- and tri-specific, antibodies formed from antibody fragments (C. A. K Borrebaeck, editor (1995) Antibody Engineering (Breakthroughs in Molecular Biology), Oxford University Press; R. Kontermann & S. Duebel, editors (2001)
30 Antibody Engineering (Springer Laboratory Manual), Springer Verlag). An antibody other than a "multi-specific" or "multi-functional" antibody is understood to have each of its binding sites identical. The F(ab')₂ or Fab may be engineered to minimize or completely remove the intermolecular disulfide interactions that occur between the CH1 and CL domains.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

10 Variants of the antibodies or antigen-binding antibody fragments contemplated in the invention are molecules in which the binding activity of the antibody or antigen-binding antibody fragment is maintained.

"Binding proteins" contemplated in the invention are for example antibody mimetics, such as Affibodies, Adnectins, Anticalins, DARPins, Avimers, Nanobodies (reviewed by Gebauer M. et al., Curr. Opinion in Chem. Biol. 2009; 13:245-255; Nuttall S.D. et al., Curr. Opinion in Pharmacology 2008; 8:608-617).

A "human" antibody or antigen-binding fragment thereof is hereby defined as one that is not chimeric (e.g., not "humanized") and not from (either in whole or in part) a non-human species. A human antibody or antigen-binding fragment thereof can be derived from a human or can be a synthetic human antibody. A "synthetic human antibody" is defined herein as an antibody having a sequence derived, in whole or in part, in silico from synthetic sequences that are based on the analysis of known human antibody sequences. In silico design of a human antibody sequence or fragment thereof can be achieved, for example, by analyzing a database of human antibody or antibody fragment sequences and devising a polypeptide sequence utilizing the data obtained there from. Another example of a human antibody or antigen-binding fragment thereof is one that is encoded by a nucleic acid isolated from a library of antibody sequences of human origin (e.g., such library being based on antibodies taken from a human natural source). Examples of human antibodies include antibodies as described in Söderlind et al., Nature Biotech. 2000, 18:853-856.

30 A "humanized antibody" or humanized antigen-binding fragment thereof is defined herein as one that is (i) derived from a non-human source (e.g., a transgenic mouse which bears a heterologous immune system), which antibody is based on a human germline sequence; (ii) where amino acids of the framework regions of a non-human antibody are partially exchanged to human amino acid sequences by genetic engineering or (iii) CDR-grafted, wherein the

CDRs of the variable domain are from a non-human origin, while one or more frameworks of the variable domain are of human origin and the constant domain (if any) is of human origin.

A "chimeric antibody" or antigen-binding fragment thereof is defined herein as one, wherein the variable domains are derived from a non-human origin and some or all constant domains are derived from a human origin.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the term "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The term "monoclonal" is not to be construed as to require production of the antibody by any particular method. The term monoclonal antibody specifically includes chimeric, humanized and human antibodies.

An "isolated" antibody is one that has been identified and separated from a component of the cell that expressed it. Contaminant components of the cell are materials that would interfere with diagnostic or therapeutic uses of the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes.

An "isolated" nucleic acid is one that has been identified and separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

As used herein, an antibody "binds specifically to", is "specific to/for" or "specifically recognizes" an antigen of interest, e.g. a tumor-associated polypeptide antigen target, is one that binds the antigen with sufficient affinity such that the antibody is useful as a therapeutic agent in targeting a cell or tissue expressing the antigen, and does not significantly cross-react with other proteins or does not significantly cross-react with proteins other than orthologs and variants (e.g. mutant forms, splice variants, or proteolytically truncated forms) of the aforementioned antigen target. The term "specifically recognizes" or "binds specifically to" or is "specific to/for" a particular polypeptide or an epitope on a particular polypeptide target as used

herein can be exhibited, for example, by an antibody, or antigen-binding fragment thereof, having a monovalent K_D for the antigen of less than about 10^{-4} M, alternatively less than about 10^{-5} M, alternatively less than about 10^{-6} M, alternatively less than about 10^{-7} M, alternatively less than about 10^{-8} M, alternatively less than about 10^{-9} M, alternatively less than about 10^{-10} M, alternatively less than about 10^{-11} M, alternatively less than about 10^{-12} M, or less. An antibody "binds specifically to," is "specific to/for" or "specifically recognizes" an antigen if such antibody is able to discriminate between such antigen and one or more reference antigen(s). In its most general form, "specific binding", "binds specifically to", is "specific to/for" or "specifically recognizes" is referring to the ability of the antibody to discriminate between the antigen of interest and an unrelated antigen, as determined, for example, in accordance with one of the following methods. Such methods comprise, but are not limited to surface plasmon resonance (SPR), Western blots, ELISA-, RIA-, ECL-, IRMA-tests and peptide scans. For example, a standard ELISA assay can be carried out. The scoring may be carried out by standard color development (e.g. secondary antibody with horseradish peroxidase and tetramethyl benzidine with hydrogen peroxide). The reaction in certain wells is scored by the optical density, for example, at 450 nm. Typical background (=negative reaction) may be 0.1 OD; typical positive reaction may be 1 OD. This means the difference positive/negative is more than 5-fold, 10-fold, 50-fold, and preferably more than 100-fold. Typically, determination of binding specificity is performed by using not a single reference antigen, but a set of about three to five unrelated antigens, such as milk powder, BSA, transferrin or the like.

"Binding affinity" or "affinity" refers to the strength of the total sum of non-covalent interactions between a single binding site of a molecule and its binding partner. Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1 : 1 interaction between members of a binding pair (e.g. an antibody and an antigen). The dissociation constant " K_D " is commonly used to describe the affinity between a molecule (such as an antibody) and its binding partner (such as an antigen) i.e. how tightly a ligand binds to a particular protein. Ligand-protein affinities are influenced by non-covalent intermolecular interactions between the two molecules. Affinity can be measured by common methods known in the art, including those described herein. In one embodiment, the " K_D " or " K_D value" according to this invention is measured by using surface plasmon resonance assays using suitable devices including but not limited to Biacore instruments like Biacore T100, Biacore T200, Biacore 2000, Biacore 4000, a Biacore 3000 (GE Healthcare Biacore, Inc.), or a ProteOn XPR36 instrument (Bio-Rad Laboratories, Inc.).

"Epitope tag" refers to polypeptide sequences which are chosen because high-affinity antibodies can be reliably produced in many different species. These are often derived from viral genes, which explain their high immunoreactivity. Epitope tags include V5-tag, Myc-tag,

HA-tag and NE-tag but also include for example the antibody Fc-region or the GST-protein. These tags are particularly useful for western blotting, immunofluorescence and immunoprecipitation experiments, although they also find use in antibody purification.

"Percent (%) sequence identity" with respect to a reference polynucleotide or polypeptide sequence, respectively, is defined as the percentage of nucleic acid or amino acid residues, respectively, in a candidate sequence that are identical with the nucleic acid or amino acid residues, respectively, in the reference polynucleotide or polypeptide sequence, respectively, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Conservative substitutions are not considered as part of the sequence identity. Preferred are un-gapped alignments. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Sequence homology" indicates the percentage of amino acids that either is identical or that represent conservative amino acid substitutions.

The term "vector", as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors."

As used herein, the terms "fluorescence label" and "fluorophore" used interchangeably and refer to any substance that emits electromagnetic energy such as light at a certain wavelength (emission wavelength) when the substance is illuminated by radiation of a different wavelength (excitation wavelength) and is intended to encompass a chemical or biochemical molecule or fragments thereof that is capable of interacting or reacting specifically with an analyte of interest in a sample to provide one or more optical signals.

The terms "host cell", "host cell line", and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants", "transformed cells", "transfectants", "transfected cells", and "transduced cells", which include the primary transformed/transfected/transduced cell and progeny derived therefrom without regard to the number of passages. Progeny may not be

completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

5 The term "eukaryotic" refers to a nucleated cell or organism, and includes insect cells, plant cells, mammalian cells, animal cells and lower eukaryotic cells.

The term "lower eukaryotic cells" includes yeast and filamentous fungi. Yeast and filamentous fungi include, but are not limited to *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia*
10 *stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium lucknowense*, *Fusarium* sp., *Fusarium gramineum*, *Fusarium venenatum*, *Physcomitrella patens* and *Neuraspora crassa*. *Pichia* sp., any *Saccharomyces* sp., *Hansenula*
15 *polymorpha*, any *Kluyveromyces* sp., *Candida albicans*, any *Aspergillus* sp., *Trichoderma reesei*, *Chrysosporium lucknowense*, any *Fusarium* sp. and *Neurospora crassa*.

DESCRIPTION OF THE INVENTION

The present invention provides a method for expressing and displaying proteins on the surface of a lower eukaryote in a form that is accessible for detection. Combining this method for
20 example with fluorescence-activated cell sorting (FACS) provides a means for selecting cells that express proteins with increased or decreased affinity for another molecule, catalytic activity, altered specificity, or conditional binding. The method is particularly useful for constructing and screening antibody libraries in lower eukaryotes such as yeast or filamentous fungi.

25 Display System

The present invention provides a protein display system that is capable of displaying diverse proteins on the surface of a eukaryotic host cell such as a lower eukaryote host cell (e.g., yeast or filamentous fungal cells). The compositions and methods are particularly useful for the display of collections of proteins (libraries of proteins) in the context of discovery (that is,
30 screening) or molecular evolution protocols. A salient feature of the method is that it provides a display system in which proteins of interest can be displayed on the surface of a host cell without having to express the protein of interest as a fusion protein in which the protein of interest is fused to a surface anchor protein.

The system comprises at least three components:

- I. The first component is a nucleotide acid sequence that encodes for a protein of interest or libraries of which the protein of interest (POI) is to be selected (for example, a library of vectors encoding for antibodies or fragments thereof). The nucleotide acid sequence encodes the proteins of interest as fusion proteins in which a first binding moiety comprising one partner of the FimGT/DsF interaction pair is fused to the N- or C-terminus of the proteins of interest. The nucleotide acid sequence may be a vector.
- II. The second component is a capture moiety (second binding moiety) that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety wherein the capture moiety can be attached to the cell surface of a host cell.
- III. The third component is a host cell which expresses the protein of interest and has the capture moiety at least temporarily on its surface.

I. General Characteristics of the Protein of Interest and the Nucleotide Acid Sequence that encodes for it.

The invention embraces any engineered protein, protein domain, or functional part thereof as protein of interest. Engineered proteins that are particularly embraced by the invention are engineered proteins that are functional proteins, binding proteins, antibodies, scaffold proteins or enzymes. However, in some embodiments, an engineered protein of the invention may be a structural protein, a storage protein, or any other protein of interest. A protein or enzyme of the invention may be, but is not limited to, a therapeutic polypeptide, polymerase, ligase, restriction enzyme, topoisomerase, kinase, phosphatase, metabolic enzyme, catalytic enzyme, therapeutic enzyme, pharmaceutical enzyme, environmental enzyme, industrial enzyme, pharmaceutical polypeptide, environmental polypeptide, industrial polypeptide, binding protein, antibody, antibody fragment, single antibody chain, chimeric antibody, scaffold protein, immunotoxin, antibody-like polypeptide, signaling molecule, cytokine or a receptor.

In preferred embodiments, proteins of interest and / or engineered proteins are antibodies, antibody chains or antibody fragments. In other preferred embodiments proteins of interest and / or engineered proteins are "Binding proteins" like for example antibody mimetics, such as Affibodies, Adnectins, Anticalins, DARPins, Avimers, and Nanobodies.

A first binding moiety comprising FimGT or DsF (one partner of the FimGT/DsF interaction pair) is fused to the N- or C-terminus of the protein of interest.

