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(54) Title: MUTANT STREPTAVIDIN WITH IMPROVED FLUORESCENT BRIGHTNESS AND USES THEREOF

(57) Abstract: The present invention provides a mutant streptavidin subunit which comprises at least two amino acid mutations compared to a wild-type streptavidin subunit, wherein said mutations comprise: (a) a substitution of the lysine residue at the position equivalent to position 121 of SEQ ID NO. 2; and (b) a substitution of at least one of the lysine residues at positions equivalent to positions of 80 and 132 of SEQ ID NO. 2 and/or a substitution or deletion of the lysine residue at the position equivalent to position of 134 of SEQ ID NO. 2, wherein the mutant streptavidin subunit is conjugated to a fluorescent label via reaction with one or more amine groups and wherein a streptavidin protein comprising four of said subunits has a higher fluorescent brightness than a streptavidin protein comprising four wild-type streptavidin subunits in which the fluorescent label is conjugated via the amino groups.

Mutant streptavidin with improved fluorescent brightness and uses thereof

The present invention relates to a mutant streptavidin polypeptide subunit with improved properties, and in particular to a subunit that, when labelled with a fluorescent dye and comprised in a streptavidin protein (i.e. a tetramer of subunits as described below), results in improved fluorescence brightness of the protein. The subunit and protein comprising said subunit can advantageously be used in a variety of imaging and labelling techniques, such as fluorescence microscopy and flow cytometry. The protein may also find utility in therapeutic and diagnostic methods and uses. The present invention also provides a streptavidin protein comprising said mutant streptavidin subunit. Nucleic acid molecules encoding said subunit, vectors comprising said nucleic acid molecules and host cells comprising said vectors and nucleic acid molecules are also provided. A kit comprising said polypeptides, proteins, nucleic acid molecules/vectors and/or host cells is also provided. A method of producing said mutant subunit and the uses of the polypeptides and proteins of the invention are also provided.

Streptavidin, a protein produced by *Streptomyces avidinii,* binds to the water-soluble vitamin biotin with a very high affinity. Streptavidin is a tetrameric protein and the interaction between biotin and streptavidin is one of the strongest and most widely used protein-ligand interactions in biological research. The binding of biotin by streptavidin or avidin is a paragon of molecular recognition, achieving exceptional stability despite a small contact surface area. Streptavidin is used in imaging, purification, immobilisation, immunoassays and nano-assembly, while also showing success in cancer clinical trials for drug targeting. However, high affinity is not the only important feature of the streptavidin-biotin interaction.

Chemical modification provides diverse opportunities to harness and extend protein function. One of the most common chemical modifications of proteins is fluorescent labelling, particularly for microscopy, flow cytometry and diagnostics. However, dye modification of proteins is more complicated than it first appears. Apart from the potential to sterically disrupt protein-ligand interactions, dye conjugation can alter binding patterns of proteins to cells both in culture and *in vivo.* Increased non-specific binding correlates particularly with dye hydrophobicity and can alter mobility in single-particle tracking, signal to noise, and membrane insertion.

Dye-dye photophysical interactions must also be considered when labelling a protein with a fluorescent dye. Such interactions include homo-fluorescence

resonance energy transfer (homo-FRET, radiationless transfer up to 10 nm and dependent on overlap of excitation and emission spectra) and exciton coupling, when dyes can physically contact each other. These effects lead to major changes in both absorption spectrum and quantum yield, which can impact on the brightness of the signal. Brightness is a precious commodity for signal detection amidst biological autofluorescence, for lowering diagnostic detection limits, and enabling cell-culture or whole-body imaging with enhanced temporal and spatial resolution (especially in super-resolution microscopy).

The vast majority of dyes are provided as N-hydroxysuccinimide (NHS) esters or sulfo-NHS esters (hereafter grouped together as NHS). NHS can react with amine groups on proteins to form a stable amide bond. These amines include the a-amino group and lysine ε-amine. Lysine is one of the most abundant surface residues on proteins and so amine-based conjugation allows multiple modification sites per protein. For example, there are approximately 80 lysines per IgG antibody. Accordingly, it can be difficult to control the conjugation of dyes to proteins.

Whilst it may be possible to control dye labelling more precisely by titrating down dye-NHS levels, this requires extensive case-by-case optimisation, given the sensitivity of NHS reaction efficiency to pH, buffer additives, protein concentration and the individual dye. The second most commonly available reactive dyes are maleimide-dyes, designed to target low abundance surface cysteine residues. The precision of maleimide-dye labelling is often useful, but it takes time to clone and express proteins with a new surface cysteine. Furthermore, new cysteine residues can interfere with folding of proteins containing disulfide bonds. Also, maleimide-protein conjugates can re-arrange, hydrolyse or exchange in the presence of other thiols.

Thus, there is a need and desire for new proteins, particularly streptavidin mutants, with improved characteristics for dye modification.

The present inventors have determined that fluorescent labelling of streptavidin can severely disrupt its interaction with biotin. Furthermore, as shown in the experiments described in the Examples below, efforts to control dye labelling of wild-type streptavidin by reducing the dye:protein ratio did not avoid damage to biotin binding and did not affect fluorescence brightness. Accordingly, the inventors have engineered streptavidin mutant subunits which surprisingly have increased fluorescence brightness, when labelled with a fluorescent dye and present in tetrameric form, relative to wild-type streptavidin labelled with the same dye. In

particular, the inventors have determined that, contrary to expectations, reducing the number of amines where fluorescent dyes can attach to the streptavidin subunits results in a significant improvement in fluorescence brightness when comprised in the tetrameric form. Thus, the mutant streptavidin subunits, and proteins comprising said subunits, are extremely useful in applications where signal brightness is limiting, e.g. for the detection of antigens present at extremely low levels. Notably, the modifications required to achieve the improvement in fluorescent brightness do not substantially affect or impair the capacity of streptavidin to assemble into a tetramer or to bind to biotin. The properties of the mutants of the present invention are particularly surprising in view of the determination that fluorescent brightness of other fluorescently-labelled proteins, such as antibodies, typically increases to a plateau as additional dye molecules are conjugated to the protein, i.e. decreasing, particularly minimising, the number of fluorescent dye molecules attached to a protein would be expected to decrease fluorescent brightness. It was also unexpected that the fluorescent brightness of labelled streptavidin could be improved, without substantial damage to its functional properties, by spatial manipulation of the amine organisation, especially in light of the fact that streptavidin is a highly optimized system and it is easy for mutations to dramatically damage assembly or ligand binding.

As discussed in detail in the Examples below, the inventors have surprisingly determined that it is possible to improve the fluorescence brightness of a streptavidin protein labelled with a fluorescent dye by reducing the number of fluorescent dye units (labels or molecules) attached to each subunit. In particular, the inventors have found that the number of fluorescent dyes conjugated to a streptavidin subunit, and subsequently present in a streptavidin protein (i.e. tetramer), may be controlled by reducing the number of amino groups to which the fluorescent dye may be conjugated. Specifically, the inventors have shown that fluorescent brightness may be maximised by limiting the reactive sites for amine labelling to one, i.e. one reactive site for an amine-reactive dye may be optimal for streptavidin fluorescent brightness.

As mentioned above, fluorescent dyes typically are provided in a form that is able to react with amine groups on proteins to form a stable amide bond, e.g. as N-hydroxysuccinimide (NHS) esters or sulfo-NHS esters. The amine groups present in a protein with which the dyes can react include the a-amino group and lysine ε-amine. The wild-type streptavidin subunit (e.g. the core streptavidin subunit as

shown in SEQ ID NO. 3) comprises five amines to which a fluorescent dye may be conjugated via an amide bond, i.e. the a-amino group at the N-terminus and the ε-amino groups present on four lysine residues, which correspond to residue positions 80, 121, 132 and 134 of the mature wild-type streptavidin subunit as set forth in SEQ ID NO. 2. The inventors have determined that mutation of the lysine residues, to amino acids to which a fluorescent dye cannot be readily conjugated via an amide bond, does not alter significantly the interaction between streptavidin and biotin, e.g. the on and off rates of streptavidin comprising the mutant subunits are equivalent (e.g. functionally equivalent, comparable or similar) to the rates of the wild-type protein.

Thus, in one aspect, the present invention therefore provides a mutant streptavidin subunit which comprises at least two amino acid mutations compared to a wild-type streptavidin subunit, wherein said mutations comprise:

(a) a substitution at the lysine residue at the position equivalent to position 121 of SEQ ID NO. 2; and

(b) a substitution of at least one of the lysine residues at positions equivalent to positions of 80 and 132 of SEQ ID NO. 2 and/or a substitution or deletion of the lysine residue at the position equivalent to position of 134 of SEQ ID NO. 2, and

wherein a streptavidin protein comprising four of said subunits each conjugated to a fluorescent label via the amino groups has a higher fluorescent brightness than a streptavidin protein comprising four wild-type streptavidin subunits in which the fluorescent label is conjugated via the amino groups.

Thus, in the mutant streptavidin subunit of the invention, a fluorescent dye can be conjugated via a covalent bond, e.g. an amide bond, to the a-amino group at the N-terminus and/or any of the ε-amino groups present in the remaining (i.e. unmutated, e.g. unsubstituted) lysine residues. For instance, in some embodiments the mutant streptavidin subunit comprises substitutions at positions 121 and 80, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus and the ε-amino groups of lysine residues at positions 132 and 134.

In other embodiments, the mutant streptavidin subunit comprises substitutions at positions 121 and 132, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus and the ε-amino groups of lysine residues at positions 80 and 134.

In yet other embodiments, the mutant streptavidin subunit comprises substitutions at positions 121 and 134 or a substitution at position 121 and deletion

of position 134, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus and the ε-amino groups of lysine residues at positions 80 and 132.

In some embodiments, the mutant streptavidin subunit comprises mutations at three lysine residues. Thus, the mutant streptavidin subunit may comprise substitutions at three lysine residues or substitutions at two lysine residues (a first substitution at position 121 and a second substitution at one of positions 80 and 132) and deletion of the third lysine residue (position 134). Thus, for instance, the mutant streptavidin subunit may comprise substitutions at positions 121 , 132 and 134 or substitutions at positions 121 and 132 and deletion of position 134, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus and the ε-amino groups of the lysine residue at position 80.

In other embodiments, the mutant streptavidin subunit may comprise substitutions at positions 121 , 80 and 134 or substitutions at positions 121 and 80 and deletion of position 134, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus and the ε-amino groups of the lysine residue at position 132.

In yet other embodiments, the mutant streptavidin subunit may comprise substitutions at positions 121, 80 and 132, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus and the ε-amino groups of the lysine residue at position 134.

In a preferred embodiment, the mutant streptavidin subunit comprises mutations at all four lysine residues, i.e. the mutant streptavidin subunit may comprise substitutions at positions 80, 121, 132 and 134 or substitutions at positions 80, 121 and 132 and deletion of position 134, i.e. the fluorescent dye can conjugate via an amide bond to the a-amino group at the N-terminus.

Thus, alternatively viewed, in some embodiments the mutant streptavidin subunit may be capable of being conjugated to one, two or three fluorescent dye units (labels) via an amide bond (i.e. each fluorescent label can conjugate to the protein via a separate amide bond).

In some embodiments, the mutant streptavidin subunit is conjugated to a fluorescent dye (label). In particular, the fluorescent dye (label) is conjugated to the mutant streptavidin subunit via reaction with an amine group, e.g. to form an amide bond. The mutant streptavidin subunit may be conjugated to one, two or three fluorescent dye units (i.e. labels or molecules).

Thus, a mutant streptavidin subunit conjugated to a fluorescent label via the amino groups will comprise one, two or three fluorescent labels. However, a wild-type streptavidin subunit conjugated to a fluorescent label via the amino groups may comprise more than three fluorescent labels, i.e. four or five fluorescent labels.

The term "streptavidin" or "streptavidin protein" as used herein refers to streptavidin comprising four streptavidin subunits. Particularly, streptavidin may be a tetrameric protein comprising four streptavidin subunits where the individual subunits multimerise (e.g. tetramerise) together to form streptavidin. For the tetrameric protein, a nucleic acid sequence may encode an individual subunit(s) which is then translated into a single subunit polypeptide. The individual subunits then multimerise as discussed above to form the tetrameric protein.

"Wild-type streptavidin" refers to the wild-type streptavidin protein comprising four wild-type streptavidin subunits as defined further below. Wild-type streptavidin may be in the form of the tetrameric protein formed by the multimerisation of four wild-type streptavidin subunits.

Reference to a "streptavidin subunit" or "streptavidin polypeptide" as used herein refers to a single streptavidin subunit which is not multimerised and is not present in tetrameric form. However, a "streptavidin subunit" or "streptavidin polypeptide" as used herein generally refers to a single streptavidin subunit which is capable of multimerisation, e.g. tetramerisation.

Thus, the invention further provides a mutant streptavidin protein comprising at least one mutant streptavidin subunit of the invention.

The "mutant streptavidin" or "mutant streptavidin protein" of the invention comprises at least one mutant streptavidin subunit of the invention. The mutant streptavidin may therefore comprise 2, 3 or 4 mutant streptavidin subunits of the invention. Where the mutant streptavidin comprises 1, 2 or 3 mutant subunits of the invention, the other 3, 2, or 1 subunits, respectively present may be wild-type or comprise mutations which affect other characteristics of the streptavidin protein, e.g. the capacity of streptavidin to bind to biotin, such as off rate or on rate for biotin.

The mutant streptavidin may be a tetrameric protein formed by the multimerisation of four streptavidin subunits, at least one of which is a mutant streptavidin subunit of the invention.

As discussed in more detail below, a variety of mutant streptavidin subunits are known, which alter the properties of the streptavidin protein comprising said

mutant subunits, e.g. increased on rate or reduced off rate for biotin. Thus, it is envisaged that known mutant subunits may be combined with the subunit of the invention to produce so-called chimeric streptavidin proteins, i.e. a streptavidin protein in which at least two of the subunits have different amino acid sequences.

In some embodiments, one or more of the mutations in the known mutant streptavidin subunits that result in improved or altered properties of the resultant streptavidin protein may be combined with, i.e. introduced or incorporated into, the mutant streptavidin subunit of the present invention. Thus, a "mutant streptavidin subunit" refers to a mutant streptavidin subunit comprising the lysine mutations (i.e. substitutions and deletion) described above and optionally one or more additional mutations.

The term "wild-type streptavidin subunit" as used herein includes all forms of wild-type streptavidin subunits including full length and truncated sequences as discussed below. The sequence of the full length streptavidin subunit as encoded in the genome of *Streptomyces avidinii* is well known in the art and consists of an amino acid sequence as set forth in SEQ ID NO. 1. This full length sequence is usually truncated at the N- terminus to remove the signal peptide and the production of streptavidin by *Streptomyces avidinii* may result in the production of a mixture of protein chains from the endogenous cleavage of full length streptavidin subunits.

In particular, the full length sequence may be truncated to produce the mature streptavidin subunit sequence which is 159 amino acids in length and begins at amino acid position 25 in the full length sequence (SEQ ID NO. 1). The universal numbering of residues within the streptavidin subunit begins at the first residue of the mature streptavidin subunit sequence, which is set forth in SEQ ID NO. 2. Thus, alternatively viewed, residue 25 in the full length subunit sequence (SEQ ID NO. 1) is defined as residue 1.