In some embodiments, the first binding partner (FimGT or DsF) is connected to the protein of interest through a linker peptide. The linker peptide can be of any length, preferably 1 to 100 amino acid residues in length. The linker peptide can be composed of any amino acid. In a preferred embodiment the linker polypeptide comprises at least one Gly, Ser, Arg, Leu, Cys, Ala, Leu and/or Lys residue. In a more preferred embodiment the linker polypeptide comprises Gly and Ser residues. A further preferred embodiment is a linker peptide which comprises Gly and Ser residues and has a ratio of Gly to Ser of at least 3 to 1.

In a highly preferred embodiment of the invention the protein of interest is an antibody which is fused with the heavy chain at its C-terminal end to FimGT or DsF (see Figure 1 and Figure 5), optionally through a linker peptide.

A nucleotide acid sequence encodes the proteins of interest as fusion proteins in which a first binding moiety comprising one partner of the FimGT/DsF interaction pair is fused to the N- or C-terminus of the proteins of interest. In some embodiments the nucleotide acid sequence comprises additional regulatory sequences.

Regulatory sequences which may be used in the practice of the methods disclosed herein include signal sequences, promoters, and transcription terminator sequences. Examples of signal sequences include those of *Saccharomyces cerevisiae* invertase; the *Aspergillus niger* amylase and glucoamylase; human serum albumin; *Kluyveromyces fragilis* inulinase; and *Pichia pastoris* mating factor and Kar2. Signal sequences shown herein to be useful in yeast and filamentous fungi include, but are not limited to, the alpha mating factor presequence and preprosequence from *Saccharomyces cerevisiae*; and signal sequences from numerous other species.

Examples of promoters include promoters from numerous species, including but not limited to alcohol-regulated promoter, tetracycline-regulated promoters, steroid-regulated promoters (e.g., glucocorticoid, estrogen, ecdysone, retinoid, thyroid), metal-regulated promoters, pathogen-regulated promoters, temperature-regulated promoters, and light-regulated promoters. Specific regulatable promoter systems well-known in the art include the tetracycline-regulatable systems (See for example, Berens & Hillen, *Eur J Biochem* 270: 3109-3121 (2003)), RU 486-inducible systems, ecdysone-inducible systems, and kanamycin regulatable system. Lower eukaryote-specific promoters include but are not limited to the *Saccharomyces cerevisiae* TEF-1 promoter, *Pichia pastoris* GAPDH promoter, *Pichia pastoris* GUT1 promoter, PMA-1 promoter, *Pichia pastoris* PCK-1 promoter, and *Pichia pastoris* AOX-1 and AOX-2 promoters.

In some embodiments, the nucleotide acid sequences are located on a nucleic acid vector (e.g., containing one or more additional sequences useful for replication, selection, etc., or any combination thereof). It should be appreciated that the invention covers any vector comprising nucleic acids including plasmid, phage, viruses, etc., or any other suitable nucleic acid vector.

- 5 The vectors can be introduced into a host cell prior to expression of the engineered protein using well known techniques. The vector may contain a variety of regulatory elements for maintenance of the cell or expression of the engineered protein. Regulatory elements include promoters and markers that allow for positive identification of cells that have taken up the vector. The regulatory elements may be species specific. Regulatory elements are known in
10 the art and the invention embraces any single or combination of regulatory elements needed or desired to express the engineered proteins.

II. General Characteristics of the Capture Moiety

- The second binding moiety is the capture moiety. The capture moiety comprises the other partner of the FimGT/DsF interaction pair (which means if the protein of interest comprises
15 FimGT the capture moiety comprises DsF, and if the protein of interest comprises DsF the capture moiety comprises FimGT) and is capable of specifically interacting with the first binding moiety. The capture moiety can be attached to the cell surface of a host cell.

- It should be appreciated that the second binding moiety may be attached to cell surface either in vitro or in vivo, directly or indirectly, by a variety of methods. Suitable in vitro methods
20 include but are not limited to direct coupling to amino groups or coupling to thiols or indirect coupling (through for example biotin or an antibody). Alternatively, the second binding moiety may be expressed and secreted by the host cell. In some embodiments, the second binding moiety is fused to an anchoring motif and displayed at the cell surface.

- In some embodiments the second binding moiety is attached to the cell surface through a set
25 of binding partners. In some embodiment the cell surface displays a third binding moiety. In some embodiments the second binding moiety is linked to a fourth binding moiety, which can bind to the third binding moiety, thereby displaying the second binding moiety on the cell surface.

- In certain preferred embodiments of the invention the second and fourth binding moiety
30 comprise biotin, while the third binding moiety comprises or is streptavidin or avidin (see for an example Figure 3).

The display of biotin on the cell surface can be established for instance by biotinylation of the cell surface proteins in vitro by, for example the use of NHS-PEG4–biotin (ThermoFischer

Scientific), sulpho-NHS-biotin (Pierce Chemical Co.) or biotin-sulfosuccinimidyl ester (Invitrogen). However, one should appreciate that as cells divide, the daughter cells do not retain biotin. In some embodiments, biotinylation of the cell surface proteins may be accomplished in vivo.

- 5 In another preferred embodiment the second binding moiety comprises a cell surface anchoring protein or cell wall binding protein that is capable of binding or integrating to the surface of the host cell fused at its N- or C-terminus to FimGT or DsF capable of binding the first binding moiety. The FimGT or DsF is located at the end of the cell surface anchoring protein that is exposed to the extracellular environment such that the FimGT or DsF is capable
10 of interacting with the first binding moiety. In a preferred embodiment of the invention the second binding moiety is expressed by the host cell, optionally under the control of an inducible promoter (see Figure 5).

Glycosylphosphatidylinositol-anchored (GPI) proteins provide a suitable means as cell surface anchoring protein for tethering the second binding moiety (capture moiety) to the surface of the
15 host cell. GPI proteins have been identified and characterized in a wide range of species from humans to yeast and fungi. Thus, in particular aspects of the methods disclosed herein, the cell surface anchoring protein is a GPI protein or fragment thereof that can anchor to the cell surface. Lower eukaryotic cells have systems of GPI proteins that are involved in anchoring or tethering expressed proteins to the cell wall so that they are effectively displayed on the cell
20 wall of the cell from which they were expressed. For example, 66 putative GPI proteins have been identified in *Saccharomyces cerevisiae* (See, de Groot et al., *Yeast* 20: 781-796 (2003)). The cell surface anchoring protein may be selected from the list consisting of an agglutinin (α -agglutinin, α -agglutinin, alpha-agglutinin), Cwp1p, Cwp2p, Gas1p, Yap3p, Flo1p, Crh2p, Pir1p, Pir4p, Sed1p, Tip1p, Wpip, Hpwp1p, Als3p, and Rbt5p. α -agglutinin (α -agglutinin) is a
25 highly preferred cell surface anchoring protein if the host cell is yeast.

In some embodiments, the second binding moiety (the capture moiety) is connected to the cell surface through a spacer. The second binding moiety can be covalently bound to the spacer or the second binding moiety can be bound to the spacer through other binding interactions, including binding interactions based on affinity. In some embodiments, the spacer comprises a
30 biotin moiety and is referred to as a biotin spacer. In some embodiments, spacers, including the biotin spacer, can be of any length and comprise any kind of linker, including, alkanes, PEG, etc., or any combination thereof. In some embodiments the linker is attached to the cell surface, through the action of cell wall binding protein. Preferably the spacer is chemically inert and does not react with any other component of the host cell, engineered protein or agent of
35 the cellular environment of the host cell. Molecules that are suitable for linkers in spacers are

known in the art. A spacer may be connected to the cell surface and the invention embraces the linking of the spacer to any cell surface moiety, including sugars, amino acids and lipids. In some embodiments, the spacer is linked to an amino acid side chain (e.g., through the action of an N-succinimidyl ester).

5 III. General Characteristics of the Host Cell

In general any eukaryote can be used to express the protein of interest. Preferred are lower eukaryotes such as yeast or filamentous fungi which can be used for expression of the proteins, particularly glycoproteins because they can be economically cultured, give high yields, and when appropriately modified are capable of suitable glycosylation. Highly preferred
10 are *Pichia* and *Saccharomyces* cells like *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp.

While in general the host cells used to practice the present invention are lower eukaryote host
15 cells (e.g., yeast), it is envisioned that the methods herein can be adapted to use higher eukaryote cells. Thus, in particular embodiments, the cell systems used for recombinant expression and display of the protein of interest can also be any higher eukaryote cell, tissue, organism from the animal kingdom, for example transgenic goats, transgenic rabbits, CHO cells, insect cells, and human cell lines. Examples of animal cells include, but are not limited to
20 CHO cells, COS cells, SC-I cells, LLC-MK cells, CV-1 cells, murine cells, human cells, HeLa cells, 293 cells, VERO cells, MDBK cells, MDCK cells, MDOK cells, CRFK cells, 5 RAF cells, TCMK cells, LLC-PK cells, PK15 cells, WI-38 cells, MRC-5 cells, T-FLY cells, BHK cells, SP2/0, NS0 cells, and derivatives thereof. Insect cells include cells of *Drosophila melanogaster* origin.

25 In particular embodiments, the higher eukaryote cell, tissue, organism can also be from the plant kingdom, for example, wheat, rice, corn, tobacco, and the like.

The methods disclosed herein can be adapted for use in mammalian, insect, and plant cells. The regulatable promoters selected for regulating expression of the expression cassettes in mammalian, insect, or plant cells should be selected for functionality in the cell-type chosen.
30 Examples of suitable regulatable promoters include but are not limited to the tetracycline-regulatable promoters (See for example, Berens & Hillen, Eur. J. Biochem. 270: 3109-3121 (2003)), RU 486-inducible promoters, ecdysone-inducible promoters, and kanamycin-regulatable systems. These promoters can replace the promoters exemplified in the

expression cassettes described in the examples. The capture moiety can be fused to a cell surface anchoring protein suitable for use in the cell-type chosen.

Transformation methods for making stable and transiently transfected mammalian, insect, plant host cells are well known in the art.

5 **Methods for Displaying Proteins**

In general, provided is a method of capturing recombinantly expressed secreted proteins on a cell surface by using the interaction pair FimGT/DsF.

Provided is a method for the immobilization of recombinantly expressed secreted proteins on a eukaryote host cell surface where an interaction of FimGT and DsF is formed.

- 10 Further provided is a method for displaying proteins on a eukaryote host cell surface, which comprises a step of immobilization and / or capturing a protein of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair (which means FimGT or DsF), wherein said POI is expressed and secreted by said host cell and wherein a second binding moiety that comprises the other partner of the
- 15 FimGT/DsF interaction pair (which means if the first binding moiety comprises FimGT the second binding moiety comprises DsF and if the first binding moiety comprises DsF the second binding moiety comprises FimGT) is immobilized on said host cell surface.

- Further provided is a method for displaying proteins on a eukaryote cell surface, comprising (i) transforming a host cell with a nucleic acid encoding a protein of interest (POI) fused to a first
- 20 binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, and (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety.

- 25 Further provided is a method for selecting proteins for displayability on a eukaryote cell surface, comprising: (i) transforming a host cell with nucleic acids encoding proteins of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, (iii) capturing the POI on the cell surface with a second binding moiety
- 30 that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety, and (iv) contacting the host cells with a detection means that specifically binds to POI that are displayed on the surface of the host cell and does not bind to proteins that are not displayed on the surface of the host cell; and

isolating the host cells with which the detection means is bound, wherein the presence of the detection means bound to a protein on the surface of the host cells indicates the proteins are displayable on the eukaryote cell surface.

Further provided is a method for selecting a desired protein or a recombinant eukaryote host cells that display a desired protein on the surface of the host cells, comprising: (i) transforming a host cell with nucleic acids encoding proteins of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety, and (iv) contacting the host cells with a detection means that specifically binds to proteins that are displayed on the surface of the host cell and isolating the host cells with which the detection means is bound to select the host cells that display the desired protein.

Further provided is a library method for identifying and selecting a particular member of a specific binding pair including but not limited to antibodies and antibody fragments (e.g. Fab fragments) or cells that produce a particular member of a specific binding pair including but not limited to antibodies and antibody fragments (e.g. Fab fragments). Therefore, in further aspects, a method of producing a protein that is a member of a specific binding pair, wherein the specific binding pair member is an antibody or antibody fragment, comprising an antibody VH domain and an antibody VL domain, and having an antigen binding site with binding specificity for an antigen of interest. The method comprises providing a library of lower eukaryote host cells displaying on their surface a specific binding pair member, which specific binding pair member is an antibody or antibody fragment comprising a synthetic human antibody VH domain and a human antibody VL domain. The library is created by providing lower eukaryote host cells that express a capture moiety comprising a cell surface anchoring protein fused to a first binding moiety and providing a library of nucleic acid sequences encoding a genetically diverse population of the specific binding pair member, wherein the VH domains of the genetically diverse population of the specific binding pair member are biased for one or more VH gene families and wherein the specific binding pair member includes a second binding moiety that is capable of specifically interacting with the first binding moiety fused to the cell surface anchoring protein. The library of nucleic acid sequences is expressed in the lower eukaryote host cells so that each specific binding pair member is displayed at the surface of a lower eukaryote host cell. Then, cells that produce one or more specific binding pair members having a binding specificity for the antigen of interest are selected by binding the one or more specific binding pair members with the antigen of interest. The further aspects, the specific binding pair member comprises a synthetic human antibody VH domain and a

synthetic human antibody VL domain and wherein the synthetic human antibody VH domain and the synthetic human antibody VL domain comprise framework regions and hyper variable loops, wherein the framework regions and first two hyper variable loops of both the VH domain and VL domain are essentially human germ line, and wherein the VH domain and VL domain have altered CDR3 loops. In further still aspects in addition to having altered CDR3 loops, the human synthetic antibody VH and VL domains contain mutations in other CDR loops. In further aspects, each human synthetic antibody VH domain CDR loop is of random sequence. In further still aspects, the human synthetic antibody VH domain CDR loops are of known canonical structures and incorporate random sequence elements. The binding pair member can be a full-sized or whole antibody or a fragment such as a single-chain Fv antibody fragment.

Detection of host cells that express the desired protein of interest can be achieved by labeling the host cells with a first label, wherein the first label associates with or binds to the protein of interest and does not associate with or bind to host cells which do not express the protein of interest. For example, in the case when the protein of interest is an antibody, the first label can be an antigen that is specifically recognized by the antibody of interest. The host cells with which the first label is associated are selected and the amount of first label associated with the host cell is quantitated. A high occurrence of the first label indicates the protein of interest has desirable binding properties and a low occurrence of the first label indicates the protein of interest does not have desirable binding properties.

A further aspect includes the steps of labeling the above host cells with a second label, wherein the second label associates with or binds to host cells expressing an epitope tag fused to the protein of interest and does not associate with or bind to host cells which do not express the epitope tag. The amount of second label associated with the host cells is quantitated. The amount of the second label associated with the host cell indicates a number of expressed copies of the epitope-tagged protein of interest on the host cell surface and by comparing the quantitation of the first label to the quantitation of the second label enables the amount of the first label normalized for the amount of the second label, wherein a high occurrence of the first label relative to the occurrence of the second label indicates the protein to be tested has desirable binding properties.

Another aspect includes the steps of labeling the above host cells with a third label that competes with the first label for binding to the protein of interest. In this aspect, the host cells are labeled with the first label and the amount of first label associated with host cells is quantitated. Then the host cells are labeled with the second label and the amount of second label associated with host cells is quantitated. Comparing the quantitation of the first label to

the quantitation of the second label is performed to determine the occurrence of the first label normalized for the occurrence of the second label, wherein a low occurrence of the first label relative to the occurrence of the second label indicates the protein of interest has desirable binding properties.

- 5 In further aspects, the first label is a fluorescent label attached to a ligand specific for the protein of interest and the second label is a fluorescent label attached to an antibody specific for the protein of interest. When the labels are fluorescent, the quantitation step is performed by flow cytometry or confocal fluorescence microscopy. In a further still aspect, the first label is a fluorescent label attached to a ligand specific for the protein of interest and fluorescence
10 activated cell sorting (FACS) is used to separate the host that express the protein of interest from host cells that do not produce the protein of interest.

Representative fluorophores for use in the methods provided herein include, for example, green fluorescent protein, blue fluorescent protein, red fluorescent protein, fluorescein, fluorescein 5-isothiocyanate (FITC), cyanine dyes (Cy3, Cy3.5, Cy5, Cy5.5, Cy7), Bodipy dyes
15 (Invitrogen) and/or Alexa Fluor dyes (Invitrogen), dansyl, Dansyl Chloride (DNS-C1), 5-(iodoacetamida) fluorescein (5-IAF, 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), 7-nitrobenzo-2-oxa-1,3,-diazol-4-yl chloride (NBD-Cl), ethidium bromide, Lucifer Yellow, rhodamine dyes (5-carboxyrhodamine 6G hydrochloride, Lissamine rhodamine B sulfonyl chloride, rhodamine-B-isothiocyanate (RITC (rhodamine-B-isothiocyanate), rhodamine 800);
20 tetramethylrhodamine 5-(and 6-) isothiocyanate (TRITC)), Texas Red™, sulfonyl chloride, naphthalamine sulfonic acids including but not limited to 1-anilinonaphthalene-8-sulfonic acid (ANS) and 6-(ptoluidinyl) naphthalen-e-2-sulfonic acid (TNS), Anthroyl fatty acid, DPH, Parinaric acid, TMA-DPH, Fluorenyl fatty acid, Fluorescein-phosphatidylethanolamine, Texas red-phosphatidylethanolamine, Pyrenyl- phophatidylcholine, Fluorenyl-phosphotidylcholine,
25 Merocyanine 540, Naphtyl Styryl, 3,3'dipropylthiadicarbocyanine (diS-C3-(5)), 4-(p-dipentyl aminostyryl)-1-methylpyridinium (di-5-ASP), Cy-3 Iodo Acetamide, Cy-5-N-Hydroxysuccinimide, Cy-7-Isothiocyanate, IR-125, Thiazole Orange, Azure B, Nile Blue, A1 Phthalocyanine, Oxaxine 1, 4', 6-diamidino-2-phenylindole. (DAPI), Hoechst 33342, TOTO, Acridine Orange, Ethidium Homodimer, N(ethoxycarbonylmethyl)-6-methoxyquinolinium
30 (MQAE), Fura-2, Calcium Green, Carboxy SNARF-6, BAPTA, coumarin, phytofuors, Coronene, and metal-ligand complexes.

Preferred Embodiments of this invention are:

1. A method for displaying proteins on a eukaryote host cell surface, which comprises a step of immobilization and / or capturing a protein of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, wherein said POI is expressed and secreted by said host cell and wherein a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair is immobilized on said host cell surface.
2. The method for displaying proteins on a eukaryote cell surface according to embodiment 1, comprising the steps of (i) transforming a host cell with a nucleic acid encoding a protein of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, and (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety.
3. A method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface of the host cells using the method according to embodiment 1 or 2.
4. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to embodiment 3 comprising: (i) transforming a host cell with nucleic acids encoding proteins of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety, and (iv) contacting the host cells with a detection means or a first label that specifically binds to desired proteins that are displayed on the surface of the host cell and isolating the host cells with which the detection means or the first label is bound to select the host cells that display the desired protein.
5. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to embodiment 3 or 4,

wherein the desired protein is an antibody or a functional fragment thereof or a binding protein.

- 5 6. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to one or more of embodiments 3 to 5 which comprises the steps of labeling the above host cells with a second label, wherein the second label associates with or binds to host cells expressing an epitope tag fused to the protein of interest and does not associate with or bind to host cells which do not express the epitope tag.
- 10 7. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to one or more of embodiments 3 to 6, wherein the first label is a fluorescent label attached to a ligand specific for the protein of interest and fluorescence activated cell sorting (FACS) is used to separate the host that express the protein of interest from host cells that do not produce the protein of interest.
- 15 8. A method for selecting proteins for displayability on a eukaryote cell surface using a method according to embodiment 1 or 2.
- 20 9. The method for selecting proteins for displayability on a eukaryote cell surface according to embodiment 8 comprising: (i) transforming a host cell with nucleic acids encoding proteins of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety, and (iv) contacting the host cells with a
25 detection means that specifically binds to POI that are displayed on the surface, wherein the presence of the detection means bound to a protein on the surface of the host cells indicates the proteins are displayable on the eukaryote cell surface.
10. The method according to one or more of embodiments 1 to 9, wherein the host cell is a lower eukaryote.

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11. The method according to one embodiment 10, wherein the lower eukaryote cells are Pichia or Saccharomyces cells, preferably Pichia pastoris or Saccharomyces cerevisiae cells.
- 5 12. The method according to one or more of embodiments 1 to 11, wherein the host cells have been transformed with a nucleic acid library encoding proteins of interest (POI).
13. The method according to one or more of embodiments 1 to 12, wherein FimGT or DsF comprised in the second binding moiety is chemically linked to an amino acid side chain of a cell surface protein or fused to a cell surface anchoring protein.
- 10 14. The method according to embodiment 13 wherein said cell surface anchoring protein is alpha-agglutinin.
- 15 15. The method according to one or more of embodiments 1 to 14, wherein the cell surface displays a third binding moiety and the second binding moiety is linked to a fourth binding moiety, which can bind to the third binding moiety, thereby displaying the second binding moiety on the cell surface.
16. The method according to embodiment 15 wherein the second and fourth binding moiety comprise biotin, and wherein the third binding moiety comprises or is streptavidin or avidin.
17. The method according to one or more of embodiments 1 to 16, wherein the first binding moiety comprises DsF and the second binding moiety comprises FimGT.

EXPERIMENTAL PROCEDURES

Experiment 1

The aim of the experiment was the confirmation of the interaction of an IgG-FimGT fusion molecule and a DsF comprising peptide as a coating agent. Therefore, as a proof of concept, a first, three-layer, avidin based approach was tested as depicted in Figure 3. The first layer consists of NHS-biotin, the second layer of Streptavidin-APC and the third layer of DsF-biotin. All three layers were applied to the yeast cells in separate steps. The yeast strain used for this experiment was yscETF42 producing the soluble IgG anti-TWEAK-R B01 fused to FimGT.

The expression plasmid pETF67 used in this experiment 1 is based on the bicystronic, yeast epitope tagging vector pESC-Leu (#217452) from Agilent Technologies. Heavy (SEQ ID NO:4) and light chain sequences (SEQ ID NO:3) of an anti-TWEAK-B01 antibody were integrated into the multiple cloning sites under the control of the bicystronic GAL1/GAL10 promoter (SEQ ID NO:1). Both chains were genetically fused to the leader peptide app8 (SEQ ID NO:2), respectively. The anti-TWEAK-B01 antibody light chain was fused to a myc affinity tag (SEQ ID NO:8) and the corresponding anti-TWEAK-B01 antibody heavy chain was fused to a 3xHA (SEQ ID NO:5) and a FLAG tag (SEQ ID NO:6) followed by the fusion protein FimGT (SEQ ID NO:7) (Figure 2). The expression plasmid pETF67 was transformed into the yeast strain vwk18gal⁻ (derived from CEN.PK102-3A with a gal1 deletion; *MATa MAL2-8c, SUC2 leu2-3,112, ura3-52, gal1::URA3*) by using the Frozen-EZ Yeast Transformation II Kit™ of ZYMOGEN research according to the manufactures instruction resulting in the strain yscETF42.