This mature sequence may be further truncated and particularly may be truncated to produce the core streptavidin subunit sequence (also known as core streptavidin). The core streptavidin subunit sequence begins at residue 13 of the mature streptavidin subunit sequence and is a fragment of the mature sequence consisting of residues 13 to 139 of SEQ ID NO. 2. The sequence of core streptavidin subunit is set forth in SEQ ID NO. 3. Further truncations can be made from the core sequence, e.g. fragments consisting of residues 14 to 138 and 16 to 133 of the mature streptavidin subunit sequence have been produced and shown to

function. Notably, the experiments described in the Examples below are based on polypeptides comprising the core streptavidin subunit and mutants thereof, i.e. polypeptides comprising an amino acid sequence as set forth in SEQ ID NO. 3 and mutants thereof.

Thus, in a preferred aspect of the invention, the mutant streptavidin subunit of the invention is a core streptavidin subunit, i.e. a mutant of SEQ ID NO. 3. However, as noted above, convention dictates that the numbering of the sequence is based on the mature sequence, which is set forth in SEQ ID NO. 2. Thus, in some embodiments, the mutant subunit of the invention is a mutant of the core streptavidin subunit and comprises at least two amino acid mutations compared to the wild-type core streptavidin subunit, wherein said mutations comprise:

(a) a substitution of the lysine residue at the position equivalent to position 121 of SEQ ID NO. 2; and

(b) a substitution of at least one of the lysine residues at positions equivalent to positions of 80 and 132 of SEQ ID NO. 2 and/or a substitution or deletion of the lysine residue at the position equivalent to position of 134 of SEQ ID NO. 2.

Thus, alternatively viewed, the mutant subunit of the invention is a mutant of the core streptavidin subunit and comprises at least two amino acid mutations compared to the wild-type core streptavidin subunit, wherein said mutations comprise:

(a) a substitution of the lysine residue at the position equivalent to position 109 of SEQ ID NO. 3; and

(b) a substitution of at least one of the lysine residues at positions equivalent to positions of 68 and 120 of SEQ ID NO. 3 and/or a substitution or deletion of the lysine residue at the position equivalent to position of 122 of SEQ ID NO. 3.

The mutant streptavidin subunit of the present invention thus comprises two or more mutations compared to a wild-type streptavidin subunit, i.e. compared to SEQ ID NOs 1, 2 or 3, at residue positions in the mutant streptavidin subunit which are equivalent to positions 80, 121, 132 and 134 in SEQ ID NO. 2. Although other modifications, i.e. mutations, may be made to the mutant streptavidin subunit compared to the wild-type sequences in addition to the mutations to any one or more of the residues at positions 80, 121, 132 and 134, a mutant streptavidin protein (i.e. comprising four mutant subunits of the invention) in which each subunit is conjugated to a fluorescent label via the amino groups must have a higher fluorescent brightness than a streptavidin protein comprising four wild-type

streptavidin subunits in which the fluorescent label is conjugated via the amino groups. Thus, the mutant subunit may have other mutations, i.e. insertions, deletions, substitutions etc. as compared to the wild-type subunit sequence in addition to the mutations at positions 80, 121, 132 and 134 described above. Further, the mutant streptavidin subunit may have N- and/or C-terminal extensions and may thus have a longer sequence than a wild-type streptavidin subunit. For example, the mutant streptavidin subunit may contain a tag to facilitate its purification, e.g. a His-tag or hexaglutamate tag, or conjugation to other molecules, e.g. a peptide tag such as SEQ ID NO. 10.

As discussed above, the mutant streptavidin subunit of the invention comprises two or more amino acid mutations at amino acid residue positions equivalent to positions 121 and at least one of positions 80, 132 and 134 of SEQ ID NO. 2. In a preferred embodiment, said two or more amino acid mutations are substitutions. Thus, any two or more of the wild-type amino acid residues (i.e. lysine residues) found at positions 80, 121 , 132 and 134 (or the equivalent positions) may be substituted with any other natural or non-natural amino acid residue. In a preferred embodiment, the amino acids found at positions 80, 121, 132 and 134 (or the equivalent positions) are substituted with any other natural amino acid residue, i.e. any amino acid other than lysine.

Thus, for example, any of the 19 conventional amino acid residues other than lysine (Ala (A), Cys (C), Asp (D), Glu (E), Phe (F), Gly (G), His (H), lie (I), Leu (L), Met (M), Asn (N), Pro (P), Gin (Q), Arg (R), Ser (S), Thr (T), Val (V), Trp (W) and Tyr (Y)) can be substituted into any of positions 80, 121 , 132 and 134.

In some embodiments, the substituting amino acid at positions 80, 132 and/or 134 may be polar and/or charged (i.e. basic or acidic) or alanine (A). Thus, in some embodiments the substituting amino acid at positions 80, 132 and 134 may be independently selected from any one of R, N, D, E, A, H, Q, S or T. In some embodiments, the substituting amino acid is not a non-polar neutral amino acid. Thus, is some embodiments, the substituting amino acid is not G, I, L, F, P, W, Y, M or V. In some embodiments, the substituting amino acid is not C. In some embodiments, the substituting amino acid is not E. In a particularly preferred embodiment, positions 80 and 134 are not both substituted with E. Alternatively viewed, only one of positions 80 and 134 may be E. In a particularly preferred embodiment, at least one of positions 80, 132 and 134 is substituted with R or A. Thus, in some embodiments, two or more of positions 80, 132 and 134 are

substituted with R or A, e.g. 80 and 132 or 80 and 134. In some embodiments at least one of positions 80 and 132 is substituted with R or A and the lysine at position 134 is deleted. In still further embodiments, all three positions 80, 132 and 134 are substituted with R or A, preferably R.

In some embodiments, at position 121, the substituting amino acid is not G, Q or E. Thus, in some embodiments the substituting amino acid may be a non-polar, basic or neutral amino acid other than glycine. Hence, the substituting amino acid may be selected from R, H, A, I, L, V, W, Y, P and F. In a particularly preferred embodiment, the substituting amino acid is R or A, preferably R.

Thus, in some embodiments, position 121 is substituted with R or A and positions 80, 132 and 134 are substituted with R. In some embodiments, position 121 is substituted with R or A, positions 80 and 132 are substituted with R and the lysine at position 134 is deleted. In a particularly preferred embodiment, all of the lysine residues in the streptavidin subunit (i.e. positions 80, 121, 132 and 134) are substituted with arginine.

Thus, in one embodiment, the mutant streptavidin subunit comprises an amino acid sequence as set forth in SEQ ID NO. 4. In an alternative embodiment, the mutant streptavidin subunit comprises an amino acid sequence with at least 70% sequence identity to SEQ ID NO. 4 (more particularly at least 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO. 4), wherein the amino acid residues at positions equivalent to positions 80, 121, 132 and 134 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 4 (i.e. the amino acid residues at positions 80, 121, 132 and 134 are arginine).

In another embodiment, the mutant streptavidin subunit comprises an amino acid sequence as set forth in SEQ ID NO. 5. In an alternative embodiment, the mutant streptavidin subunit comprises an amino acid sequence with at least 70% sequence identity to SEQ ID NO. 5 (more particularly at least 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO. 5), wherein the amino acid residues at positions equivalent to positions 121, 132 and 134 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 5 (i.e. the amino acid residues at positions 121, 132 and 134 are arginine).

In yet another embodiment, the mutant streptavidin subunit comprises an amino acid sequence as set forth in SEQ ID NO. 6. In an alternative embodiment, the mutant streptavidin subunit comprises an amino acid sequence with at least 70% sequence identity to SEQ ID NO. 6 (more particularly at least 75, 80, 85, 90,

95, 96, 97, 98 or 99% sequence identity to SEQ ID NO. 6), wherein the amino acid residues at positions equivalent to positions 80 and 121 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 6 (i.e. the amino acid residues at positions 80 and 121 are arginine).

As discussed above, inventors have determined that the mutation of the lysine residues of the streptavidin subunit as described above does not significantly affect the ability of a streptavidin protein comprising said subunit to bind to biotin or a biotin conjugate. Thus, in some embodiments of the invention, a streptavidin protein comprising four of the mutant streptavidin subunits of the invention has an off rate for biotin or for a biotin conjugate that is equivalent to, or lower than, a streptavidin protein comprising four wild-type streptavidin subunits under the same conditions.

However, as discussed herein, in some embodiments it may be advantageous to introduce additional mutations to the mutant streptavidin subunit of the invention to alter the characteristics of the streptavidin protein. For instance, it may be useful to introduce mutations which reduce or eliminate the biotin binding capacity of a subunit, e.g. to facilitate the production of a monovalent streptavidin protein. Thus, it is not essential that a streptavidin protein comprising four of the mutant streptavidin subunits of the invention has an off rate for biotin or a biotin conjugate that is equivalent to, or lower than, wild-type streptavidin (i.e. comprising four wild-type subunits) under the same conditions.

The term "off rate" as used herein refers to the rate of dissociation of a ligand from its binding partner. Particularly, the term "off rate" as used herein refers to the rate of dissociation of biotin or a biotin conjugate from streptavidin (or mutant forms of streptavidin). The off rate may be measured in absolute terms in units of s^{-1}. Thus, the smaller the off rate figure, the less biotin is released from the particular streptavidin form over time, e.g. per second. The off rate of biotin from wild-type streptavidin has been measured at about 2.9×10^{-5} s^{-1}, based on measurements at 37°C in NaCl/phosphate buffer pH 7.0 (Hyre et al., 2000, Protein Sci., 9, 878-885).

The off rate may also be conveniently calculated in relative terms, e.g. the off rate of a mutant streptavidin protein may be measured relative to the off rate of the wild-type streptavidin protein. For instance, biotin-4-fluorescein (B4F) may be used as an efficient read-out of ligand binding to streptavidin or a streptavidin mutant. Biotin-4-fluorescein fluorescence is quenched by ~90% upon streptavidin binding. Thus, biotin-4-fluorescein's dissociation, induced by adding excess free

biotin, can be continuously monitored from the recovery of biotin-4-fluorescein's fluorescence upon dissociation from streptavidin's binding pocket.

Exemplary conditions for measuring the dissociation rate include combining 10 µl of 1 µM streptavidin (mutant or wild-type) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HP0_4, 1.8 mM KH_2P0_4, pH 7.4) with 170 µl of 24 nM B4F in PBS, followed by incubation at 37 °C for 1 h. Addition of excess biotin (e.g. 20 µl of 1 mM biotin in PBS) followed by immediate measurement of fluorescence (using 484 nm excitation and 535 nm emission) at regular time points at 37 °C will provide a measure of the dissociation rate, i.e. off rate. Control experiments may include a quenched control and a B4F-only control. In the quenched control, 10 µl of 1 µM streptavidin (mutant or wild-type) in PBS may be combined with 170 µl of 24 nM B4F in PBS, followed by incubation at 37 °C for 1 h and subsequent addition of 20 µl of PBS. In the B4F-only control, 10 µl PBS may be combined with 170 µl of 24 nM B4F in PBS, followed by incubation at 37 °C for 1 h and subsequent addition of 20 µl of 1 mM biotin in PBS. Notably, unless specified otherwise, the streptavidin concentrations herein are stated as the concentration of subunits, not the concentration of the tetramer.

The dissociation can be calculated as a percentage: 100* [(streptavidin with B4F)-(quenched control)]/[(B4F-only control)-(quenched control)] and the measurement of this dissociation over time provides a dissociation rate, i.e. off rate. The dissociation rates of different streptavidin proteins (e.g. wild-type versus mutant) measured under the same reaction conditions, e.g. temperature, concentration etc. as exemplified above, can be readily compared to determine whether the off rate for each protein is higher, lower or equivalent.

A mutant streptavidin with an off rate that is "equivalent" to the off rate of the wild-type streptavidin may have an off rate that is similar (e.g. functionally equivalent or comparable) to the off rate of wild-type streptavidin, i.e. such that the practical applications of the mutant streptavidin are not substantially affected, e.g. within a margin of experimental error. Thus, an equivalent off rate means that the mutant streptavidin does not dissociate from biotin or a biotin conjugate at a substantially higher rate than the wild-type streptavidin under the same conditions.

Thus, the off rate of the mutant streptavidin for biotin or biotin conjugate may be 140% or less, e.g. 135, 130, 125, 120% or less, such as 115, 110, 109, 108, 107, 106, 105, 104, 103, 102, 101% or less of the off rate of wild-type streptavidin for biotin or a biotin conjugate. Alternatively viewed, the off rate of the mutant

streptavidin for biotin or a biotin conjugate may be no more than 40% higher than the off rate of wild-type streptavidin for biotin or a biotin conjugate, e.g. no more than 35, 30, 25 or 20% higher, e.g. no more than 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% higher than the off rate of wild-type streptavidin for biotin or a biotin conjugate. Thus, in absolute terms, the off rate of the mutant streptavidin for biotin or biotin conjugate may be about 4.1 x 10^{-5} s^{-1} or less, e.g. 4.0 x 10^{-5} s^{-1}, 3.9 x 10^{-5} s^{-1}, 3.8 x 10^{-5} s^{-1}, 3.7 x 10^{-5} s^{-1}, 3.6 x 10^{-5} s^{-1}, 3.5 x 10^{-5} s^{-1}, 3.4 x 10^{-5} s^{-1}, 3.3 x 10^{-5} s^{-1} or less, such as about 3.2 x 10^{-5} s^{-1}, 3.1 x 10^{-5} s^{-1} or 3.0 x 10^{-5} s^{-1} or less.

In some embodiments, e.g. when the mutant streptavidin subunit contains additional mutations that improve (i.e. decrease) the off rate of the mutant streptavidin relative to the wild-type streptavidin, the off rate of the mutant streptavidin for biotin or biotin conjugate may be lower than the off rate of wild-type streptavidin. Thus, for instance, the off rate of the mutant streptavidin for biotin may be at least 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 or 95 % less than the off rate of wild-type streptavidin for biotin or a biotin conjugate. Alternatively viewed, the off rate of the mutant streptavidin for biotin may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400 or 500 fold lower than the off rate of wild-type streptavidin for biotin or a biotin conjugate.

The off rate may further be described in terms of the half-life in hours for the dissociation of streptavidin from biotin or a biotin conjugate. The half-life for conjugated biotin and wild-type streptavidin is approximately 6-100 hours (depending on the conjugate) and the half-life of free biotin and wild-type streptavidin is approximately 6.6 hours.

Thus the mutant streptavidin of the present invention (e.g. comprising four mutant subunits of the present invention) may have an equivalent (e.g. similar) or higher half-life for biotin (either free or conjugated) than wild-type streptavidin. The half-life of the mutant streptavidin of the invention for a biotin conjugate may therefore be at least 5, 5.5, 6, 6.5, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60 or 70 hours and/or the half-life of mutant streptavidin of the invention for free biotin may be at least 5, 5.5, 6, 6.5, 7, 8, 9, 10, 15 or 20 hours. Alternatively viewed, the half-life of the mutant streptavidin subunit of the present invention is increased by at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 95% of the half-life value of wild-type streptavidin for biotin when the mutant subunit is assembled into streptavidin.

Biotin is alternatively known as vitamin H, vitamin B7 and cis-hexahydro-2-oxo H-thieno[3,4] imidazole-4-pentanoic acid. A biotin conjugate refers to biotin

covalently linked to another molecule. Preferably the linkage is through an amide bond or through an ester bond. Thus, biotin may be conjugated to any other molecule or entity, particularly to a nucleic acid, a protein/polypeptide (e.g. antibody), a fluorophore, a cell, a virus, a virus-like particle, a bead or to any other surface. For example, biotin may be conjugated to oligonucleotides, oligopeptides, fluorescent molecules, enzymes, organic polymers, carbon nanotubes, small molecule drugs etc.