A single colony of the strain yscETF42 was inoculated into 10 ml SC-leu + 2% raffinose and incubated at 30 °C with shaking overnight. With this pre-culture 10 ml of YP-Induction media (2 % Galactose + 0,025 % BSA) were inoculated to OD₆₀₀ = 5 and incubated at 20 °C with shaking overnight. 2 x10⁷ cells were washed two times with 0,1% BSA/PBS. The pellet was resuspended in fresh 20 µl 2 mg/ml NHS-PEG4-biotin (solved in carbonate buffer: 4.2% NaHCO₃ and 0.034% Na₂CO₃, pH= 8.4; 2 mg NHS-PEG4 – biotin linker dissolved in 100 µl carbonate buffer; a 1:10 dilution was prepared) (Thermo scientific, Pierce, EZ-Link NHS-PEG4-Biotin, No-Weigh Format, 8x2mg). Cells were incubated for 30 min at RT and vortexed every 10 min. Cells were washed 1 time with 1 ml 0,1 % BSA/PBS. Cells were resuspended in 20 µl of 5 mg/ml Strep-APC or 5 mg (PerkinElmer SureLight™ Allophycocyanin-streptavidin (APC-SA))/ml Strep (Roche; Cat. No. 11 721 674 001 5 mg package) and incubated at RT for 20 min in the dark. Cells were washed 1 time with 1 ml 0,1% BSA/PBS and incubated with biotinylated DsF (1,1 mg/ml). Cells were washed 1 time with 1 ml 0,1% BSA/PBS. Cells were resuspended in 1 ml YP induction medium + 10 % PEG (YP, 2 % Galactose, 10 % BSA) and

incubated in a 6well plate (1 ml per well) at 20 °C without shaking in the dark. 100µl per sample were taken for OD₆₀₀ measurement and FACS analysis to verify the coating efficiency. After 22h the display level via FACS and the cell density of all samples were analyzed. All yeast cells were harvested and washed 1 time using PBS. 10⁷ cells were stained using goat-
5 anti-h FC PE conjugated antibody (Jackson Immuno Research 109-116-088 Lot. 93263) in a 1:100 dilution at RT in the dark for 20 min. Cells were washed 1 time in PBS and analyzed via FACS using a BD FACS AriaIII cell sorter.

First, FACS analysis of the cells revealed an efficient labeling of the yeast cells via the APC signal of streptavidin-APC. Second, captured IgG-FimGT fusions were detected via an anti-
10 human Fc-PE conjugated antibody (Figure 4). The FACS analysis revealed an efficient labeling of 99 % of all analyzed cells and also the capturing of IgG-FimGT fusions on the surface (Table 1). Thus, the data show a functional interaction between DsF as coating agent and soluble expressed IgG-FimGT fusion proteins.

Experiment 2

15 The aim of the experiment was to underline the feasibility of the interaction of FimGT and DsF in a yeast display setting in a bait prey system because the approach could also be envisioned by using FimGT as the catching moiety and DsF fused to a full IgG molecule at the heavy chain (Figure 5).

Therefore, FimGT was displayed via alpha-agglutinin (DNA sequence is SEQ ID NO:9) (BAC-
20 system). FimGT (SEQ ID NO:7) was introduced into the multiple cloning site of the plasmid under the control of the Gal7 promoter (SEQ ID NO:11) and integrated downstream to the alpha mating factor leader sequence (SEQ ID NO:10) and upstream to a myc tag (SEQ ID NO:8) and the membrane anchor molecule alpha-agglutinin (SEQ ID NO:9) The resulted expression plasmid pETF65 was transformed into the yeast strain *vwk18^{gal-}* (derived from
25 CEN.PK102-3A with a *gal1* deletion; *MATa MAL2-8c, SUC2 leu2-3,112, ura3-52, gal1::URA3*) by using the Frozen-EZ Yeast Transformation II Kit™ of ZYMOGEN research according to the manufactures instruction resulting in the strain yscETF40 encoding for FimGT fused to alpha-agglutinin.

A single colony of the strain yscETF40 and yscETF13 (carrying the empty plasmid pEsc-leu
30 instead the expression plasmid pETF65) was inoculate into 20 ml SC-leu + 2% raffinose and incubated at 30 °C with shaking overnight. With this pre-culture 20 ml of SC-LEU-Induction media (2 % Galactose, 10 % PEG) were inoculated to OD₆₀₀ = 0,5 and incubated at 20 °C with shaking for 3 days. Cells were harvested and washed once with PBS. 10⁷ cells were resuspended in 50 µl of staining solution of the primary antibody (anti-myc Sigma C3956-

m2MG (1:50 in PBS)) for 40 min. at 4 °. Cells were harvested and washed once with PBS. 10⁷ cells were resuspended in 50 µl of staining solution of the secondary antibody (anti-rabbit-APC Jackson Immuno Research, 111-136-144, Lot: 11947; 1:100 in PBS) for 30 min. at 4 °C. Cells were washed once with PBS and analyzed via FACS using BD FACS AriaIII cell sorter.

- 5 FACS analysis confirmed a sufficient display level of FimGT via alpha-agglutinin in comparison to the strain yscETF13 carrying the empty plasmid pEsc-leu instead the expression plasmid pETF65 (Figure 4). To address the question whether displayed FimGT can be used to catch DsF-fusion proteins, FimGT displaying cells (yscETF42) were incubated with c-terminal biotin-labeled DsF peptides (peptide No: 5). In a next step, the binding of Dsf to displayed FimGT
- 10 was measured. Therefore, 10⁷ cells were stained as before to monitor the FimGT display and also coated afterwards with DsF-Biotin (incubate cells with 7 µg/ml; stock solution 1,1 mg/ml) for 30 min. at RT. The detection of bound DsF-Biotin was done via Streptavidine-PE (Life technologies; S21388; 1252780). Therefore, in the second step, caught DsF-biotin peptides on the yeast cell were labeled with streptavidin-PE (1:100 dilution in PBS) and analyzed by FACS
- 15 (Figure 6) (Table 2).

The FACS analyses clearly showed that FimGT could be displayed on the surface of yeast cells via alpha-agglutinin (see Table 2 comparing yscETF13 and yscETF40 depicting a difference in the APC signal of 53,4 %) and is capable to catch soluble DsF peptides in a yeast display experimental set-up (see Table 2 comparing yscETF13 and yscETF40 depicting

20 a difference in the PE signal of 58,2 %).

Experiment 3

The aim of the experiment was to verify the coating of yeast cells via maleimide coupled peptides as DsF and further identify the optimal conditions for this purpose.

- 25 As a prerequisite of an efficient display of IgG-FimGT fusion proteins on the surface of yeast cells the coating procedure of yeast cells via DsF-maleimide had to be optimized. Therefore, a n-terminal biotinylated DsF-maleimide peptide was used to monitor the optimal conditions for a most efficient labeling of cells. The peptide No: 2 was resolved in DMF resulting in a concentration of 11mg/ml. 10⁷ freshly grown cells (yscETF42) were coated with a 1:1000 dilution of the peptide stock solution and incubated under different conditions (Table 3).

- 30 After incubation the cells were labeled with R-phycoerythrin conjugated streptavidin (Life technologies, S21388) (1:100 dilution in PBS) and incubated at RT for 20 min. Cells were harvested and washed once with PBS. Cells were analyzed via FACS to monitor the efficiency of labeling (Figure 7).

The experiment clearly revealed that peptides coupled to maleimide could be coated on the surface of yeast cells via covalent binding between thiol groups located on the surface of the yeast cell and the functional group maleimide coupled to the peptide. As the optimal coating condition for this approach 60 minutes incubation at RT with shaking could be identified

5 (Figure 7).

TABLES

Table 1:

FACS analysis of yeast cells concerning coating efficiency (APC signal) and captured, displayed IgG-FimGT fusion on the surface (PE-signal)

Strain	APC positive (% of all cells) [Coating efficiency]	PE positive (% of all cells) [IgG display]
yscETF42 without biotin-DsF coating	99.3	4.9
yscETF42+ biotin-DsF coating	99.2	51.2

5

Table 2:

FACS analysis of yscETF13 and yscETF14 concerning FimGT display (APC signal) and DsF binding (PE signal)

Strain	APC positive (% of all cells) [FimGT display]	PE positive (% of all cells) [DsF binding]
yscETF13	1.7	1.5
yscETF40	55.1	59.7

10 **Table 3:**

Tested conditions to identify the optimal conditions for yeast cells coating via maleimide coupled peptide DsF

Duration	shaking	
30 min	yes	no
60 min	yes	no
120 min	yes	no
No coating	yes	no

Table 4:

Sequences

Name	SEQ ID NO:	Sequence
GAL1/Gal10 promoter	SEQ ID NO:1	g tt ga at t c ga at t t t t c a a a a a t t c t t a c t t t t t t t t t t g g a t g g a c g c a a a g a a g t t t a a t a a t c a t a t t a c a t g g c a t t a c c a c c a t a t a c a t a t c c a t a t a c a t a t c c a t a t c t a a t c t t a c t t a t a t g t t g t g g a a a t g t a a a g a g c c c c a t t a t c t t a g c c t a a a a a a c c t t c t c t t t g g a a c t t t c a g t a a t a c g c t t a a c t g c t c a t t g c t a t a t t g a a g t a c g g a t t a g a a g c g c g a g c g g g t g a c a g c c c t c c g a a g g a a g a c t c t c c t c c g t g c g t c c t c g t c t t c a c c g g t c g c g t t c c t g a a a c g c a g a t g t g c c t c g c g c c g c a c t g c t c c g a a c a a t a a g a t t c t a c a a t a c t a g c t t t t a t g g t t a t g a a g a g g a a a a a t t g g c a g t a a c c t g g c c c c a c a a a c c t t c a a a t g a a c g a a t c a a a t t a a c a a c c a t a g g a t g a t a a t g c g a t t a g t t t t t t a g c c t t a t t t c t g g g g t a a t t a a t c a g c g a a g c g a t g a t t t t t g a t c t a t t a a c a g a t a t a t a a a t g c a a a a a c t g c a t a a c c a c t t t a a c t a a t a c t t t c a a c a t t t t c g g t t t g t a t t a c t t c t t a t t c a a a t g t a a t a a a g t a t c a a c a a a a a t t g t t a a t a t a c c t c t a t a c t t t a a c g t c a a
prepro leader app8	SEQ ID NO:2	a a a t a a t c t t t t a t c t a a t t g c c c t t c t t c t t t a g c a g c a a t g c t g g c a a t a g t a g t a t t a g t a g a a g a t a a c c c g t t a t t t g t g c t g t t g g a t a a t g g c a a a g c a g c a g c a t c g a a a t c c c c t t c t a a a t c t g a g t a a t c g a t g a c a g c t t c a g c c g g a a t t t g t g c c g t t t c a t c t t c t g t t g t a g t g t t a g c g a c a g c a g c t a a t g c g g a g g a t g c t g c g a a t a a a a c g g c a g t a a a a a t t g a a g g a a a t c t c a t
mature Lc TWEAK-B01	SEQ ID NO:3	g a c a t c c a g a t g a c c c a g t c t c c a g c c a c c c t g t c t g c a t c t g t a g g a g a c a g a g t c a c c a t c a c t t g c g g g c a a g t c a g a g c a t t a g c a g c t a t t t a a a t t g g t a t c a g c a g a a a c c a g g g a a a g c c c c t a a g c t c c t g a t c t a t g c t g c a t c c a g t t t g c a a a g t g g g g t c c c a t c a a g g t t c a g t g g c a g t g g a t c t g g g a c a g a t t t c a c t c t c a c c a t c a g c a g t c t g c a a c c t g a a g a t t t t g c a a c t t a c t a c t g t c a a c a g a g c t a c t c t a g t c c a g g g a t c a c t t t c g g c c c t g g g a c c a a g g t g g a g a t c a a a c g a a c t g t g g c t g c a c c a t c t g t c t t c a t c t t c c c g c c a t c t g a t g a g c a g t t g a a a t c t g g a a c t g c c t c t g t t g t g t g c c t g c t g a a t a a c t t c t a t c c c a g a g a g g c c a a a g t a c a g t g g a a g g t g g a t a a c g c c c t c c a a t c g g g t a a c t c c c a g g a g a g t g t c a c a g a g c a g g a c a g c a a g g a c a g c a c c t a c a g c c t c a g c a g c a c c c t g a c g c t g a g c a a g c a g a c t a c g a g a a c a c a a a c t c t a c g c c t g c g a a g t c a c c c a t c a g g g c c t g a g c t c g c c c g t c a c a a a g a g c t t c a a c a g g g g a g a g t g t
mature Hc TWEAK-B01	SEQ ID NO:4	g c c g g g g c t c a g g c t c a g g g a c t t c t g g g t g t a g t g a t t g t g c a g g g c c t c g t g c a t c a c g c t g c a g c t g a a c a c g t t g c c c t g c t g c c a c c g g c t c t t g t c c a c g g t c a g c t t g g a g t a c a g g a a g a a t g a g c c g t c g c t g t c c a g c a c a g g g g g g t g g t c t t g t a a t t g t t c t c g g g c t g g c g t t g c t c t c c c a t t c c a c g g c g a t a t c g c t g g g g t a g a a g c c t t t c a c c a g a c a g g t c a g g g a c a c c t g g t t c t t g g t c a t c t c t t c c c g g c t g g g g g c a g t g t g t a c a c c t g a g g c t c g c g g g c t g g c c c t t g g c c t t g c t g a t g g t t t t c t c g a t g g g g c a g g c a g g g c c t t g t t g g a g a c c t t g c a c t t g t a c t c t t t g c g t t c a g c c a g t c c t g g t g c a g c a c g g t c a g c a c g g a c a c c a c c g g t a g g t g c t g g c g t a c t g t t c c t c t c t g g g c t t g g t c t t g g c g t t a t g c a c t t c c a c g c c g t c c a c g t a c c a a t t a a a c t t c a c t t c t g g g t c c t c g t g g g a c a c g t c c a c c a c c a c g c a g g t c a c t t c g g g g g t c c g g c t g a t c a t c a g

Name	SEQ ID NO:	Sequence
		ggtgtccttgggctttggggggaacaggaacacgctgggtcc gcccagcagttcaggggcagggcaggggggacaggtgtgggt cttgtcgcagctcttgggttccaccgccttgtccaccttgggt gttgtctgggcttgtgggttcacgttgcagatgtaggctctgggt tcccaggctgctgctgggcactgtcaccacgctgctcaggct gtacaggccgctgctctgcagcacggcggggaagggtatgcac tccgctggtcagggcgccagagttccaggacacgggtcacggg ctgggggaagtagtctttcaccaggcagcccaggggcggtgt tccgccgctgggtgctcttctgctgctgggtgctagcggggaagac cgatggggcccttgggtggaggcgcttgagacgggtgaccagggt tccctggccccagtagtcaaagtagtgcgaaataaccatcacc cctctcgcacagtaatacacggccgtgtcctcagcccttaa gctgttcatctgcaagtagagagtattcttagagttgtctct agagatagtgaagcgacctttaacggagtcagcataatgagt cttgccaccagaaggagagatataagaaacccactccaaacc tttaccaggagcttggcgaaacccacatcatagggttaaggaga gaaagtgaatccggaagcagcgcaagaaagacgtaaaagaacc accaggctgaacaagaccgccaccagactctaacaattgaac ttc
3x HA tag	SEQ ID NO:5	agcatagtctgggacgtcatatggataagcatagtctgggac gtcatatggata
FLAG tag	SEQ ID NO:6	cttatcgctcgatccttctaatac
FimGT fusion	SEQ ID NO:7	gctataggtataggtaatgctaataactgctgaatgggtgcc ctgggttgcaccaccattaacgggtcagtgacgaacctgcag cggaaaatgtgcgctctgggtgctatcatcaacctgaacgggt tttgggtgcaccgggtattcagggtattaccgctatcatcctg cagttccagctgaatattctgtgcggtgccctgatttttgta ataaccgggtgctatcgggtgcaccgctaaagctggcggtaac acgggtggtgccaaccggacaattgggtcagttccagtgcaac atcatgccatgcgcttctgctgcaccggcactcatcaggctaaa gctatacagatcaccagatcaacgggttgcatgtgggtgct aacgggtacacgggttttgc
myc tag	SEQ ID NO:8	gaacagaagttgattttccgaagaagacctcg
Alpha agglutinine	SEQ ID NO:9	gctagcgccaaaagctcttttatctcaaccactactactgat ttaacaagtataaacactagtgcgtattccactggatccatt tccacagtagaaacagggaatcgaactacatcagaagtgatc agtcatgtgggtgactaccagcacaaaactgtctccaactgct actaccagcctgacaattgcacaaaccagtagtattcttact gactcaaatatcacagtaggaacagatattcacaccacatca gaagtgattagtgatgtggaaccattagcagagaaacagct tcgaccgtttagcgctccaacctcaacaactggatggaca ggcgctatgaatacttacatcccgaattttacatcctcttct ttcgcaacaatcaacagcacaccaataatctcttcacagca gtatttgaaacctcagatgcttcaattgtcaatgtgcacact gaaaatatcacgaatactgctgctgttccatctgaagaaccc acttttgtaaatgccacgagaaactccttaaatccttttgc agcagcaaacagccatccagtcctcatcttatacgtcttcc ccactcgtatcgctccctctccgtaagcaaaacattactaagc accagttttacgccttctgtgccaacatctaatacatatatac aaaacggaaaatacgggttactttgagcacacggctttgaca acatcttcagttggccttaattcttttagtgaaacagcactc tcatctcaggggaacgaaaattgacaccttttttagtgatcc ttgatcgcatatccttcttctgcatcaggaagccaattgtcc ggtatccaacagaatttcacatcaacttctctcatgatttca acctatgaaggtaaagcgtctatatttttctcagctgagctg ggttcgatcatttttctgcttttctgctacctgctattc

Name	SEQ ID NO:	Sequence
Mating factor alpha leader	SEQ ID NO:10	atgatgagatttccttcaatttttactgccgttttattcgca gcatcctccgcattagctgctccagtcacactacaacagaa gatgaaacggcacaattccggctgaagctgtcatcggttac tcagatttagaaggggatttcgatgttgctgttttgccattt tccaacagcacaataaacgggttattgtttataaatactact attgccagcattgctgctaagaagaaggggtatctc
Gal 7 promoter	SEQ ID NO:11	tctagctatacttcgcagcactgttgagcgaaggctcattag atataattttctgtcattttccttaacccaaaaataagggaga ggatccaaaaagcgctcggacaactgttgaccgtgatccgaa ggactggctatacagtggttcacaaaatagccaagctgaaaat aatgtgtagccttttagctatgttcagttagtttggttagcaa agatataaaagcaggtcggaaatatttatgggcattattatg cagagcatcaacatgataaaaaaaaaacagttgaatattccgag
FimGT	SEQ ID NO:12	AKPCTVSTTNATVDLGDLYSFSLSMAGAASAWHDVALELTNC PVGTSRVTSFSGAADSTGYKNGQTAQNIQLELQDDSGNTL NTGATKTVQVDDSSQSAHFPLQVRALTVNGGATQGTIQAVIS ITYTYS
DsF	SEQ ID NO:13	ADSTITIRGYVRDNG

Table 5:

Peptides used

Name	Peptide No	Description
DsF-Ttds-Lys-maleimide	peptide No: 1	ADSTITIRGYVRDNG-Ttds-Lys-maleimide
Biotin-Ttds-DsF-Lys-Maleimide	peptide No: 2	Biotin-Ttds-ADSTITIRGYVRDNG-Ttds-Lys-maleimide
DsF-Ttds-Lys-biotin	peptide No: 5	ADSTITIRGYVRDNG-Ttds-Lys-biotin

5 **Table 6:**

Yeast Strains

Name	Description
yscETF13	CEN.PK102-3A + pEsc-Leu (MATa MAL2-8c, SUC2 leu2-3,112, ura3-52, gal1::URA3)
yscETF14	CEN.PK102-3A + pETF35 (MATa MAL2-8c, SUC2 leu2-3,112, ura3-52, gal1::URA3)
yscETF40	CEN.PK102-3A + pETF65 (MATa MAL2-8c, SUC2 leu2-3,112, ura3-52, gal1::URA3)
yscETF42	CEN.PK102-3A + pETF67 (MATa MAL2-8c, SUC2 leu2-3,112, ura3-52, gal1::URA3)

CLAIMS

1. A method for displaying proteins on a eukaryote host cell surface, which comprises a step of immobilization and / or capturing a protein of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, wherein said POI is expressed and secreted by said host cell and wherein a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair is immobilized on said host cell surface.
2. The method for displaying proteins on a eukaryote cell surface according to claim 1, comprising the steps of (i) transforming a host cell with a nucleic acid encoding a protein of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, and (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety.
3. A method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface of the host cells using the method according to claim 1 or 2.
4. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to claim 3 comprising: (i) transforming a host cell with nucleic acids encoding proteins of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically interacting with the first binding moiety, and (iv) contacting the host cells with a detection means or a first label that specifically binds to desired proteins that are displayed on the surface of the host cell and isolating the host cells with which the detection means or the first label is bound to select the host cells that display the desired protein.

5. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to claim 3 or 4, wherein the desired protein is an antibody or a functional fragment thereof or a binding protein.
- 5 6. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to one or more of claims 3 to 5 which comprises the steps of labeling the above host cells with a second label, wherein the second label associates with or binds to host cells expressing an epitope tag fused to the protein of interest and does not associate with or bind to host cells which do not express the epitope tag.
- 10 7. The method for selecting a desired protein and / or a recombinant eukaryote host cell that displays a desired protein on the surface according to one or more of claims 3 to 6, wherein the first label is a fluorescent label attached to a ligand specific for the protein of interest and fluorescence activated cell sorting (FACS) is used to separate the host that express the protein of interest from host cells that do not produce the
15 protein of interest.
8. A method for selecting proteins for displayability on a eukaryote cell surface using a method according to claim 1 or 2.
9. The method for selecting proteins for displayability on a eukaryote cell surface according to claim 8 comprising: (i) transforming a host cell with nucleic acids
20 encoding proteins of interest (POI) fused to a first binding moiety wherein the first binding moiety comprises one partner of the FimGT/DsF interaction pair, (ii) incubating the host cells under conditions resulting in secretion of the POI, (iii) capturing the POI on the cell surface with a second binding moiety that comprises the other partner of the FimGT/DsF interaction pair and is capable of specifically
25 interacting with the first binding moiety, and (iv) contacting the host cells with a detection means that specifically binds to POI that are displayed on the surface, wherein the presence of the detection means bound to a protein on the surface of the host cells indicates the proteins are displayable on the eukaryote cell surface.
- 30 10. The method according to one or more of claims 1 to 9, wherein the host cell is a lower eukaryote.

11. The method according to one claim 10, wherein the lower eukaryote cells are *Pichia* or *Saccharomyces* cells, preferably *Pichia pastoris* or *Saccharomyces cerevisiae* cells.
- 5 12. The method according to one or more of claims 1 to 11, wherein the host cells have been transformed with a nucleic acid library encoding proteins of interest (POI).
13. The method according to one or more of claims 1 to 12, wherein FimGT or DsF comprised in the second binding moiety is chemically linked to an amino acid side chain of a cell surface protein or fused to a cell surface anchoring protein.
- 10 14. The method according to claim 13 wherein said cell surface anchoring protein is alpha-agglutinin.
- 15 15. The method according to one or more of claims 1 to 14, wherein the cell surface displays a third binding moiety and the second binding moiety is linked to a fourth binding moiety, which can bind to the third binding moiety, thereby displaying the second binding moiety on the cell surface.
16. The method according to claim 15 wherein the second and fourth binding moiety comprise biotin, and wherein the third binding moiety comprises or is streptavidin or avidin.
17. The method according to one or more of claims 1 to 16, wherein the first binding moiety comprises DsF and the second binding moiety comprises FimGT.

FIGURES

Fig. 1

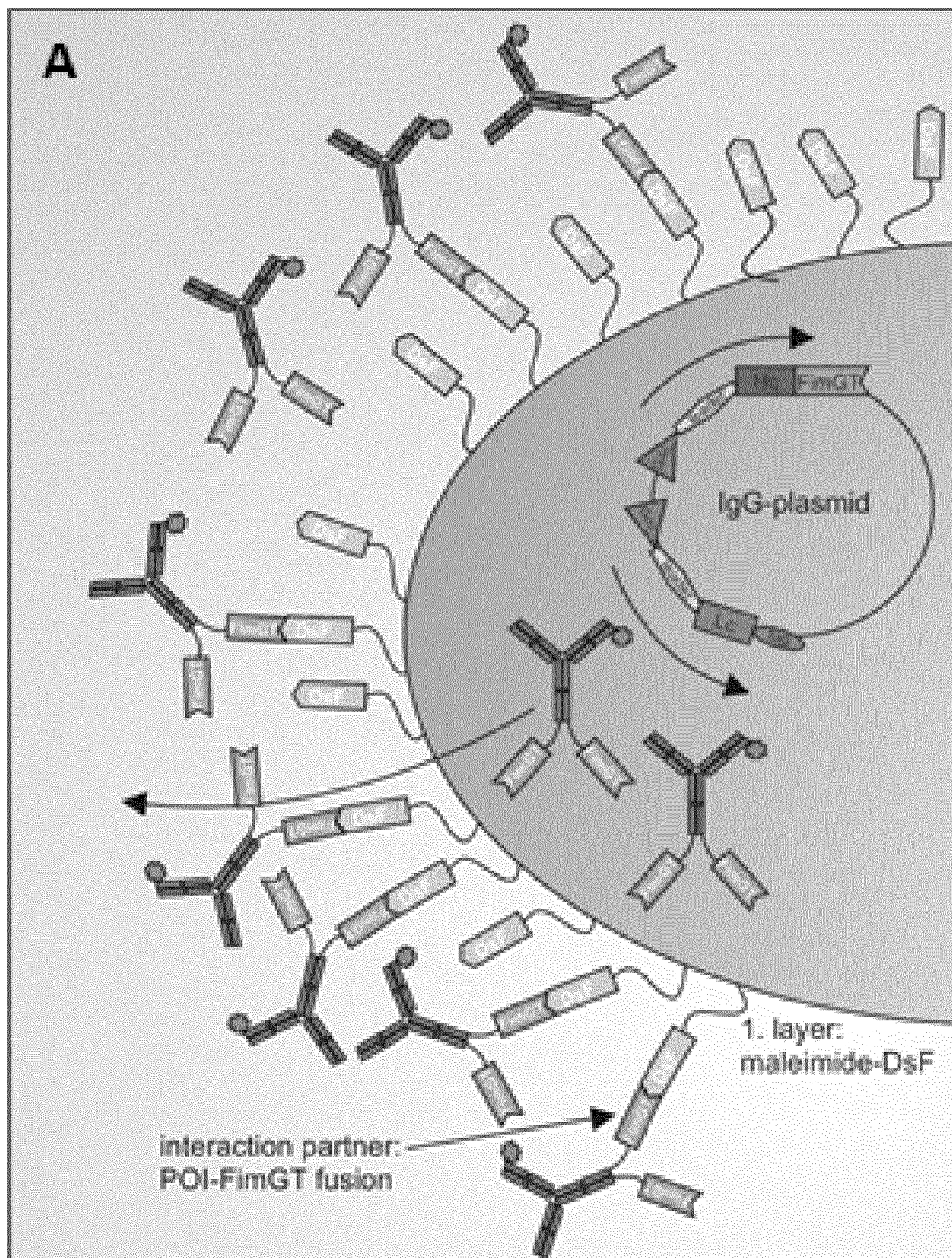