In some embodiments, the streptavidin mutant of the present invention may also bind to biotin analogues. For instance, the off rate of the mutant streptavidin of the invention may be equivalent to, or lower than, the off rate of the wild-type streptavidin for various biotin analogues. Biotin analogues include, but are not limited to, desthiobiotin (also known as dethiobiotin), selenobiotin, oxybiotin, homobiotin, norbiotin, iminobiotin, diaminobiotin, biotin sulfoxide, biotin sulfone, epibiotin, 5-hydroxybiotin, 2-thiobiotin, azabiotin, carbobiotin, methylated derivatives of biotin, and/or ketone biotin. Biotin analogues may be free or in conjugated form.

The mutant streptavidin subunit of the invention may have other functional properties. For instance, a streptavidin protein comprising four mutant streptavidin subunits of the invention may have an on rate for biotin or for a biotin conjugate that is equivalent to a streptavidin protein comprising four wild-type streptavidin subunits under the same conditions.

The term "on rate" as used herein refers to the rate of association of a ligand to its binding partner. Particularly, the term "on rate" as used herein refers to the rate of association of biotin or a biotin conjugate to streptavidin (or mutant forms of streptavidin). The on rate may be measured in absolute terms in units of $M^{-1}s^{-1}$. Thus, the smaller the on rate figure, the slower biotin binds to the particular streptavidin form over time, e.g. per second. The on rate of biotin for wild-type streptavidin has been measured at about 6.7×10^7 $M^{-1}s^{-1}$.

The on rate may also be calculated in relative terms, similarly to the off rate described above, i.e. the on rate of a mutant streptavidin protein may be measured relative to the on rate of the wild-type streptavidin protein. For instance, biotin-4-fluorescein's association with streptavidin can be continuously monitored from the quenching of biotin-4-fluorescein's fluorescence upon association to streptavidin's binding pocket.

Exemplary conditions for measuring the dissociation rate include combining 100 µl of 500 pM streptavidin (mutant or wild-type) in PBS with 100 µl of 100 pM

B4F in PBS, followed by immediate measurement of fluorescence (using 485 nm excitation and 520 nm emission) at regular time points at 37 °C. Control experiments may include a streptavidin-only control and a B4F-only control. In the streptavidin-only control, 100 µl of 500 pM streptavidin (mutant or wild-type) in PBS may be combined with 100 µl of PBS. In the B4F-only control, 100 µl of PBS may be combined with 100 µl of 100 pM B4F in PBS.

The association can be calculated as a percentage: 100* [(streptavidin + B4F signal)-(streptavidin-only control signal)]/[(B4F-only control signal)-(streptavidin-only control signal)] and the measurement of this association over time provides an association rate, i.e. on rate. The association rates of different streptavidin proteins (e.g. wild-type versus mutant) measured under the same reaction conditions, e.g. temperature, concentration etc. as exemplified above, can be readily compared to determine whether the on rate for each protein is higher, lower or equivalent.

A mutant streptavidin with an on rate that is "equivalent" to the on rate of the wild-type streptavidin may have on rate that is similar (i.e. comparable) to the on rate of wild-type streptavidin, i.e. such that the practical applications of the mutant streptavidin are not significantly affected, e.g. within a margin of experimental error. Thus, an equivalent on rate means that the mutant streptavidin does not associate with biotin or a biotin conjugate at a substantially lower rate than the wild-type streptavidin under the same conditions.

Thus, the on rate of the mutant streptavidin for biotin or biotin conjugate may be at least 60%, e.g. at least 70, 75, 80, 85 or 90% of the on rate of wild-type streptavidin for biotin or a biotin conjugate, such as at least 91, 92, 93, 94, 95, 96, 97, 98 or 99% of the on rate of wild-type streptavidin for biotin or a biotin conjugate. Alternatively viewed, the on rate of the mutant streptavidin for biotin or a biotin conjugate may be no more than 40% lower than the on rate of wild-type streptavidin for biotin or a biotin conjugate, e.g. no more than 35, 30, 25 or 20% lower than the on rate of wild-type streptavidin for biotin or a biotin conjugate, such as no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% lower than the on rate of wild-type streptavidin for biotin or a biotin conjugate. Particularly, in absolute terms, the on rate of the mutant streptavidin for biotin or biotin conjugate may be about at least 3.0×10^7 $M^{-1}s^{-1}$, such as at least 3.2×10^7 $M^{-1}s^{-1}$, 3.4×10^7 $M^{-1}s^{-1}$, 3.6×10^7 $M^{-1}s^{-1}$, 3.8×10^7 $M^{-1}s^{-1}$, 4.0×10^7 $M^{-1}s^{-1}$, e.g. about at least 4.5×10^7 $M^{-1}s^{-1}$, 5.0×10^7 $M^{-1}s^{-1}$, 5.2×10^7 $M^{-1}s^{-1}$, 5.5×10^7 $M^{-1}s^{-1}$, 5.7×10^7 $M^{-1}s^{-1}$, 6.0×10^7 $M^{-1}s^{-1}$ or 6.2×10^7 $M^{-1}s^{-1}$.

In some embodiments, the on rate of the mutant streptavidin for biotin may be reduced compared to the on rate of wild-type streptavidin. Hence, the mutant streptavidin of the present invention may associate more slowly with biotin than wild-type streptavidin. Hence, the on rate of the mutant streptavidin of the present invention may be at least 10, 20, 30, 40, 50, 60, 70, 80 or 90% less than the on rate of wild-type streptavidin. Alternatively viewed, the on rate of mutant streptavidin for biotin may be 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 fold less than the on rate of wild-type streptavidin. Particularly, in absolute terms, the on rate of the mutant streptavidin may be less than $6.7x10^7$ $M^{-1}s^{-1}$, for example equal or less than $6.0x10^7M^{-1}s^{-1}$, $5.0x10^7M^{-1}s^{-1}$, $4.0x10^7M^{-1}s^{-1}$, $3.0x10^7M^{-1}$, $2.0x10^7M^{-1}s^{-1}$, $1x10^7M^{-1}s^{-1}$, $9.0x\ 10^6\ M^{-1}s^{-1}$, $8.0x\ 10^6\ M^{-1}s^{-1}$, $7.0x\ 10^6\ M^{-1}s^{-1}$, e.g. equal to or less than $6.7\ x\ 10^6\ M^{-1}s^{-1}$.

The on rates and off rates described above refer to the properties of the mutant streptavidin, i.e. comprising at least one mutant streptavidin subunit of the invention, in which the mutant streptavidin subunit is not conjugated to a fluorescent dye. However, in a preferred embodiment of the invention, the mutant streptavidin of the invention has on and off rates described above even after the mutant streptavidin subunit has been conjugated to a fluorescent dye.

As discussed above, the inventors have surprisingly determined that the mutant streptavidin subunit of the invention, conjugated to a fluorescent dye via the amino groups, imparts improved fluorescent properties on a streptavidin protein comprising said subunit. In particular, the inventors have demonstrated that a streptavidin protein comprising four of the mutant streptavidin subunits, each conjugated to a fluorescent label via the amino groups (i.e. where each free amine group in the subunit may be conjugated to a fluorescent label via a covalent bond, e.g. an amide bond) has a higher fluorescent brightness when compared to (i.e. relative to) a streptavidin protein comprising four wild-type streptavidin subunits in which the same fluorescent label is conjugated via the amino groups ((i.e. where each free amine group in the subunit may be conjugated to a fluorescent label via a covalent bond, e.g. an amide bond). Alternatively viewed, a streptavidin protein comprising four of the mutant streptavidin subunits, each conjugated to a fluorescent label via the amino groups has an increased fluorescent brightness relative to a streptavidin protein comprising four wild-type streptavidin subunits in which the same kind of fluorescent label is conjugated via the amino groups.

Whilst not wishing to be bound by theory, it is hypothesised that reducing the number of fluorescent dyes (labels or molecules) conjugated to each subunit prevents interference and quenching effects caused by close proximity of dye molecules to each other on the streptavidin protein. In this respect, a wild-type streptavidin protein in which a fluorescent dye is conjugated to the free amine groups in each subunit may contain up to 20 dye molecules, i.e. 5 per subunit as described above. In contrast, the mutant streptavidin protein of the invention, e.g. comprising four subunits of the invention, may comprise a maximum of 12 dye molecules (e.g. 3 per subunit) and as few as 4 dye molecules (one per subunit). In some embodiments, the mutant streptavidin protein of the invention may contain fewer than 4 dye molecules, e.g. in chimeric proteins which contain unlabelled subunits that contain only one N-terminus amine group, i.e. only the a-amino group. In preferred embodiments, the mutant streptavidin protein of the invention comprises 4-12 fluorescent dye molecules, e.g. 4, 5, 6, 7, 8, 9, 10, 11 or 12 molecules, such as 12 or fewer, 10 or fewer, 8 or fewer, 7 or fewer or 6 or fewer.

Fluorescent brightness or intensity is quantitatively dependent on a variety of parameters including the molar extinction coefficient, optical path length, solute concentration, fluorescence quantum yield of the dye, excitation source intensity and fluorescence collection efficiency of the instrument used to measure the fluorescent signal (i.e. emission). For instance, in dilute solutions or suspensions, fluorescence or brightness intensity is linearly proportional to these parameters. However, in concentrated samples, e.g. where absorbance exceeds about 0.05 in a 1 cm pathlength, the relationship may become non-linear and measurements may be distorted by artefacts such as self-absorption and the inner-filter effect.

The fluorescence quantum yield (QY) of a dye is the fraction of photons absorbed resulting in emission of fluorescence. The QY of a fluorophore is determined relative to a reference compound of known QY, preferably using the same excitation wavelength, gain and slit bandwidths. It is advantageous to have a reference molecule resembling the molecule for which the QY is to be measured.

The molar extinction coefficient (also known as the molar attenuation coefficient or molar absorptivity) is a measurement of how strongly a molecule attenuates light at a given wavelength, i.e. the absorbance. The absorbance of a material that has only one attenuating species depends on the pathlength and the concentration of the species, according to the Beer-Lambert law, $A=\varepsilon.\alpha I$ (wherein

A is absorbance (unitless), ε is the extinction coefficient ($M^{-1}cm^{-1}$) and l is pathlength (cm)).

When measuring the fluorescent brightness of large molecules, such as proteins, e.g. fluorescent proteins, specific values may be derived from the product of the molar extinction coefficient and quantum yield, divided by the value for a reference molecule, such as Green Fluorescent Protein (GFP). However, such absolute values are of limited use in practice because spectral profiles and wavelength maxima may vary due to environmental effects, such as pH, ionic concentration, and solvent polarity, as well as fluctuations in the concentration of the molecule to be measured. Therefore, absolute values of fluorescent brightness are relevant only if the specific conditions under which the various measurements were obtained are provided. Accordingly, measurements of fluorescence brightness typically are based on relative measurements, e.g. comparison to a fluorescent reference standard (which may be structurally distinct from the molecule or substance to be measured) or to a molecule to which the molecule to be measured is comparable, e.g. in terms of structure.

Thus, the term "fluorescent brightness" as used herein refers to the relative brightness or intensity of the fluorescent signal (i.e. emission) from two, preferably comparable, sources. For instance, fluorescent brightness of a mutant streptavidin protein of the invention labelled with (i.e. conjugated to) a fluorescent dye may be determined by measuring the fluorescent brightness (e.g. the number of photons emitted following excitation) of said mutant streptavidin protein and comparing it to the brightness of a wild-type streptavidin protein labelled with (i.e. conjugated to) the same fluorescent dye, under the same conditions. Alternatively, fluorescent brightness of a mutant streptavidin protein of the invention labelled with (i.e. conjugated to) a fluorescent dye may be determined by separately measuring the fluorescent brightness (e.g. the number of photons emitted following excitation) of said mutant streptavidin protein and a wild-type streptavidin protein labelled with (i.e. conjugated to) the same fluorescent dye (under the same conditions) and comparing the brightness of each protein to a fluorescent reference standard, e.g. the free fluorescent dye, i.e. not conjugated to a protein, or the fluorescent dye conjugated to a reference protein, e.g. bovine serum albumin (BSA).

Thus, "higher" or "increased" fluorescent brightness means that the test molecule, e.g. the mutant streptavidin protein labelled with a fluorescent dye, emits more fluorescence photons upon excitation than the reference molecule, e.g. the

wild-type streptavidin protein labelled with the same fluorescent dye, under the same conditions, e.g. excitation conditions.

Thus, in some embodiments the fluorescent brightness of the mutant streptavidin protein of the invention is at least 50% higher than the wild-type streptavidin protein conjugated to the same fluorescent dye (measured under the same conditions). For instance, the fluorescent brightness of the mutant streptavidin protein of the invention is at least 75%, 100%, 125%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500% or higher than the wild-type streptavidin protein conjugated to the same fluorescent dye (measured under the same conditions). Alternatively viewed, the fluorescent brightness of the mutant streptavidin protein of the invention is increased by at least 1.5 times relative to the wild-type streptavidin protein conjugated to the same fluorescent dye (measured under the same conditions). For instance, the fluorescent brightness of the mutant streptavidin protein of the invention is increased by at least 1.75, 2, 2.25, 2.5, 3, 3.5, 4, 4.5 or 5 times relative to the wild-type streptavidin protein conjugated to the same fluorescent dye (measured under the same conditions).

Fluorescent brightness may be measured using any suitable means, such as a spectrofluorometer.

As noted above, the mutant streptavidin subunit of the invention may be conjugated to a fluorescent dye (label or tag). In this respect, the terms "fluorescent dye" or "fluorescent label" are used interchangeably herein and refer to fluorophores, i.e. any fluorescent chemical compound (molecule) that can re-emit light upon light excitation. Fluorophores often comprise a number of aromatic groups. Other typical fluorophores are planar or cyclic molecules with multiple π bonds.

Any suitable fluorophore may find utility in the present invention. In this respect, a fluorophore must be capable of being conjugated, directly or indirectly, to a protein, particularly a streptavidin subunit or mutant thereof, via the amino groups, e.g. via an amide linkage (i.e. an amide bond), e.g. via NHS chemistry as discussed below. Fluorophores have been identified with excitation and emission spectra ranging from UV to near IR wavelengths. Thus, the fluorophore may have an excitation and/or emission wavelength in the UV, visible or IR spectral range.

The fluorophore may be a protein, peptide, small organic compound, synthetic oligomer or synthetic polymer. In some embodiments the fluorophore is a small organic compound, e.g. an organic compound with a molecular weight of

5000 Da or less. Thus, in some embodiments, the fluorophore has a molecular weight of 4000 Da or less, such as 3500 Da, 3000 Da, 2500 Da, 2250 Da, 2000 Da, 1900 Da, 1800 Da, 1700 Da, 1600 Da, 1500 Da or less.

Thus, the fluorophore may be a xanthene derivative (e.g. fluorescein, rhodamine, Oregon green, eosin, Texas red), a cyanine derivative (e.g. cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, merocyanine), a squaraine derivative (e.g. ring-substituted squaraine, Seta, SeTau), a naphthalene derivative (e.g. dansyl or prodan derivative), a coumarin derivative, an oxadiazole derivative (such as pyridyloxazole, nitrobenzoxadiazole and benzoxadiazole), an anthracene derivative (such as an anthraquinone, including DRAQ5, DRAQ7 and CyTRAK Orange), a pyrene derivative (e.g. cascade blue), an oxazine derivative (e.g. Nile red, Nile blue, cresyl violet, oxazine 170), an acridine derivative (such as proflavin, acridine orange, acridine yellow), an arylmethine derivative (e.g. auramine, crystal violet, malachite green) or a tetrapyrrole derivative (such as porphyrin, phthalocyanine, bilirubin).