Fig. 1B

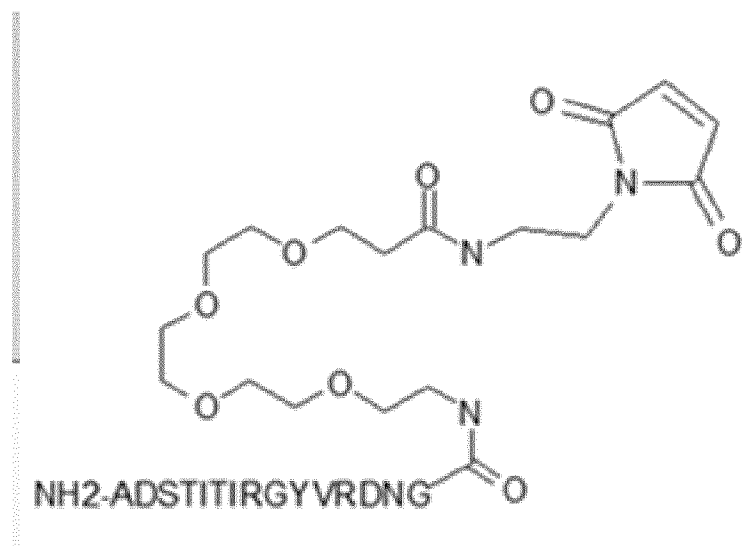


Fig. 2

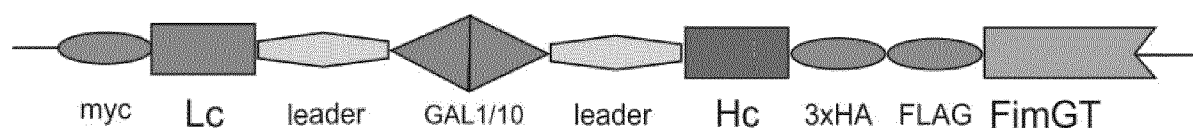


Fig. 3

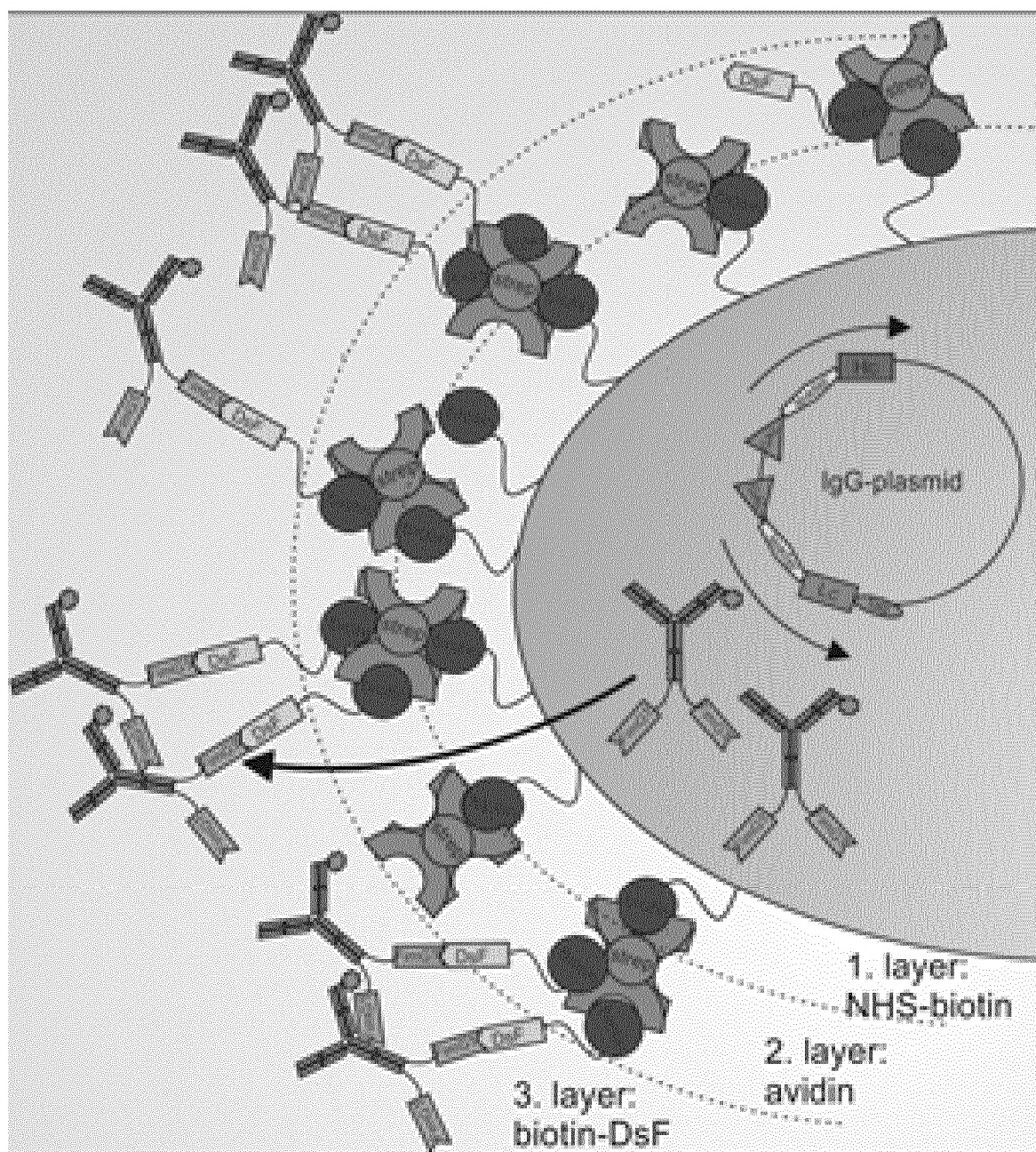


Fig. 4

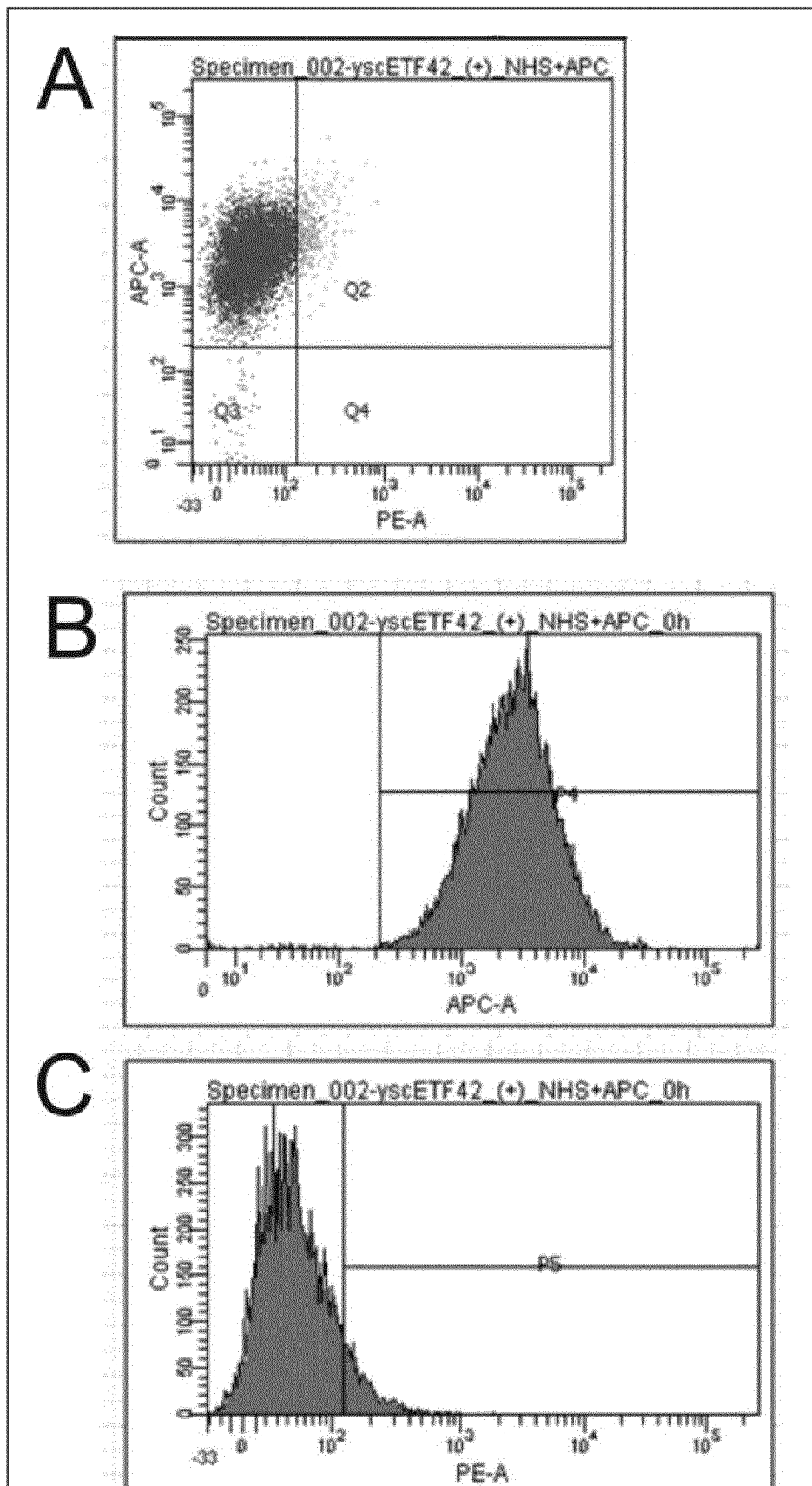


Fig. 4

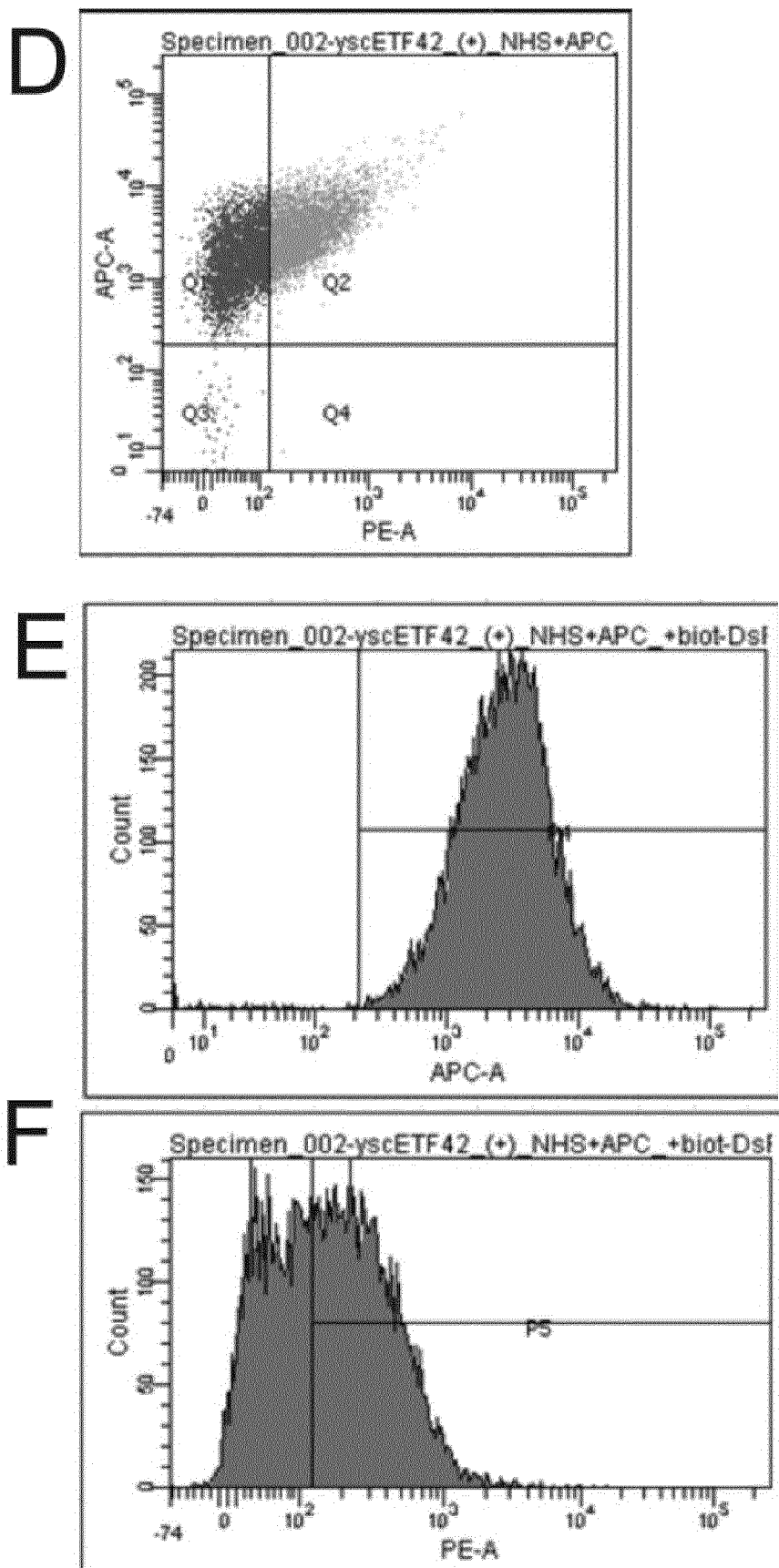


Fig. 5

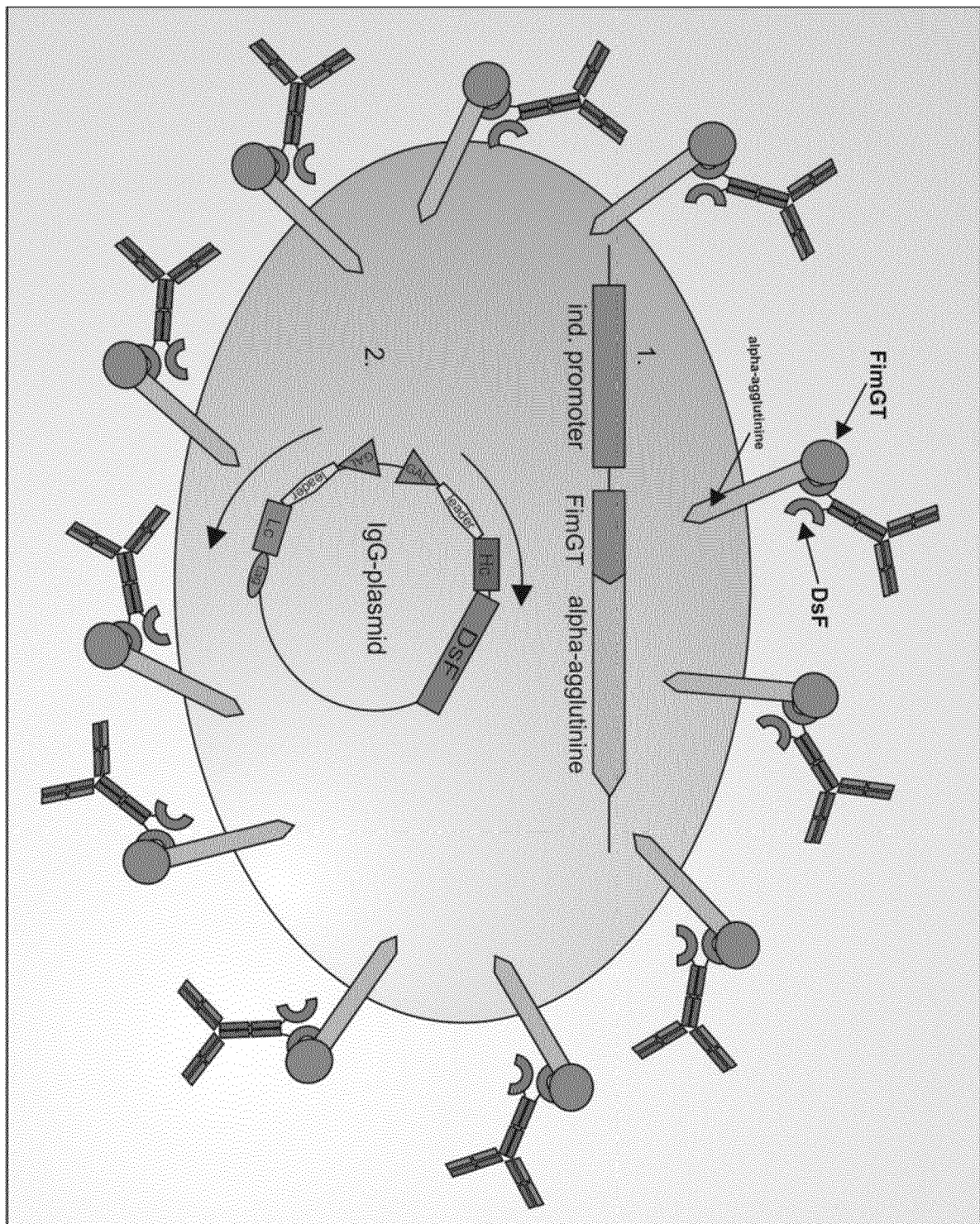


Fig. 6

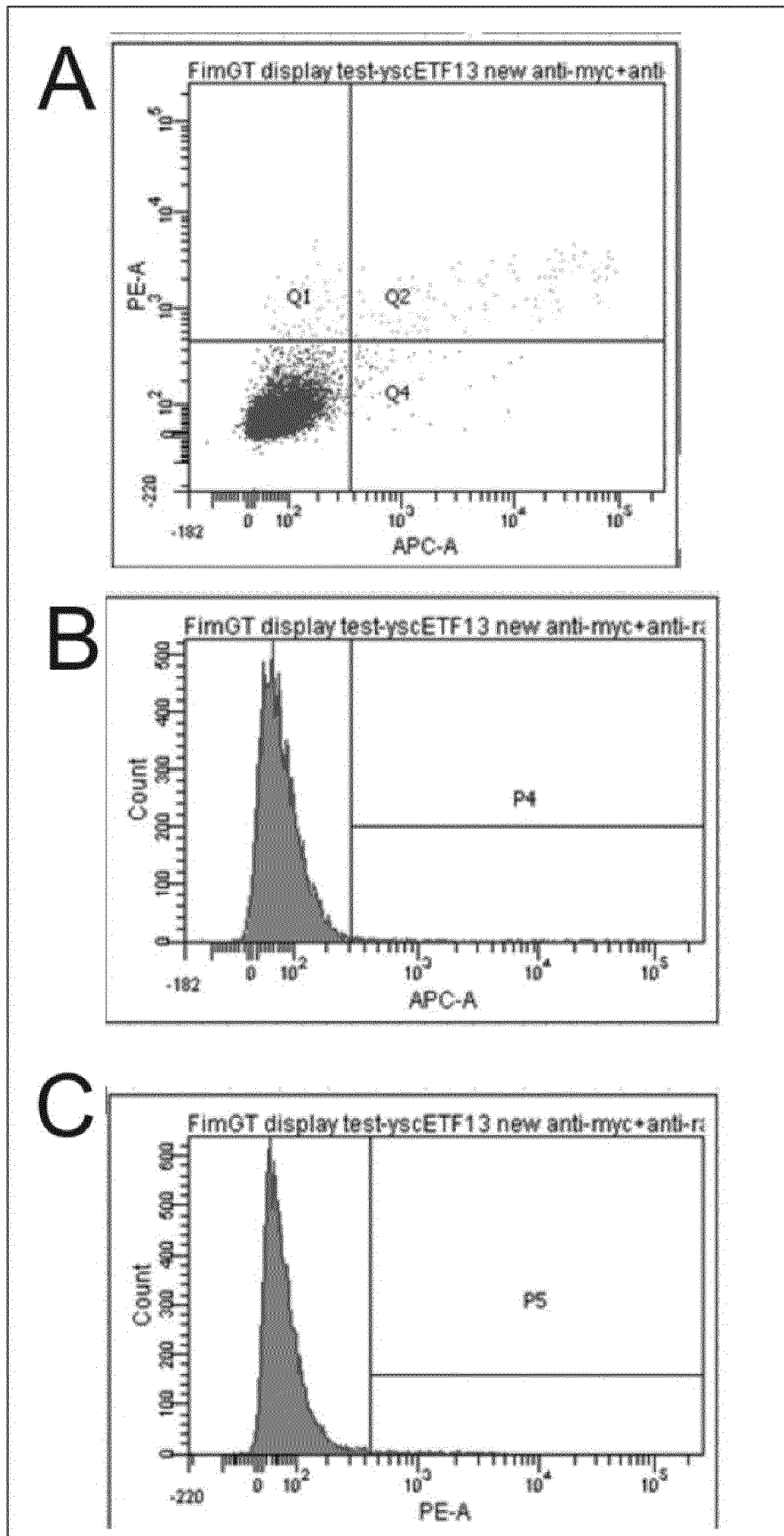


Fig. 6

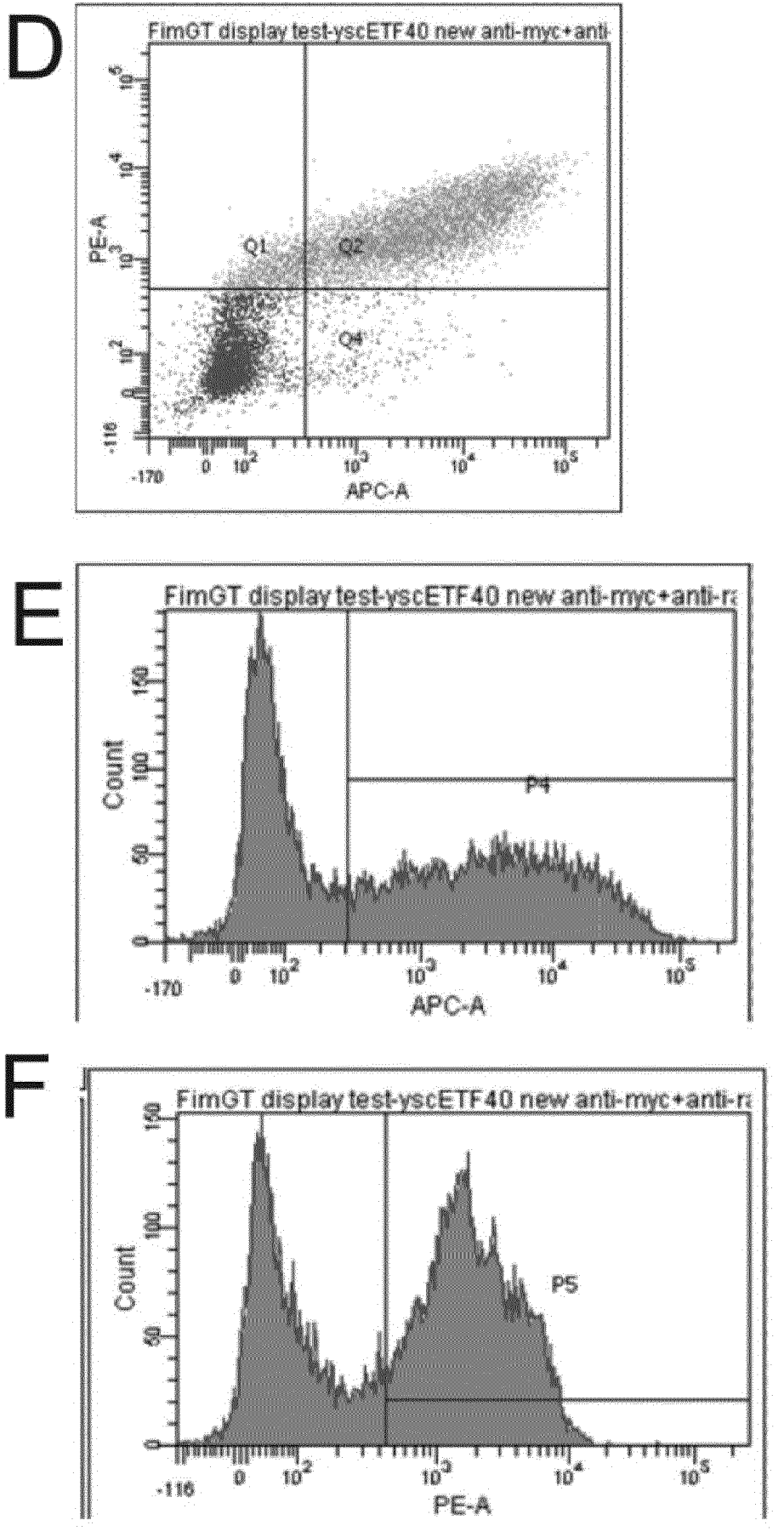
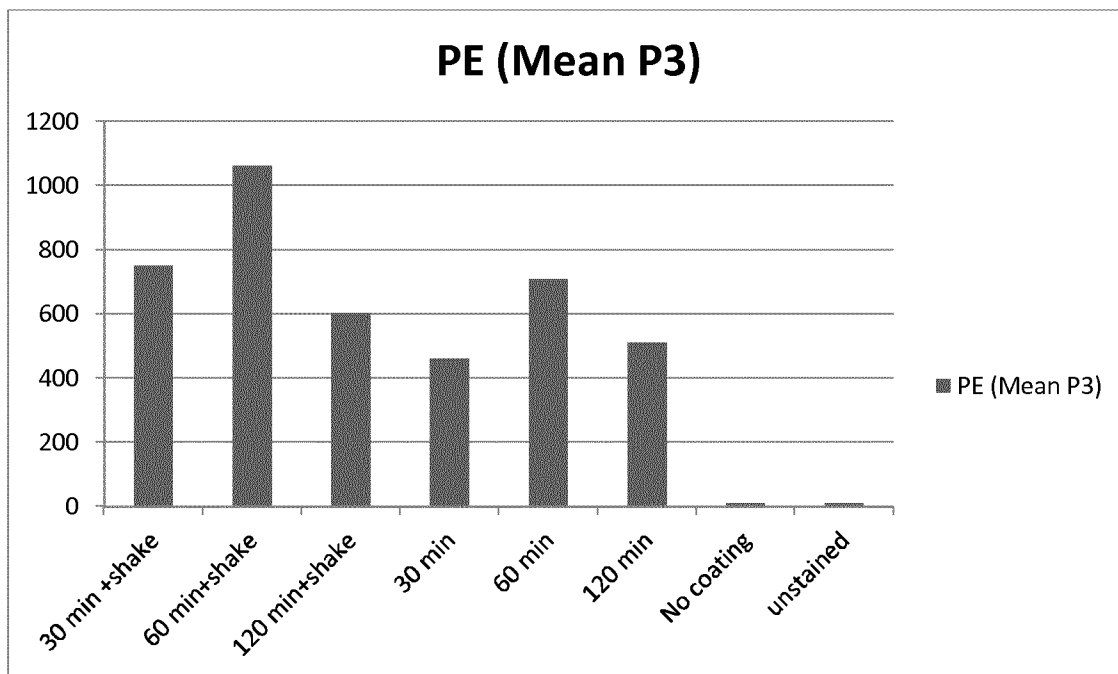


Fig. 7



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/071444

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10
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)
C12N C07K

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 practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHRISTOPH GIESE ET AL: "The Most Stable Protein-Ligand Complex: Applications for One-Step Affinity Purification and Identification of Protein Assemblies", ANGEWANDTE CHEMIE INTERNATIONAL EDITION, vol. 51, no. 18, 27 April 2012 (2012-04-27), pages 4474-4478, XP055414261, ISSN: 1433-7851, DOI: 10.1002/anie.201108747 the whole document, in particular p.4474, right-hand column, first paragraph and p.4478, left-hand column, first paragraph ----- -/--	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

11 October 2017

Date of mailing of the international search report

23/10/2017

Name and mailing address of the ISA/

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Authorized officer

Bassias, Ioannis

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/071444

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/028697 A1 (ETH ZUERICH INST OF MOLECULAR BIOLOGY AND BIOPHYSICS [CH]; ROCHE DIAGN) 8 March 2012 (2012-03-08) the whole document, in particular claims and figures -----	1-17
Y	WO 2008/118476 A2 (CODON DEVICES INC [US]; RAKESTRAW JAMES A [US]; LIPOVSEK DASA [US]) 2 October 2008 (2008-10-02) cited in the application the whole document, in particular claims and figures -----	1-17
Y	RAKESTRAW J A ET AL: "Secretion-and-capture cell-surface display for selection of target-binding proteins", PROTEIN ENGINEERING, DESIGN AND SELECTION, OXFORD JOURNAL, LONDON, GB, vol. 24, no. 6, 1 June 2011 (2011-06-01), pages 525-530, XP002718703, ISSN: 1741-0126, DOI: 10.1093/PROTEIN/GZR008 [retrieved on 2011-03-14] the whole document, in particular Abstract and Fig. 1 -----	1-17
A	LAURA RHIEL ET AL: "REAL-Select: Full-Length Antibody Display and Library Screening by Surface Capture on Yeast Cells", PLOS ONE, vol. 9, no. 12, 12 December 2014 (2014-12-12), page e114887, XP055410882, DOI: 10.1371/journal.pone.0114887 -----	1-17
A	WO 2016/066260 A1 (MERCK PATENT GMBH [DE]) 6 May 2016 (2016-05-06) -----	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/071444

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