Specific examples of fluorophores or fluorophore series that may find utility in the present invention include Alexa Fluors (such as Alexa Fluor 488, Alexa Fluor 647 etc.), Atto, Cyanine (Cy), indocyanine, sulfocyanine, DyLight, Abberior STAR, Chromeo, Oregon Green, Fluorescein, Texas red, Rhodamine, Silicon-rhodamine (SiR), Squaraine, FluoProbes, Tetrapyrrole, Bodipy, HiLyte, Quasar, CAL fluor, Coumarin, Seta, CF, Tracy, IRDye, CruzFluor, Tide Fluor, Oyster, iFluor, Chromis and Brilliant Violet and fluorescent derivatives or analogues thereof.

The term "conjugated to a fluorescent label via the amino groups" means that the fluorescent dye (label or tag) is conjugated to the (mutant or wild-type) streptavidin subunit via (i.e. following) a reaction with one or more amine groups on the streptavidin subunit, i.e. the fluorescent dye is linked to the subunit via a reaction with the a-amino group (at the N-terminus of the subunit) and optionally to one or two ε-amino groups of lysine residues that have not been mutated, i.e. substituted or deleted, wherein the reaction results in the formation of a covalent bond. Thus, the fluorescent dye is linked to the mutant subunit of the invention by a covalent bond via a reaction with the a-amino group (at the N-terminus of the subunit) and optionally to one or two lysines at positions equivalent to positions 80, 132 or 134 of SEQ ID NO. 2.

The fluorescent dye may be conjugated to the mutant streptavidin subunit via reaction with one or more amine groups by any suitable means. In this respect,

the fluorophore may be conjugated directly by means of a reactive or functional group or label on the fluorophore that is able to react with a-amino and ε-amino groups to form a covalent bond, e.g. an amide bond, an isothiourea bond, an isourea bond, a sulfonamide bond or a secondary amine bond. In some embodiments, the fluorophore may be conjugated indirectly by means of a linker group covalently attached to the fluorophore which contains a reactive or functional group or label that is able to react with a-amino and ε-amino groups to form a covalent bond, e.g. an amide bond, an isothiourea bond, an isourea bond, a sulfonamide bond or a secondary amine bond. Thus, in some embodiments, the fluorophore or linker is conjugated to the subunit via a direct reaction with the amine groups to form a covalent bond, e.g. an amide bond, an isothiourea bond, an isourea bond, a sulfonamide bond or a secondary amine bond.

In other embodiments, the amine groups may be specifically modified to enable the fluorophore to be conjugated to the subunit via a different type of bond, e.g. a thioether bond. For instance, the streptavidin subunit may be reacted with a heterobifunctional cross-linker that is capable of reacting specifically with the free amine groups on the subunit and comprises another reactive group, e.g. a thiol-reactive group, such as a maleimide group, to which a fluorophore may be conjugated (directly or indirectly).

By way of example, the subunit may be reacted with succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), such that the cross-linker is conjugated to the free amine groups on the subunit, i.e. the free amine groups are modified to comprise the cross linker. The modified subunit may subsequently be reacted with a fluorophore (or other soluble reagent or solid-phase e.g. resin, surface or nanoparticle, as described below) comprising a thiol group. In this respect, the fluorophore itself does not need to contain a thiol group, e.g. the fluorophore may be conjugated to another molecule containing a thiol group (e.g. a small molecule, thiol-modified oligonucleotide, protein, phosphorothioate DNA probe etc.) that is able to react with the thiol-reactive group (e.g. maleimide group) on the cross-linker molecule attached to the streptavidin subunit via the a-amino and ε-amino groups, to form a thioether bond.

In a further example, the subunit may be reacted with a molecule that reacts specifically with the free amine group(s) to generate a free thiol group(s), e.g. the subunit may be reacted with N-succinimidyl S-acetylthioacetate (SATA) or iminothiolane (2-iminothiolane). The modified subunit may subsequently be reacted

with a fluorophore (or other soluble reagent or solid phase, e.g. resin, surface or nanoparticle) comprising a thiol-reactive group, such as a maleimide group. In this respect, the fluorophore itself does not need to contain a thiol reactive group, e.g. the fluorophore may be conjugated to another molecule containing a thiol-reactive group (e.g. a small molecule, thiol-reactive oligonucleotide, maleimide-bearing protein, resin, surface, nanoparticle etc.) that is able to react with the thiol group on the streptavidin subunit.

Thus, in some embodiments, the fluorophore (or other soluble reagent or solid-phase, e.g. resin, surface or nanoparticle) or linker is conjugated to the streptavidin subunit via an indirect reaction with the amine groups, i.e. the amine groups are modified to comprise a thiol group or thiol-reactive group, such that the fluorophore (or other soluble reagent or solid-phase, e.g. resin, surface or nanoparticle) is conjugated to the subunit (directly or indirectly) via a covalent bond, e.g. a thioether bond.

Suitable reactive or functional groups that may be present on a fluorophore or linker covalently attached to said fluorophore include N-hydroxysuccinimide (NHS), sulfo-N-hydroxysuccinimide (sulfo-NHS), Tetrafluorophenyl ester (TFP), sulfo-tetrafluorophenyl ester (SFP), pentafluorophenyl ester (PFP), Acyl azide, Imidoester, Anhydride, Isothiocyanate, Isocyanate, Sulfonyl chloride, Aldehyde (optionally wherein the conjugate stabilized by further reaction with a reducing agent after the reaction with an amine, a process known as reductive amination) and Epoxide. In some preferred embodiments, the reactive or functional group that may be present on a fluorophore or linker covalently attached to said fluorophore is selected from N-hydroxysuccinimide (NHS), sulfo-N-hydroxysuccinimide (sulfo-NHS), Tetrafluorophenyl ester (TFP), sulfo-tetrafluorophenyl ester (SFP) and pentafluorophenyl ester (PFP).

In some embodiments it may be advantageous to mutate other residues (i.e. residues in addition to the lysine residues described above) in the mutant streptavidin subunit of the present invention. For instance, such additional mutations may impart desired properties on the subunit and/or resultant mutant streptavidin protein. Such desired properties may include a lower off rate for biotin or a biotin conjugate, a higher on rate for biotin or a biotin conjugate, improved thermostability and/or mechanostability. Additionally, mutations may be introduced to prevent biotin binding, e.g. to facilitate the assembly of a monovalent, divalent or trivalent streptavidin protein. For example, for monovalent streptavidin, one subunit

of the tetramer would have biotin binding properties equivalent to wild-type streptavidin, while three subunits of the tetramer would have weak or negligible biotin binding properties. Further mutations may include the insertion of tags or linkers, e.g. to facilitate the precise linkage of streptavidin proteins to other components. In preferred embodiments, the additional mutations are substitutions and/or insertions.

Thus, the mutant streptavidin subunit of the present invention particularly may be at least 70% identical to the wild-type streptavidin subunit sequences as set forth in SEQ ID NOs 1, 2 or 3 and more particularly is at least 75, 80, 85, 90, 95, 96, 97, 98 or 99% identical to SEQ ID NOs 1, 2 or 3. In other embodiments, the mutant streptavidin subunit of the present invention particularly may be at least 70% identical to the mutant streptavidin subunit sequences as set forth in any one of SEQ ID NOs 4-8, 11 and 12, and more particularly is at least 75, 80, 85, 90, 95, 96, 97, 98 or 99% identical to SEQ ID NOs 4-8, 11 and 12, preferably wherein the mutant streptavidin subunit comprises the specific mutations of SEQ ID NOs. 4-8, 11 and 12 relative to the wild-type sequence, as described below. The mutant streptavidin subunit thus encompasses mutant forms of streptavidin homologue subunits which are structurally similar to streptavidin subunits and are able to interact with biotin when tetramerised. In cases where a mutant streptavidin homologue subunit is produced or where the mutant streptavidin subunit comprises other mutations, e.g. deletions or insertions, in addition to the at least two mutations at positions 80, 121, 132 and 134, the mutations at the at least two amino acid residue positions 80, 121, 132 and 134 are carried out at equivalent amino acid residues in the homologue or mutant subunit sequences.

Thus a mutant streptavidin subunit of the present invention may differ from the wild type streptavidin subunit sequences by for example 2 to 20, 2 to 15, 2 to 10, 2 to 8, 2 to 6, 2 to 5, 2 to 4 or 2 to 3 amino acid substitutions, insertions and/or deletions, preferably substitutions. In some embodiments, any other mutations that are present in the mutant streptavidin subunit of the present invention may be conservative amino acid substitutions. A conservative amino acid substitution refers to the replacement of an amino acid by another which preserves the physiochemical character of the polypeptide (e.g. D may be replaced by E or vice versa, N by Q, or L or I by V or vice versa). Thus, generally the substituting amino acid has similar properties, e.g. hydrophobicity, hydrophilicity, electronegativity, bulky side chains etc. to the amino acid being replaced when such substitution is

made to a residue other than the lysines at positions 80, 121, 132 and 134. Isomers of the native L-amino acid e.g. D-amino acids may be incorporated.

Sequence identity may be determined by any suitable means known in the art, e.g. using the SWISS-PROT protein sequence databank using FASTA pep-cmp with a variable pamfactor, and gap creation penalty set at 12.0 and gap extension penalty set at 4.0, and a window of 2 amino acids. Other programs for determining amino acid sequence identity include the BestFit program of the Genetics Computer Group (GCG) Version 10 Software package from the University of Wsconsin. The program uses the local homology algorithm of Smith and Waterman with the default values: Gap creation penalty - 8, Gap extension penalty = 2, Average match = 2.912, Average mismatch = -2.003.

Preferably said comparison is made over the full length of the sequence, but may be made over a smaller window of comparison, e.g. less than 120, 100, 80 or 50 contiguous amino acids.

Preferably such sequence identity-related proteins are functionally equivalent to the polypeptides which are set forth in the recited SEQ ID NOs. As referred to herein, "functional equivalence" refers to homologues of the streptavidin subunits discussed above that, when present in a streptavidin protein, may show some reduced efficacy in binding biotin (e.g. higher off rate, lower on rate or lower fluorescent brightness) relative to the parent molecule (i.e. the molecule with which it shows sequence homology), but preferably are as efficient or are more efficient.

Hence, any modification or combination of modifications may be made to a wild-type streptavidin subunit to produce the mutant streptavidin subunit of the invention, provided that the mutant comprises at least two amino acid mutations at amino acid residue positions equivalent to positions 80, 121, 132 and 134 of SEQ ID NO. 2 as defined above and retains the functional characteristics defined above, i.e. it results in a streptavidin protein with a higher fluorescent brightness relative to a wild-type streptavidin protein conjugated to the same fluorescent label via the amino groups and optionally has an equivalent or lower off rate for biotin or a biotin conjugate relative to wild-type streptavidin.

Alternatively, other modifications which can be made to the wild-type streptavidin subunit include further truncations of the subunit. For example, truncated streptavidin subunits consisting of residues 16-133 and 14-138 of the mature sequence of SEQ ID NO. 2 can be used or other truncations which result in the production of a mutant subunit having the properties required by the present

invention. In this respect, the skilled person will understand that nucleic acid molecules encoding the mutant streptavidin subunit of the invention may include the initiating methionine residue (an N-terminal methionine residue) relative to some of the sequences disclosed herein, e.g. SEQ ID NOs. 4-8, but this may not be present in the expressed protein, e.g. because it is removed when the protein is expressed in bacteria.

Thus, in some embodiments the mutant streptavidin subunit of the invention may also comprise the mutations N23A, S27D and S45A (wherein the first letter refers to the amino acid present in the wild-type sequence using the single letter code, the number refers to the position of the amino acid using the number of SEQ ID NO. 2, and the second letter refers to the substituting amino acid in the mutant streptavidin subunit using the single letter code). Mutant streptavidin subunits comprising the mutations N23A, S27D and S45A do not bind to biotin and may facilitate the production of a monovalent streptavidin protein, i.e. a protein in which only one of the streptavidin subunits binds to biotin.

Thus, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in any one of SEQ ID NOs. 4-6 or an amino acid sequence with at least 70% sequence identity to an amino acid sequence as set forth in any one of SEQ ID NOs. 4-6, wherein the amino acid residues at positions equivalent to positions 80, 121, 132 and 134 in SEQ ID NO. 2 are as set forth in one of SEQ ID NOs. 4-6 and the amino acid residues at positions equivalent to positions 23, 27 and 45 in SEQ ID NO. 2 are A, D and A, respectively.

Thus, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in SEQ ID NO. 7. In an alternative embodiment, the mutant streptavidin subunit comprises an amino acid sequence with at least 70% sequence identity to SEQ ID NO. 7 (more particularly at least 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO. 7), wherein the amino acid residues at positions equivalent to positions 23, 27, 45, 80, 121, 132 and 134 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 7.

In some embodiments the mutant streptavidin subunit of the invention may also comprise the mutations S52G and R53D (wherein the first letter refers to the amino acid present in the wild-type sequence using the single letter code, the number refers to the position of the amino acid using the number of SEQ ID NO. 2, and the second letter refers to the substituting amino acid in the mutant streptavidin subunit using the single letter code). Mutant streptavidin subunits comprising the

mutations S52G and R53D result in a streptavidin protein (tetramer) with a lower off rate for biotin and increased mechanostability relative to wild-type streptavidin.

Thus, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in any one of SEQ ID NOs. 4-6 or an amino acid sequence with at least 70% sequence identity to an amino acid sequence as set forth in any one of SEQ ID NOs. 4-6, wherein the amino acid residues at positions equivalent to positions 80, 121 , 132 and 134 in SEQ ID NO. 2 are as set forth in one of SEQ ID NOs. 4-6 and the amino acid residues at positions equivalent to positions 52 and 53 in SEQ ID NO. 2 are G and D, respectively.

Thus, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in SEQ ID NO. 8. In an alternative embodiment, the mutant streptavidin subunit comprises an amino acid sequence with at least 70% sequence identity to SEQ ID NO. 8 (more particularly at least 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity to SEQ ID NO. 8), wherein the amino acid residues at positions equivalent to positions 52, 53, 80, 121, 132 and 134 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 8.

In some embodiments it may be useful to incorporate a peptide tag into the mutant streptavidin subunit of the invention, e.g. to facilitate the linkage of the mutant streptavidin subunit to other molecules, e.g. other proteins, such as other modified or mutant streptavidin subunits as described in herein. Thus, in some embodiments, the mutant streptavidin subunit of the invention comprises a peptide linker. Preferably, the peptide linker does not contain any lysine residues. The peptide linker may be incorporated in any part of the mutant streptavidin subunit of the invention, preferably at the N-terminus or C-terminus, most preferably at the C-terminus. The peptide tag may be attached or conjugated (e.g. covalently linked) to the mutant streptavidin subunit directly (i.e. as an N-terminal or C-terminal extension of the polypeptide) or indirectly via a linker, e.g. a peptide linker, such as the amino acid sequence as set forth in SEQ ID NO. 9. In some embodiments, the peptide tag comprises the amino acid sequence as set forth in SEQ ID NO. 10.

Thus, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in any one of SEQ ID NOs. 4-8 or an amino acid sequence with at least 70% sequence identity to an amino acid sequence as set forth in any one of SEQ ID NOs. 4-8, wherein the amino acid residues at positions equivalent to positions 23, 27, 45, 52, 53, 80, 121, 132 and 134 in SEQ ID NO. 2 are as set forth in one of SEQ ID NOs. 4-8, and wherein said amino acid sequence

comprises a peptide tag with an amino acid sequence as set forth in SEQ ID NO. 10 at the N or C terminus.

In a particularly preferred embodiment, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in SEQ ID NO. 11 or an amino acid sequence with at least 70% sequence identity to an amino acid sequence as set forth in SEQ ID NO. 11, wherein the amino acid residues at positions equivalent to positions 23, 27, 45, 80, 121 , 132 and 134 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 11.

In another particularly preferred embodiment, the mutant streptavidin subunit of the invention may comprise an amino acid sequence as set forth in SEQ ID NO. 12 or an amino acid sequence with at least 70% sequence identity to an amino acid sequence as set forth in SEQ ID NO. 12, wherein the amino acid residues at positions equivalent to positions 80, 121, 132 and 134 in SEQ ID NO. 2 are as set forth in SEQ ID NO. 11.

In some embodiments the mutant streptavidin subunit of the invention may have wild-type residues present at positions 23, 27, 43, 45, 49, 79, 88, 90, 92, 108, 110 and 128, i.e. identical residues found at these positions in SEQ ID NO. 2 are present at equivalent positions in the mutant streptavidin subunit (or conservative amino acid substitutions of such residues which do not affect the formation of the biotin binding site). The residues at positions 23, 27, 43, 45, 49, 79, 88, 90, 92, 108, 110 and 128 in SEQ ID NO. 2 are as follows:

N23, S27, Y43, S45, N49, W79, S88, T90, W92, W108, and D128 and thus these amino acids should preferably be found at equivalent positions in the mutant streptavidin subunit, except where alternative residues are required according to the specific embodiments described above.

These amino acid residues are important for the formation of the biotin binding site in the mutant streptavidin subunit. Additionally, when the subunit is comprised within a mutant streptavidin protein, e.g. in tetrameric form, a further residue which is provided by an adjacent subunit in streptavidin may help form the biotin binding site, namely W120. Thus, in streptavidin each subunit provides a W120 residue for an adjacent subunit. In some embodiments, for a tetravalent streptavidin protein, all subunits may comprise a wild-type W120 residue or have only a conservative amino acid substitution at this position.

Preferably therefore, when the mutant streptavidin subunit of the invention is comprised within a mutant streptavidin protein, e.g. is in tetrameric form, a wild-type

residue is present at a position equivalent to position 120 in SEQ ID NO. 2, in another adjacent subunit in the streptavidin to allow stable biotin binding with the mutant streptavidin subunit. Thus, as discussed above, an adjacent streptavidin subunit preferably contributes a W120 residue to the biotin binding site of the mutant streptavidin subunit in the tetramer. The particular adjacent subunit which provides the W120 residue to the mutant streptavidin subunit depends on the positioning of the mutant subunit in streptavidin. Therefore, when the mutant streptavidin subunit is comprised in a mutant streptavidin protein, preferably at least an adjacent subunit has a wild-type residue at a position equivalent to position 120 of SEQ ID NO. 2. Further, the other subunits may also have a wild-type residue at a position equivalent to position 120 of SEQ ID NO. 2, particularly, where the streptavidin is divalent, trivalent or tetravalent i.e. has 2, 3, or 4 biotin binding sites. Thus, for tetravalent streptavidin, all subunits, including the mutant streptavidin subunits of the invention may have a wild-type residue at a position equivalent to position 120 of SEQ ID NO. 2.

Further, the wild-type residues at positions 23, 27, 43, 45, 49, 79, 88, 90, 92, 108, 110 and 128 occur at equivalent amino acid positions in the homologue or mutant subunit sequences. An equivalent position is determined by reference to the amino acid sequence of SEQ ID NO. 2, i.e. the mature streptavidin subunit sequence which dictates the numbering of the amino acid residues in streptavidin. The homologous or corresponding position can be readily deduced by lining up the sequence of the homologue or mutant subunits and the sequence of SEQ ID NO. 2 based on the homology or identity between the sequences, for example using a BLAST algorithm.

Thus, the amino acid position numbering referred to with respect to the mutant streptavidin subunit may not be the actual numbering of amino acids within that subunit. The numbering used relates to the position of amino acids in the mature streptavidin sequence of SEQ ID NO. 2 and as discussed above, equivalent residue positions are intended in the mutant subunits of the invention. Therefore, although the at least two amino acid mutations may occur at residue positions 80, 121, 132 and 134 in the mutant streptavidin subunit, e.g. if its sequence is the same length as that of SEQ ID NO. 2, if the sequence is a different length to SEQ ID NO. 2, the amino acid mutation will take place at the equivalent residue. For example, if the mutant streptavidin subunit is based on the core streptavidin sequence which has a 12 amino acid truncation at the N terminus compared to the mature sequence

of SEQ ID NO. 2, then the at least two amino acid mutations will occur at positions 68, 109, 120 and 122 in that sequence which correspond to positions 80, 121, 132 and 134 of SEQ ID NO. 2. Thus reference herein to amino acid residue positions 80, 121, 132 and 134 of the mutant streptavidin subunit refers to amino acid residue positions in the mutant streptavidin subunit which are equivalent to those at positions 80, 121, 132 and 134 in SEQ ID NO. 2.

Similarly, although the wild-type residues may occur at positions 23, 27, 43, 45, 49, 79, 88, 90, 92, 108, 110 and 128 in the mutant streptavidin subunit if this has a sequence the same length as SEQ ID NO. 2, if the sequence of the mutant subunit is a different length, then the equivalent residues at those positions in the mutant subunit will be wild-type.

As discussed above, the streptavidin protein usually occurs in tetrameric form. However, monomeric streptavidin can be generated which is unable to multimerise, i.e. tetramerise. Thus, reference to streptavidin in monomeric form or to monomeric streptavidin refers to a streptavidin subunit which is unable to tetramerise and which only occurs as individual subunits. Monomeric streptavidin may be unable to tetramerise if used at high temperatures or may be produced by mutating the wild-type streptavidin subunit sequence at one or more residues. Typically, monomeric streptavidin is produced by making one or more amino acid substitutions at residue positions equivalent to positions 76, 125, 55 or 109 in SEQ ID NO. 2. Preferably, monomeric streptavidin may comprise the mutations; T76R, V125R, V55T and L109T. Thus, the mutant streptavidin subunit of the invention when in monomeric form will be unable to form tetramers and may additionally comprise one or more amino acid substitutions at residues 76, 125, 55 and 109. The mutant streptavidin subunit of the invention when in monomeric form will preferably comprise T76R, V125R, V55T and L109T amino acid substitutions in addition to the amino acid substitutions discussed above, i.e. in addition to the at least two mutations at positions 80, 121, 132 and 134 and optionally substitutions at positions 52 and 53.

Thus, in a further embodiment, the invention also provides a mutant streptavidin subunit of the invention in monomeric form.

As discussed above, the mutant streptavidin protein of the invention comprises at least one mutant streptavidin subunit of the invention, preferably 2, 3 or 4 mutant streptavidin subunits of the invention. Where the mutant streptavidin comprises 1, 2 or 3 mutant subunits of the invention, the other 3, 2, or 1 subunits,

respectively present may be wild-type or mutant subunits, e.g. with a different plurality of mutations to those described herein, i.e. the mutant streptavidin protein may be a chimeric protein. For instance, the other 3, 2 or 1 subunits may be inactivated for biotin binding, e.g. one subunit may comprise an amino acid sequence as set forth in one of SEQ ID NOs. 4-6, 8, 11 or 12 (or variants thereof as described above) and the other 3 subunits may comprise an amino acid sequence as set forth in SEQ ID NO. 7 (or variants thereof as described above). Inactivating the other subunits in such a way will not affect the stability of biotin binding to the mutant streptavidin subunit in the mutant streptavidin, as long as there is no change in the subunit interface.

Thus, the mutant streptavidin may be monovalent, i.e. only has one biotin binding site (where the valency of streptavidin refers to the number of biotin binding subunits present in the protein). Monovalent mutant streptavidin may therefore comprise one mutant streptavidin subunit of the invention that is capable of binding to biotin together with 3 inactivated mutant streptavidin subunits of the invention. Divalent, trivalent, and tetravalent mutant streptavidins can also be used, which have 2, 3, and 4 biotin binding subunits, respectively, one or more of which will be the mutant streptavidin subunit of the invention.

Preferably, the mutant streptavidin of the invention comprises four mutant streptavidin subunits of the invention, wherein at least one subunit is capable of binding to biotin, or comprises 1 or 2 mutant streptavidin subunits of the invention that are capable of binding to biotin, together with 3 or 2 inactivated streptavidin subunits of the invention which cannot bind biotin, respectively.

As described in the Examples below, the mutant streptavidin protein of the present invention retained similar (i.e. comparable or equivalent) thermostability to wild-type streptavidin. Streptavidin naturally occurs in tetrameric form, i.e. made up of four streptavidin subunits. Streptavidin tetramers will however dissociate into monomeric form at high temperatures. A similar (comparable or equivalent) thermostability as used herein hence refers to the ability of mutant streptavidin to retain tetrameric form at the same (i.e. substantially the same) temperature as wild-type streptavidin or for the same (i.e. substantially the same) percentage of mutant streptavidin molecules to be in tetrameric form at a particular temperature as wild-type streptavidin. For example, thermostability can be measured in terms of the temperature at which 50% of streptavidin is in the tetrameric form and 50% is in the monomeric form, i.e. the temperature at which there is 50% dissociation. For wild-

type streptavidin, the temperature at which there is 50% dissociation is about 70-75°C in the absence of biotin. Hence, the mutant streptavidin of the present invention may show 50% dissociation at about 65-75°C, for example 66-75°C, 67-75°C, 68-75°C, 69-75°C or 70-75°C in the absence of biotin.

Thermostability can be easily determined by heating streptavidin or a mutant thereof to a particular temperature and then placing on ice. The amounts of subunits/tetramers present can be determined using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantifying band intensities using Coomassie staining.

The mutant streptavidin or mutant streptavidin subunits of the present invention may be conjugated to other molecules or entities, for example, to a nucleic acid molecule, a protein, peptide, organic compound, inorganic compound, polysaccharide, other soluble reagent or solid-phase, e.g. resin, surface or nanoparticle, or any combination of these. In some embodiments, the mutant streptavidin or mutant streptavidin subunit may be conjugated to a compound which has a therapeutic or prophylactic effect, e.g. an antibiotic, antiviral, vaccine, anti-tumour agent, e.g. a radioactive compound or isotope, cytokines, toxins, oligonucleotides and nucleic acids encoding genes or nucleic acid vaccines. The mutant streptavidin or mutant streptavidin subunit of the invention may be conjugated to a label in addition to a fluorescent label as described above, for example, a radiolabel, chemiluminescent label, a chromophore label as well as to substances and enzymes which generate a detectable substrate, e.g. horseradish peroxidase, luciferase or alkaline phosphatase. Labels for magnetic resonance imaging, Positron emission tomography (PET) probes and boron 10 for neutron capture therapy may also be conjugated to the mutant streptavidin or mutant streptavidin subunit of the invention. The other molecules or entities to which the mutant streptavidin or mutant streptavidin subunits of the invention may be conjugated can be conjugated (e.g. covalently linked) using the same methods described above to conjugate the fluorophore to the mutant streptavidin or mutant streptavidin subunits of the invention.

A further aspect of the present invention concerns a nucleic acid molecule which comprises a nucleotide sequence encoding a mutant streptavidin subunit of the present invention. Particularly, a nucleic acid molecule which encodes a mutant streptavidin subunit comprising the sequence as set forth in any one of SEQ ID NOs. 4-8, 11 or 12 is encompassed by the invention.

A vector comprising a nucleic acid molecule of the invention is also provided by the invention. Typically, the nucleic acid molecule may be operably linked to a control sequence present in the vector, e.g. a promoter which is capable of providing for the expression of the coding sequence in a host cell. Thus, in addition to the nucleic acid sequence of the invention, the vectors may comprise other elements such as a promoter, enhancer, transcription initiation site, termination site, translation initiation site, polyA site etc. Further the vector may comprise one or more selectable marker genes such as a gene providing for ampicillin resistance or kanamycin resistance. The vector may additionally comprise a signal sequence, allowing export of an expressed product outside of the host cell.

The vector is generally selected depending on the intended expression system and may be a transposon, plasmid, virus or phage vector. The vector may be typically introduced into host cells using conventional techniques such as calcium phosphate precipitation, liposomal transfection agents, DEAE-dextran transfection or electroporation.

A further aspect of the present invention concerns a recombinant host cell which is transformed or transfected with a vector or nucleic acid molecule of the invention. Thus, the cell of the invention may carry at least one copy of a nucleic acid sequence of the invention. The cell may be a prokaryotic cell such as *E. coli, Streptomyces avidinii or Bacillus subtilis* or a eukaryotic cell such as a yeast or human cell-line or CHO cell-line.

By "recombinant" is meant that the nucleic acid molecule and/or vector has been introduced into the host cell. The host cell may or may not naturally contain an endogenous copy of the nucleic acid molecule, but it is recombinant in that an exogenous or further endogenous copy of the nucleic acid molecule and/or vector has been introduced. In some embodiments, the endogenous copy of the nucleic acid molecule may be replaced with the nucleic acid molecule of the invention, e.g. replacement of the endogenous nucleic acid molecule in *S.avidinii,* e.g. by CRISPR. Thus, in some embodiments the nucleic acid molecule is integrated into the host genome. In other embodiments, the nucleic acid molecule is not integrated into the host genome, e.g. the nucleic acid molecule or vector is present as a separate (e.g. self) replicating nucleic acid molecule, e.g. plasmid.

A further aspect of the invention provides a method of preparing a mutant streptavidin subunit (polypeptide) of the invention as hereinbefore defined, which comprises culturing a host cell containing a nucleic acid molecule as defined above,

under conditions whereby said nucleic acid molecule encoding said mutant streptavidin subunit is expressed and recovering said molecule (mutant streptavidin subunit) thus produced. The expressed mutant streptavidin subunit forms a further aspect of the invention.

In some embodiments, the mutant streptavidin subunit of the invention may be generated synthetically, e.g. by ligation of amino acids or smaller synthetically generated peptides, or more conveniently by recombinant expression of a nucleic acid molecule encoding said polypeptide as described hereinbefore.

Nucleic acid molecules of the invention may be generated synthetically by any suitable means known in the art.

Thus, the mutant streptavidin subunit of the invention may be an isolated, purified, recombinant or synthesized polypeptide. As used herein the term "polypeptide" typically refers to a streptavidin subunit or monomer whereas the term "protein" typically refers to the multimerised form of streptavidin, e.g. the tetramer form. The term polypeptide typically includes any amino acid sequence comprising at least 40 consecutive amino acid residues, e.g. at least 50, 60, 70, 80, 90, 100, 150 amino acids, such as 40-1000, 50-900, 60-800, 70-700, 80-600, 90-500, 100-400 amino acids.

Similarly, the nucleic acid molecules of the invention may be an isolated, purified, recombinant or synthesized nucleic acid molecule.

Thus, alternatively viewed, the polypeptides and nucleic acid molecules of the invention are non-native, i.e. non-naturally occurring, molecules.

Standard amino acid nomenclature is used herein. Thus, the full name of an amino acid residue may be used interchangeably with one letter code or three letter abbreviations. For instance, lysine may be substituted with K or Lys, isoleucine may be substituted with I or Ile, and so on.

In another aspect of the invention, a process for the production of a mutant streptavidin subunit of the present invention is provided which comprises the following steps:

a) transforming or transfecting a suitable host cell with a vector which comprises a nucleotide sequence encoding the streptavidin mutant subunit of the invention,

b) culturing the host cell under conditions which allows expression of the streptavidin mutant subunit to take place, and

c) isolating the mutant streptavidin subunit.

The subunits may be isolated in monomeric form, e.g. where additional mutations are present preventing tetramerisation or may be isolated in tetrameric form, e.g. as mutant streptavidin.

Thus, the mutant streptavidin subunit may be produced using recombinant methodology. It will be understood that the above production process may comprise further steps, such as a step of producing the vector which comprises a nucleotide sequence encoding the streptavidin mutant subunit of the invention. Site-directed mutagenesis of a nucleic acid sequence encoding wild-type streptavidin subunit for example may be employed to produce a nucleotide sequence encoding the mutant streptavidin subunits prior to the transformation step (a). Alternatively, the nucleotide sequence encoding the mutant streptavidin subunit may be produced by chemical synthesis. Such a mutated nucleotide sequence may then be ligated into an appropriate vector for host cell transformation or transfection. The host cells transformed or transfected may be any cell as described previously and the vector may be a vector of the present invention.

Alternatively, the mutant streptavidin subunit may be synthesised using standard chemical peptide synthesis techniques. Solid phase synthesis of peptides in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids may for example be used.

Once expressed, the mutant streptavidin subunit, e.g. in monomeric or tetrameric form may be purified according to standard procedures in the art, including ammonium sulfate precipitation, affinity chromatography, ion exchange chromatography, gel filtration chromatography etc. Particularly, it may be possible to purify the mutant streptavidin subunit using iminobiotin, diaminobiotin or another ligand partner in an affinity column.

It will be understood that after synthesis, expression or purification, the mutant streptavidin subunits (either in the streptavidin protein or in monomeric form) may not be in the conformation of the native protein and thus it may be necessary to denature and reduce the streptavidin and to refold the polypeptides. Methods which can be used to achieve this effect include denaturing in urea or guanidinium hydrochloride and renaturing by slow dialysis or rapid dilution.

The present invention also provides a kit comprising a mutant streptavidin subunit of the invention. The streptavidin may be conjugated to another molecule as discussed previously or may be coated onto a nanoparticle, bead or solid

surface such as a plate, column or microspheres. Additionally the kit may comprise biotin and/or biotin analogue(s) or conjugates.

Alternatively, the kit may comprise a nucleic acid molecule comprising a nucleotide sequence encoding a mutant streptavidin subunit of the invention. Hence, the kit may comprise a vector comprising the nucleic acid molecule and/or host cells to allow expression of the molecule after transformation/transfection. Alternatively, the kit may comprise transformed or transfected host cells, i.e. recombinant host cells.

The mutant streptavidin or mutant streptavidin subunit in monomeric form of the invention may be used for any purpose for which wild-type streptavidin or monomeric streptavidin may be used. Thus, the mutant streptavidin or mutant streptavidin subunit in monomeric form may be used for any method involving streptavidin binding to biotin conjugates. The mutant streptavidin or mutant streptavidin subunit of the invention are however particularly useful in methods which require increased fluorescent brightness, e.g. flow cytometry, fluorescent microscopy, particularly for labelling molecules that are present in low amounts.

The mutant streptavidin of the present invention may therefore be used in labelling or tagging applications, e.g. where mutant streptavidin is used to label biotinylated entities, e.g. molecules, cells etc. For instance, the biotinylated entity may be an antibody capable of binding to a specific antigen and, once bound to its antigen, e.g. cell surface protein, may be stably labelled by the fluorescently labelled mutant streptavidin and then be detected, e.g. by flow cytometry or fluorescence microscopy. In this way, it may be possible to detect an antigen which is present at low abundance in a sample by virtue of its binding to the labelled mutant streptavidin of the invention.

Thus, the invention provides a method of labelling a biotinylated entity, e.g. a molecule, cell, biological complex (one component of which is biotinylated), virus (including bacteriophage), organelle, nanoparticle or other nano-assembly, or liposome comprising the step of contacting said biotinylated entity with a fluorescently labelled mutant streptavidin of the present invention. In some embodiments, the method may further comprise a step of detecting the labelled biotinylated entity, e.g. by exciting the fluorescent label conjugated to the mutant streptavidin of the present invention and detecting, and optionally amplifying, the fluorescent signal emitted from the fluorescent label.

Thus, the present invention further provides the use of the mutant streptavidin protein of the invention in the labelling and/or detection of a biotinylated entity, e.g. a molecule, cell, biological complex (one component of which is biotinylated), virus (including bacteriophage), organelle, nanoparticle or other nano-assembly, or liposome. Additionally, the present invention further provides the use of the mutant streptavidin protein of the invention in imaging, e.g. cell imaging, such as fluorescence microscopy or flow cytometry.

The mutant streptavidin of the present invention may also be used in preparative or purification applications, where mutant streptavidin immobilized on a solid support is used to capture biotinylated entities, e.g. molecules, cells (particularly immune cells, such as B-cell or T-cells) etc. which are passed over the solid support. Such biotinylated entities will be stably captured by the mutant streptavidin on the solid support and may then be detected, e.g. by detection of a signal from the fluorescent label conjugated to the mutant streptavidin. In this way, it may be possible to purify a biotinylated entity from a composition/solution comprising other entities by virtue of its binding to the mutant streptavidin form of the invention.

In particular embodiments, the mutant streptavidin of the invention would facilitate the differential labelling of entities in a sample. For instance, major histocompatibility complex (MHC) proteins (class I and II) complexed with peptides are frequently tetramerised by site-specific biotinylation and then incubation with wild-type streptavidin. Such MHC-peptide tetramers are commonly used for analysis and isolation of specific T-cells (particularly for adoptive immunotherapy). Similarly antigens to which a humoral immune response could be raised are often site-specifically biotinylated and tetramerised with wild-type streptavidin. The avidity enhancement from this tetramerisation allows these antigen tetramers to be used to label and/or isolate specific B-cells displaying on their surface antibodies that recognise such antigens. Antigen tetramers may be used for analysing immune responses or to identify antibodies that could be useful as future therapies, e.g. against cancer, HIV or malaria. Thus, labelling the mutant streptavidin subunits of the invention with different fluorescent labels (e.g. each different MHC protein is conjugate to a mutant streptavidin subunit labelled with a different fluorescent tag) would facilitate the analysis of many different T-cell specificities from the same sample.

Thus, the invention provides a method of capturing and labelling an entity, e.g. a molecule, cell, biological complex, virus (including bacteriophage), organelle, nanoparticle or other nano-assembly, or liposome comprising the step of passing said entity over an immobilised mutant streptavidin of the present invention conjugated to a fluorescent label and a molecule, e.g. protein, that binds (specifically) to said entity under conditions that enable the molecule conjugated to said mutant streptavidin to bind to said entity specifically.

Alternatively viewed, the invention provides a method of capturing and labelling an entity, e.g. a molecule, cell, biological complex, virus (including bacteriophage), organelle, nanoparticle or other nano-assembly, or liposome comprising the step of passing said entity over an immobilised mutant streptavidin of the present invention, wherein said immobilised mutant streptavidin is conjugated to (i.e. covalently linked to) a fluorescent label and a molecule, e.g. protein, capable of binding (specifically) to said entity, and wherein said step of passing said entity over said immobilised mutant streptavidin is performed under conditions that enable the molecule conjugated to said mutant streptavidin to bind to said entity specifically.

Further, the invention provides a method of purifying and labelling an entity from a sample comprising the step of passing said entity over an immobilised mutant streptavidin of the invention conjugated to a fluorescent label and a molecule, e.g. protein, that binds to said entity and discarding the unbound sample.

Further, the mutant streptavidin of the invention may be advantageously used in targeting therapeutic agents to tumours and/or detecting tumour cells. It is possible to label tumour cells using biotinylated antibodies which are directed to tumour cell surface antigens. The mutant streptavidin or mutant streptavidin subunit in monomeric form of the invention conjugated to a therapeutic agent such as a radioactive isotope may then be administered to bind specifically to the biotinylated tumour cells (rather than to normal cells in the body). Furthermore, the mutant streptavidin of the present invention may advantageously be used to monitor the efficacy of the therapeutic agent when it is conjugated to a fluorescent label. Additionally or alternatively, the mutant streptavidin of the present invention may be used to diagnose cancer, e.g. by labelling cancer cells. The mutant streptavidin of the present invention is particularly suited for these uses in view of its higher fluorescent brightness than wild-type streptavidin conjugated to the same fluorescent label. Thus, the fluorescently labelled mutant streptavidin of the

invention will be detectable at lower levels than the corresponding wild-type protein ensuring that the efficacy of the therapeutic agent can be assessed more accurately or the cancer cells (or other diagnostic markers) may be detected even when they are present at extremely low levels. Thus, the mutant streptavidin of the present invention may advantageously facilitate the early detection of cancer or other disorders.

The mutant streptavidin of the invention can therefore be used to treat cancer or other diseases and/or monitor the treatment of cancer or other diseases which can be treated by the targeted delivery of a therapeutic agent to particular cells. Additionally, the mutant streptavidin of the invention can therefore be used to diagnose cancer or other diseases which can be diagnosed by the detection of particular cells or diagnostic markers, e.g. proteins, that can be specifically labelled with biotin, e.g. via a biotinylated antibody.

The invention thus provides the mutant streptavidin of the invention conjugated to a therapeutic agent and optionally conjugated to a fluorescent label for use in therapy. The invention also provides the mutant streptavidin of the invention conjugated to a fluorescent label for use in diagnosis. More particularly, the invention provides the mutant streptavidin of the invention conjugated to a therapeutic agent and optionally conjugated to a fluorescent label for treating and/or monitoring the treatment of cancer. The invention further provides the mutant streptavidin of the invention conjugated to a fluorescent label for diagnosing cancer. As discussed above, the mutant streptavidin in these aspects is used to target a therapeutic agent to a biotinylated cell or to detect a biotinylated cell or antigen.

Alternatively viewed, the invention provides a method for treating and/or monitoring the treatment of cancer comprising administering a mutant streptavidin of the invention conjugated to a therapeutic agent and optionally conjugated to a fluorescent label to a subject, wherein said subject has been pretreated to label cancerous cells with biotin. As discussed previously, the therapeutic agent may be a radioisotope.

Additionally the invention provides a method for diagnosing cancer comprising:

(i) obtaining a sample from a subject;

(ii) contacting said sample with a biotinylated entity capable of binding specifically to a cell or antigen associated with cancer under conditions that enable said biotinylated entity to bind to said cell or antigen;

(iii) simultaneously or sequentially contacting the sample with a mutant streptavidin of the invention conjugated to a fluorescent label under conditions that enable said mutant streptavidin to bind to said biotinylated entity;

(iv) optionally washing said sample to remove unbound mutant streptavidin and

(v) detecting a signal from the fluorescent label conjugated to said mutant streptavidin, wherein the detection of a signal is indicative of the presence of a cell or antigen associated with cancer (i.e. that the subject has cancer).

Alternatively viewed, the invention provides a method for diagnosing cancer comprising:

(i) administering a biotinylated entity capable of binding specifically to a cell or antigen associated with cancer to a subject;

(ii) simultaneously or sequentially administering a mutant streptavidin of the invention conjugated to a fluorescent label to said subject;

(iii) optionally waiting for unbound mutant streptavidin to clear, e.g. from the area of the subject in which cancer is suspected to be present; and

(iv) detecting a signal from the fluorescent label conjugated to said mutant streptavidin (e.g. by *in vivo* microscopy), wherein the detection of a signal is indicative of the presence of a cell or antigen associated with cancer (i.e. that the subject has cancer).

The invention will now be described in more detail in the following non-limiting Examples with reference to the drawings in which:

Figure 1 shows (A) Biotin-4-fluorescein dissociation rate from unlabelled wild-type streptavidin (WT) or after labelling with 635P-NHS (WT-dye), determined from the increase in fluorescence after excess free biotin is added; and (B) Biotin-4-fluorescein association rate to wild-type streptavidin (WT) or after labelling with 635P-NHS (WT-dye), determined from the decrease in fluorescence upon mixing. Error bars mean of triplicate ± 1 s.d.

Figure 2 shows (A) Tetramer integrity of K121 streptavidin mutants compared to wild-type streptavidin (WT), analysed by SDS-PAGE ± boiling and then Coomassie staining. (B) Biotin-4-fluorescein dissociation rates for K121 mutants compared to wild-type streptavidin (WT). (C) Biotin-4-fluorescein dissociation rates for K121 R with or without 635P-NHS labelling compared to wild-type streptavidin (WT) (mean of triplicate ± 1 s.d.). (D) Biotin-4-fluorescein

association rates for K121 R with or without 635P-NHS labeling. Error bars mean of triplicate ± 1 s.d.

Figure 3 shows (A) Amine composition of the streptavidin mutation series, 1 amine: K80R, K121R, K132R, K134R ("flavidin"), 2 amines: K121R, K132R, K134R, 3 amines: K80R, K121 R, 4 amines: K121 R, 5 amines: wild-type, 6 amines: N82K, R103K, K121R. (B) Tetramer integrity of amine series, analysed by SDS-PAGE ± boiling and then Coomassie staining. (C) Absorption spectra of amine series after labelling with 635P-NHS. (D) Fluorescent brightness of amine mutation series conjugated with 635P-NHS (mean of triplicate ± 1 s.d.).

Figure 4 shows (A) Flavidin thermostability after heating for 3 min at the indicated temperature and then analysis of tetramer integrity by SDS-PAGE with Coomassie staining. C is the control boiled in SDS. (B) Biotin-4-fluorescein dissociation rates for Flavidin with or without 635P-NHS labelling. (C) Biotin-4-fluorescein association rates for Flavidin with or without 635P-NHS labelling. Error bars are mean of triplicate ± 1 s.d.

Figure 5 shows (A) Biotin-4-fluorescein dissociation rates for wild-type streptavidin unmodified or after conjugation with Atto647N-NHS or Atto590-NHS. (B) Biotin-4-fluorescein dissociation rates for Flavidin unmodified or after conjugation with Atto647N-NHS or Atto590-NHS. Error bars are mean of triplicate ± 1 s.d.

Figure 6 shows (A) Absorption spectra and (B) Fluorescent brightness of Alexa Fluor 647-labelled amine mutation series. Error bars are mean of triplicate ± 1 s.d.

Figure 7 shows (A) Biotin-4-fluorescein off-rate at 37 °C for wild-type core streptavidin (WT) or WT labelled with 635P dye at the indicated dye:protein ratio. (B) Fluorescent brightness of WT-dye samples with varying ratios of 635P conjugated, as in (A). Error bars are mean of triplicate ± 1 s.d.

Figure 8 shows (A) Absorption spectra and (B) Fluorescent brightness of sulfo-Cy3-NHS labelled amine mutation series.

Figure 9 shows (A) Fluorescence signals from HeLa cells incubated with varying concentrations of biotinylated affibody against EGFR and then labelled with wild-type core streptavidin (WT-635P, left panel) or Flavidin-635P (right panel), detected by flow cytometry; (B) photomicrographs of spleen sections with AlexaFluor 488-conjugated anti-CD45R antibody (shown in dark grey) and biotinylated anti-CD3 antibody followed by WT-635P or Flavidin-635P (shown in

light grey). The bottom row represents the inset from the top row, showing only the 635P signal; and (C) photomicrographs of spleen sections with WT-sulfoCy3 (dark grey) or Flavidin-sulfoCy3 (light grey). Scale bars are 100 μηι (top row) or 20 μηι (bottom row).

Examples

Materials and Methods

Cloning

PCR was performed using KOD Hot Start DNA Polymerase (Merck Chemicals). Gibson Assembly Master Mix (New England BioLabs) was used according to the manufacturer's instructions. Constructs were initially cloned into chemically competent *Escherichia coli* DH5a cells. All mutants were verified by sequencing of the entire gene. pET21 core streptavidin encoding a C-terminal hexaglutamate tag (SAe, "WT" or "5-amine") (Fairhead et al., 2014a) was used as the starting template for new streptavidins .

pET21 SAe-K121R ("K121R" or "4-amine") (Addgene deposition in progress) was generated by QuikChange site-directed mutagenesis on SAe using primer 5'-GCTAACGCGTGGCGATCCACCCTGGTTGG (SEQ ID NO: 13) and its reverse complement.

pET21 SAe-K121Q was generated by QuikChange on SAe using primer 5'-GCTAACGCGTGGCAATCCACCCTGGTTGG (SEQ ID NO: 14) and its reverse complement.

pET21 SAe-K121E was generated by QuikChange on SAe using primer 5'-GCTAACGCGTGGGAATCCACCCTGGTTGG (SEQ ID NO: 15) and its reverse complement.

pET21 SAe-K121A was generated by QuikChange on SAe using primer 5'-GCTAACGCGTGGGCATCCACCCTGGTTGG (SEQ ID NO: 16) and its reverse complement.

pET21 SAe-N82K-R103K-K121 R- ("6-amine") was generated in two steps. First N82K was introduced into SAe-K121 R by Gibson assembly of two PCR products: (product 1) PCR with primers 5'-CCGTTGCTTGGAAAAACAAATACCGTAACGCTCACTCCGCTACCACC (SEQ ID NO: 17) and 5'- GATCGTTGTCAGAAGTAAGTTGGCC (SEQ ID NO: 18) (AmpB), (product 2) PCR with primers 5'- GTTTTTCCAAGCAACGGTCCAACC (SEQ ID NO: 19) and 5'-GGCCAACTTACTTCTGACAACGATC (SEQ ID NO: 20) (AmpA). In

the second step, R103Kwas introduced into pET21 SAe-N82K-K121R- by Gibson assembly of two PCR products: (product 1) PCR with primers 5'-CAGTACGTTGGTGGTGCTGAAGCTAAAATCAACACCCAGTGGTTGTTGACC (SEQ ID NO: 21) and AmpB, (product 2) PCR with primers 5'-ACCACCAACGTACTGGCCAGACC (SEQ ID NO: 22) and AmpA.

pET21 SAe-K80R-K121 R ("3-amine") was introduced into SAe-K121 R by Gibson assembly of two PCR products: (product 1) PCR with primers 5'-TCTGGGTTGGACCGTTGCTTGGCGCAACAACTACCGTAACGCTCAC (SEQ ID NO: 23) and AmpB, (product 2) PCR with primers 5'-CCAAGCAACGGTCCAACCCAGAG (SEQ ID NO: 24) and AmpA.

pET21 SAe-K121 R-K132R-K134R ("2-amine") was introduced into SAe-K121R template by Gibson assembly of two PCR products: (product 1) PCR with 5'- GGTCACGACACCTTCACCCGTGTTCGTCCGTCCGCTGCTTCCG (SEQ ID NO: 25) and AmpB, (product 2) PCR with 5'- GGTGAAGGTGTCGTGACCAACC (SEQ ID NO: 26) and AmpA.

pET21 SAe-K80R-K121 R-K132R-K134R ("1-amine", Flavidin) (Addgene deposition in progress) was introduced into SAe-K121R-K132R-K134R template by Gibson assembly using the two reactions described above for generating pET21 SAe-K80R-K121 R.

Protein expression

Overnight cultures of streptavidin variants were grown at 37 °C and 220 rpm in 10 mL LB, with 100 μg/mL ampicillin and 0.8% glucose, after picking a freshly-transformed colony of *E. coli* BL21 DE3 RIPL cells (Agilent). The overnight culture was then diluted to 1 L and grown at 37 °C and 200 rpm to OD_{600} 0.8-1 .0. Expression was induced with 0.4 mM IPTG. The culture was incubated for another 4 h at 37 °C. The cell pellet was dissolved in 15 mL 100 mM Tris pH 8.0 and frozen at -80 °C. After thawing, 15 μL 100 mg/mL lysozyme was added and the tube was rocked at 25 °C for 45 min. 750 μL 10% v/v Triton X-100 was added and the pellet was again frozen at -80 °C. After thawing in a 25 °C water bath, 15 mL milliQ water was added and vortexed for 30 s. This solution was then thrice sonicated on ice for 30 s. The cell lysate was centrifuged at 20,000 g for 20 min. The pellet was washed thrice with 25 mL milliQ water, dissolved in 10 mL 6 M guanidinium hydrochloride (pH 1.5) (GuHCl) and centrifuged at 20,000 g for 20 min. The supernatant was then added drop-by-drop to 200 mL fast-stirring PBS at 4 °C. After 16 h, folded

streptavidin was precipitated by slow addition of 120 g ammonium sulfate. The pellet was isolated by centrifugation at 20,000 g for 20 min and dissolved in 10 mL 20 mM Tris. The supernatant, containing streptavidin, was dialyzed overnight into PBS (three buffer exchanges). Concentrations of protein before dye modification were determined by absorbance at 280 nm.

SDS-PAGE and Thermostability Testing

SDS-PAGE was performed on 16% polyacrylamide gels using the XCell SureLock system (Life Technologies). Non-boiled samples were mixed with 6* SDS buffer (0.23 M Tris HCl pH 6.8, 24% v/v glycerol, 120 μM bromophenol blue, 0.23 M SDS) and directly loaded, while boiled samples were mixed with 6* SDS buffer, heated at 95 °C for 5 min, and then loaded. Gels were run at 190 V, with the gel box packed in ice to minimise denaturation of folded tetramers. Gels were stained with InstantBlue (Expedeon) and images were collected using a ChemiDoc XRS+ system with Image Lab v5.2.1 software (Bio-Rad).

To analyse tetramer stability, we incubated 4 μM streptavidin variant in PBS for 3 min at the specified temperature, followed by cooling to 10 °C, using the Bio-Rad C1000 Thermal Cycler. Samples were then mixed with 6* SDS buffer and promptly loaded on the gel.

Biotin-4-fluorescein Off-rate

Biotin-4-fluorescein (B4F) off-rate experiments were performed on a TECAN SpectraFluor Plus plate-reader, using 484 nm excitation and 535 nm emission. In clear 96-well plates, we added 10 μl 1 μM streptavidin variant in PBS to 170 μl 24 nM B4F in PBS, followed by incubation at 37 °C for 1 h. Excess biotin (20 μl 1 mM biotin in PBS) was added and fluorescence timepoints were immediately acquired at 37 °C.

Two controls were used for each experiment: quenched control and B4F-only control. In the quenched control, 10 μl 1 μM streptavidin variant in PBS was added to 170 μl of 24 nM B4F in PBS, followed by addition of 20 μl of PBS. In the B4F-only control, 10 μl PBS was added to 170 μl 24 nM B4F in PBS, followed by addition of 20 μl 1 mM biotin in PBS.

The percent B4F dissociation was calculated as: 100* [(streptavidin with B4F)-(quenched control)]/[(B4F-only control)-(quenched control)]. Data are means and standard deviations of three experiments.

Biotin-4-fluorescein On-rate

B4F on-rate experiments were performed on a PHERAstar RS plate reader using 485 nm excitation and 520 nm emission. In clear-bottom black-wall 96-well plates, 100 µl 500 pM streptavidin variant in PBS was added to 100 µl 100 pM B4F in PBS. Immediately, fluorescence readings were started.

For the streptavidin-only control, 100 µl 500 pM streptavidin variant in PBS was added to 100 µl PBS. In the B4F-only control, 100 µl of PBS was added to 100 µl 100 pM B4F in PBS. The concentration of free B4F was calculated as: [(streptavidin + B4F signal)-(streptavidin-only signal)]/[(B4F-only signal)-(streptavidin-only signal)]. Data are means and standard deviations of three experiments, with three replicates per experiment.

Fluorescent labelling

10 µl freshly-prepared 1 M $NaHCO_3$ pH 8.3 was added to 100 µl 10 µM streptavidin variant in PBS. Next, the streptavidin+ $NaHCO_3$ solution was added to 5 µl 10 mg/mL dye-NHS ester in dry DMSO.

The reaction was incubated at 25 °C with end-over-end rotation. After 4 h, the reaction was spun at 16,900 g for 5 min. The supernatant was applied to a PBS-washed 0.8 ml slurry of Sephadex G-25 resin. The first two 0.2 ml fractions were pooled and dialysed thrice in 3000 MWCO tubing into PBS at 4 °C. The labelled protein was then spun at 16,900 g for 5 min to remove any aggregates. At all steps, the sample was covered by aluminium foil.

Absorbance and fluorescence measurements

Fluorescent brightness of labelled streptavidin variants was measured in clear-bottom black-wall 96-well plates on a SpectraMax M3 (Molecular Devices) plate reader. For 635P, excitation of 620 nm, filter cut-off of 630 nm, and emission of 660 nm were used. For AlexaFluor 647, excitation of 650 nm, filter cut-off of 665 nm, and emission of 675 nm were used. Absorbance spectra were acquired on an ND-1000 Spectrophotometer (Thermo Scientific) using software version 3.8.1. Absorbance spectra and fluorescent brightness were normalized to the concentration of the protein from bicinchoninic acid (BCA) assay, using Pierce Microplate BCA Protein Assay Kit (Thermo Scientific) on a SpectraMax M3 plate

reader. BCA values were corrected for any contribution of dye absorbance by subtracting A_{562} of BCA-untreated controls.

Cell culture and flow cytometry

HeLa CCL-2 cells were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C with 5% C02 in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) supplemented with 10% v/v Fetal Bovine Serum, 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma-Aldrich).

Cells were trypsinized with 0.05% w/v Trypsin-EDTA (Thermo Fisher Scientific), washed with FACS buffer (PBS with 1% w/v bovine serum albumin and 0.1% w/v sodium azide) and hereafter maintained at 4 °C. Cells (500,000/well) were incubated for 20 min with a biotinylated affibody against EGFR in FACS buffer at 0, 3, 15, 60 or 300 nM. Cells were then washed twice with FACS buffer. Next, cells were treated with 5 nM WT-635P or Flavidin-635P (dye: protein monomer ratio of 12:1) in FACS buffer for 20 min. Cells were then washed thrice with FACS buffer. Flow cytometry was performed using a MACSQuant Analyzer 10 (Miltenyi Biotech), with excitation at 635 nm and emission at 655-730 nm. The data were analysed using FlowJo v10 (Tree Star Inc.) and the signal was normalized to the mode cell count for each cell sample.

Immunofluorescence

The spleen from a 12 week-old wild-type C57BL/6 mouse was embedded in optimal cutting temperature compound (O.C.T, Tissue Tec®, Sakura Finetek), and frozen in liquid nitrogen. Tissue sections (10 μηι) were cut using a CM3050S cryostat (Leica), dried for 30 min at 22 °C, and stored at -20 °C until use. Sections were fixed in PBS with 2% w/v formaldehyde (Sigma-Aldrich) for 4 min at 22 °C and all further steps were performed at this temperature. Cells in the tissue sections were permeabilized using PBS with 0.1% v/v Triton X-100 (Sigma-Aldrich) for 4 min. The tissue sections were then treated with avidin/biotin blocking kit (Vector laboratories, SP-2001) according to the manufacturer's protocol, followed by PBS + 0.5% w/v bovine serum albumin (Sigma-Aldrich) for 15 min. The sections were then incubated with biotinylated anti-CD3 antibody (1:50 dilution, clone 17A2, BioLegend) and AlexaFluor 488-conjugated anti-CD45R/B220 antibody (1:50 dilution, clone RA3-6B2, BioLegend) in PBS + 0.5% w/v bovine serum albumin for 1 h, followed by 635P- or sulfoCy3-labelled Flavidin or WT streptavidin (0.094 μM,

dye:protein monomer ratio of 12:1 for 635P and 17:1 for sulfoCy3) in PBS + 0.1% w/v bovine serum albumin for 30 min, with finally 4',6-diamidino-2-phenylindole (DAPI, 2 μg/mL, Sigma-Aldrich) in PBS for 2 min. Between all steps, the sections were washed thrice with PBS. Slides were mounted using ProLong Gold mounting media (Thermo Fisher Scientific). Images were acquired with a MetaSystems automated slide scanner equipped with a Zeiss Axiolmager.Z2 epifluorescence microscope using a Plan-Apochromat 40*/1.4 oil objective lens (Carl Zeiss Microscopy). For sulfoCy3, the excitation interval was 546/10 nm, the emission interval was 575/15 nm, and the exposure time was 33.2 ms. For 635P, the excitation interval was 581/10 nm, the emission interval was 640/30 nm, and the exposure time was 400 ms. The images were extracted from MetaSystems image viewer program VSViewer into Adobe Photoshop.

Example 1: Dye labelling of wild-type streptavidin via free amine groups impaired ligand binding

We used biotin-4-fluorescein as an efficient read-out of ligand binding to streptavidin. Biotin-4-fluorescein fluorescence is quenched by -90% upon streptavidin binding. Biotin-4-fluorescein's dissociation, induced by adding excess free biotin, can be continuously monitored from the recovery of biotin-4-fluorescein's fluorescence upon dissociation from streptavidin's binding pocket. Wld-type streptavidin was labelled using Abberior STAR 635P-NHS. This dye was chosen because of its high solubility and excellent photophysical characteristics (extinction coefficient, quantum yield, photostability) and also because its absorption and emission are well separated from fluorescein. After dye-NHS incubation, we removed unreacted dye from streptavidin by gel filtration and three rounds of dialysis. Dye-labelling of wild-type (WT) streptavidin induced a dramatic increase in biotin-4-fluorescein's dissociation rate (Fig. 1A). We observed similarly dramatic increases in dissociation rate after labelling streptavidin using two other commercially-available dyes with good fluorescence characteristics (Atto647N-NHS and Atto590-NHS, Fig. 5A).

To study ligand on-rate, we mixed streptavidin with biotin-4-fluorescein and followed the rate of quenching of the fluorescence of biotin-4-fluorescein. Dye-labelling of streptavidin substantially reduced the association rate 10-fold (Fig. 1B).

Example 2: Intersubunit interaction at K121 is the key to dye's effects on ligand binding

Based on the crystal structure of streptavidin, the amine with the closest proximity to the biotin binding site comes from K121 of the neighbouring subunit, at the 1,2 subunit interface. It is well established that W120 from the neighbouring subunit contributes an important interaction for biotin binding at this 1,2 interface. Therefore, we created a series of mutations at K121, which removed Lys's ε-amine, and expressed these proteins in *E. coli.* Sequence alignment of related avidin-family tetramers showed a strong preference for Lys at position 121, with Glu, Asn, Gin, and Thr as possible replacements. We selected Arg, Glu, Gin, and Ala replacements. Each streptavidin mutant was well expressed, could be refolded from inclusion bodies, and formed a tetramer stable to SDS (Fig. 2A). Comparing biotin-conjugate dissociation for each tetramer (without dye labelling of the protein), we found that the K121Q and K121E mutants had substantially accelerated biotin-conjugate dissociation, whereas the K121 R and K121A mutants retained WT-like dissociation rate (Fig. 2B). Since K121R was the most conservative mutation, retained the same net charge, and maintained good biotin binding, this mutation was employed in the remainder of the study. After 635P-NHS labelling of K121 R streptavidin, there was no loss in stability of biotin-conjugate binding (Fig. 2C) and only a small effect on biotin-conjugate association (Fig. 2D).

Example 3: Mutation of every ε-amine in streptavidin

WT core streptavidin possesses 5 amines (a-amine and four ε-amines at Lys). Each one of the four native Lys was mutated to Arg. In addition, we introduced two further Lys via N82K and R103K mutations. N82 and R103 sites were chosen because of their high surface-accessibility and because they were not adjacent to the biotin binding site. Overall, we expressed streptavidin mutants ranging from 1 amine (just the a-amine) up to 6 amines per subunit (Fig. 3A). All variants were well expressed in *E. coli* and refolded efficiently from inclusion bodies to the expected tetramers (Fig. 3B). Upon boiling in SDS, each tetramer was converted to the monomer, as characterized by SDS-PAGE (Fig. 3B).

Example 4: Spectroscopic characterization of the amine-landscaping protein-dye series

The streptavidin series with 1-6 amines was labelled with 635P-NHS, normalised by protein concentration, and the absorption spectra were overlaid (Fig. 3C). This analysis showed a general correlation between the number of amines with the intensity of the left shoulder peak (590 nm), hypsochromic to the principal peak at 630 nm. This hypsochromic peak is considered a marker of H-type dimer dye-dye interactions. We then measured the fluorescence brightness of the labelled series (Fig. 3D). Surprisingly, the degree of brightness inversely correlated with the number of amines. That is, for dye labelling of streptavidin via amine groups, "less is more": fewer amines led to brighter signals. This effect on the absorption spectrum and "less is more" trend for fluorescent brightness was also observed after labelling the 1-6 amine series with other commonly-used fluorescent dyes, Alexa Fluor 647-NHS (Fig. 6) and sulfo-Cy3 (Fig. 8).

Since the mutant with 1 amine showed the best fluorescent characteristics (SAe-K80R-K121R-K132R-K134R), we termed this variant Flavidin (Fluorescent streptavidin) and validated its behaviour.

Example 5: Biophysical characterization of Flavidin

We characterized Flavidin tetramer stability by heating at various temperatures followed by analysis via SDS-PAGE. This analysis showed that flavidin retained high thermostability similar to WT streptavidin (Fig. 4A).

Testing biotin-conjugate dissociation, Flavidin showed low off-rate like WT streptavidin (Fig. 4B). 635P-NHS labelling of Flavidin did not accelerate biotin-conjugate off-rate (Fig. 4B). Flavidin showed an on-rate for biotin-4-fluorescein comparable to WT streptavidin (Fig. 4C). 635P-NHS labelling of Flavidin caused an approximately two-fold decrease in biotin-conjugate association rate (Fig. 4C). Flavidin also showed low biotin-conjugate dissociation after labelling with Atto647N-NHS and Atto590-NHS (Fig 5B). Overall, Flavidin enabled dye-NHS labelling with maximal brightness along with minimal impairment of ligand binding kinetics.

Example 6: Effect of dye:protein ratio for labelling wild-type core streptavidin

To further validate the unexpected results described above, the inventors assessed the effect of reducing the number of dye molecules per protein on fluorescent brightness using a conventional approach. The wild-type core

streptavidin protein was labelled with 635P-NHS using different ratios of protein to dye, as shown in Figure 7.

Even the lowest ratio of dye:protein did not avoid damage to biotin binding, i.e. the off-rate was increased by dye labelling (Fig. 7A). Moreover, none of the ratios tested enhanced fluorescence brightness (Fig. 7B). These results indicate that the targeted substitution of lysine residues is required to enhance fluorescent brightness and advantageously does not impair biotin binding.

Example 7: Use of Flavidin in cellular contexts

Flavidin was tested to show that it allowed labelling with good specificity in a cellular context. The human HeLa cell-line was stained with a biotinylated affibody specific to the Epidermal Growth Factor Receptor (EGFR). The affibody was detected using flow cytometry with 635P-labeled WT streptavidin or Flavidin. Background staining with WT-635P (geometric mean fluorescence 0.80) or Flavidin-635P (geometric mean 0.76) was low and comparable for both proteins (Figure 9A), which indicated that Flavidin had no effect on non-specific binding.

For understanding how Flavidin would perform on cells bearing low levels of biotinylated target, HeLa cells were incubated with a range of affibody concentrations. At the lowest affibody concentration of 3 nM, the signal with WT-635P substantially overlapped with the background signal (geometric mean 1.7), whereas for Flavidin-635P nearly all cells were clearly resolved from the background signal (geometric mean 5.1) (Figure 9A). A substantially enhanced signal was observed with Flavidin compared to WT at all affibody concentrations. These observations confirmed that the brightness enhancement with these dyes was retained in a cellular context.

The use of Flavidin for immunohistochemistry of primary tissue samples was also evaluated. Consecutive tissue sections from mouse spleen were fixed with formaldehyde, permeabilized, and blocked. Next, the sections were incubated with a biotinylated anti-CD3 antibody (for labelling T-cell populations), as well as an AlexaFluor 488-conjugated anti-CD45R/B220 antibody (for B-cell populations), followed by staining with either Flavidin or WT streptavidin labelled with either 635P or sulfoCy3. In the case of 635P-labelled samples, it was found that the Flavidin panel showed substantially brighter staining of T cells (Figure 9B). SulfoCy3-labeled samples, both WT and Flavidin, gave intense T cell staining (Figure 9C). However, it was observed that the WT-sulfoCy3 showed undesired non-specific binding, as

shown by staining of B cells and diffuse T cell staining. In contrast, it was found that Flavidin-sulfoCy3 gave well-defined and specific staining of T-cells (Figure 9C). Thus, as well as the brightness benefit, Flavidin enhanced the overall quality of cell staining.

Sequences:

SEQ ID NO. 1 (full length streptavidin subunit)

MRKIWAAIAVSLTTVSITASASADPSKDSKAQVSAAEAGITGTWYNQLGST FIVTAGADGALTGTYESAVGNAESRYVLTGRYDSAPATDGSGTALGWTVAWKNN YRNAHSATTWSGQYVGGAEARINTQWLLTSGTTEANAWKSTLVGHDTFTKVKPS AAS IDAA KKAGVN NGN PLDAVQQ

SEQ ID NO. 2 (mature streptavidin subunit)

DPSKDSKAQVSAAEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNA ESRYVLTGRYDSAPATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEAR INTQWLLTSGTTEANAWKSTLVGHDTFTKVKPSAASIDAAKKAGVNNGNPLDAVQ Q

SEQ ID NO. 3 (core streptavidin subunit)

AEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWKSTLVGH DTFTKVKPSAAS

SEQ ID NO. 4 (one amine version of core sequence, known as "flavidin")

AEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWRNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGHDTFTRVRPSAAS

SEQ ID NO. 5 (two amine version of core streptavidin subunit)

AEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGHDTFTRVRPSAAS

SEQ ID NO. 6 (three amine version of core streptavidin subunit)

AEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWRNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGH DTFTKVKPSAAS

SEQ ID NO. 7 (flavidin core sequence + Dead streptavidin mutations)

AEAGITGTWYAQLGDTFIVTAGADGALTGTYEAAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWRNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGHDTFTRVRPSAAS

SEQ ID NO. 8 (flavidin core sequence + traptavidin mutations)

AEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAEGDYVLTGRYDS APATDGSGTALGWTVAWRNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGHDTFTRVRPSAAS

SEQ ID NO. 9 (peptide linker)

GSGSGG

SEQ ID NO. 10 (SpyTag)

AHIVMVDAYRPT

SEQ ID NO. 11 (flavidin core sequence + Dead streptavidin mutations + SpyTag)

AEAGITGTWYAQLGDTFIVTAGADGALTGTYEAAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWRNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGHDTFTRVRPSAASGSGSGGAHIVMVDAYRPT

SEQ ID NO. 12 (flavidin core sequence + SpyTag)

AEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDS APATDGSGTALGWTVAWRNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTT EANAWRSTLVGHDTFTRVRPSAASGSGSGGAHIVMVDAYRPT

Claims

1. A mutant streptavidin subunit which comprises at least two amino acid mutations compared to a wild-type streptavidin subunit, wherein said mutations comprise:

(a) a substitution of the lysine residue at the position equivalent to position 121 of SEQ ID NO. 2; and

(b) a substitution of at least one of the lysine residues at positions equivalent to positions of 80 and 132 of SEQ ID NO. 2 and/or a substitution or deletion of the lysine residue at the position equivalent to position of 134 of SEQ ID NO. 2,

wherein the mutant streptavidin subunit is conjugated to a fluorescent label via reaction with one or more amine groups and

wherein a streptavidin protein comprising four of said subunits has a higher fluorescent brightness than a streptavidin protein comprising four wild-type streptavidin subunits in which the fluorescent label is conjugated via the amino groups.

2. The mutant streptavidin subunit of claim 1, wherein the mutant comprises substitutions at positions 121 and 80.

3. The mutant streptavidin subunit of claim 1, wherein the mutant comprises substitutions at positions 121 and 132.

4. The mutant streptavidin subunit of claim 1, wherein the mutant comprises substitutions at positions 121 and 134 or a substitution at position 121 and deletion at position 134.

5. The mutant streptavidin subunit of claim 1, wherein the mutant comprises a substitution at position 121 and substitutions at two of positions 80, 132 and 134.

6. The mutant streptavidin subunit of claim 1, wherein the mutant comprises a first substitution at position 121, a second substitution at one of positions 80 and 132, and a deletion at position 134.

7. The mutant streptavidin subunit of any one of claims 1 to 6, wherein the mutant comprises substitutions at positions 80, 121, 132 and 134 or substitutions at positions 80, 121 and 132 and a deletion at position 134.

8. The mutant streptavidin subunit of any one of claims 1 to 7, wherein the substituting amino acid at position 80 is a polar and/or charged amino acid or alanine (A), preferably selected from any one of R, N, D, E, A, H, Q, S or T.

9. The mutant streptavidin subunit of any one of claims 1 to 8, wherein the substituting amino acid at position 132 is a polar and/or charged amino acid or A, preferably selected from any one of R, N, D, E, A, H, Q, S or T.

10. The mutant streptavidin subunit of any one of claims 1 to 9, wherein the substituting amino acid at position 134 is a polar and/or charged amino acid or A, preferably selected from any one of R, N, D, E, A, H, Q, S or T.

11. The mutant streptavidin subunit of any one of claims 1 to 10, wherein the substituting amino acid at position 121 is a non-polar, basic or neutral amino acid other than glycine, preferably selected from any one of R, H, A, I, L, V, W, Y, P and F.

12. The mutant streptavidin subunit of any one of claims 1 to 11, wherein at least one of positions 80, 132 and 134 is substituted with R or A.

13. The mutant streptavidin subunit of any one of claims 1 to 12, wherein position 121 is substituted with R or A.

14. The mutant streptavidin subunit of any one of claims 1 to 13, wherein position 121 is substituted with R.

15. The mutant streptavidin subunit of any one of claims 1 to 14, wherein positions 80, 121 and 132 and 134 are all substituted with R.

16. The mutant streptavidin subunit of any one of claims 1 to 15, wherein the mutant streptavidin subunit comprises an amino acid sequence with at least 70% sequence identity to one of SEQ ID NOs. 4-8, 11 or 12, wherein the amino acid residues at positions equivalent to positions 80, 121, 132 and 134 of SEQ ID NO. 2 are as set forth in one of SEQ ID NOs. 4, 5 or 6, preferably wherein the amino acid residues at positions equivalent to positions 80, 121, 132 and 134 of SEQ ID NO. 2 are as set forth in SEQ ID NO. 4.

17. The mutant streptavidin subunit of any one of claims 1 to 16, wherein a streptavidin protein comprising four of said mutant streptavidin subunits has an off rate for biotin or for a biotin conjugate that is equivalent to, or lower than, a streptavidin protein comprising four wild-type streptavidin subunits under the same conditions.

18. The mutant streptavidin subunit of any one of claims 1 to 17, wherein said fluorescent label comprises a reactive or functional group or linker that is able to react with a-amino and ε-amino groups to form an amide bond.

19. The mutant streptavidin subunit of claim 18, wherein said reactive or functional group or linker is selected from N-hydroxysuccinimide (NHS), sulfo-N-

hydroxysuccinimide (sulfo-NHS), Tetrafluorophenyl ester (TFP), sulfo-tetrafluorophenyl ester (SFP) and pentafluorophenyl ester (PFP).

20. The mutant streptavidin subunit of any one of claims 1 to 16, 18 or 19, wherein the mutant also comprises a substitution at position 23 from N to A, a substitution at position 27 from S to D and a substitution at position 45 from S to A, wherein the positions are equivalent to the positions of SEQ ID NO. 2, preferably wherein the mutant comprises an amino acid sequence as set forth in SEQ ID NO. 7.

21. The mutant streptavidin subunit of any one of claims 1 to 19, wherein the mutant also comprises a substitution at position 52 from S to G and a substitution at position 53 from R to D, wherein the positions are equivalent to the positions of SEQ ID NO. 2, preferably wherein the mutant comprises an amino acid sequence as set forth in SEQ ID NO. 8.

22. The mutant streptavidin subunit of any one of claims 1 to 21, wherein the mutant further comprises a peptide tag comprising an amino acid sequence as set forth in SEQ ID NO. 10, preferably wherein the mutant comprises an amino acid sequence as set forth in SEQ ID NO. 11 or 12.

23. A mutant streptavidin subunit which comprises at least two amino acid mutations compared to a wild-type streptavidin subunit, wherein said mutations comprise:

(a) a substitution of the lysine residue at the position equivalent to position 121 of SEQ ID NO. 2 with arginine; and

(b) a substitution of at least one of the lysine residues at positions equivalent to positions of 80 and 132 of SEQ ID NO. 2 and/or a substitution or deletion of the lysine residue at the position equivalent to position of 134 of SEQ ID NO. 2,

wherein a streptavidin protein comprising four of said subunits each conjugated to a fluorescent label via the amino groups has a higher fluorescent brightness than a streptavidin protein comprising four wild-type streptavidin subunits in which the fluorescent label is conjugated via the amino groups.

24. The mutant streptavidin subunit of claim 23, wherein the mutant is as defined in any one of claims 2 to 10, 12, 15 to 17 or 20 to 22.

25. The mutant streptavidin subunit of claim 23 or 24, wherein the mutant is conjugated to a nucleic acid molecule, protein, peptide, organic compound, inorganic compound, polysaccharide, other soluble reagent, solid-phase or any combination thereof.

26. The mutant streptavidin subunit of any one of claims 23 to 25, wherein the mutant is conjugated to a nucleic acid molecule, protein, peptide, organic compound, inorganic compound, polysaccharide, other soluble reagent, solid-phase or any combination thereof via a reaction with one or more amine groups.

27. The mutant streptavidin subunit of claim 26, wherein said nucleic acid molecule, protein, peptide, organic compound, inorganic compound, polysaccharide, other soluble reagent, solid-phase or any combination thereof comprises a reactive or functional group or linker that is able to react with a-amino and ε-amino groups to form an amide bond.

28. The mutant streptavidin subunit of claim 27, wherein said reactive or functional group or linker is selected from N-hydroxysuccinimide (NHS), sulfo-N-hydroxysuccinimide (sulfo-NHS), Tetrafluorophenyl ester (TFP), sulfo-tetrafluorophenyl ester (SFP) and pentafluorophenyl ester (PFP).

29. A mutant streptavidin protein comprising at least one mutant streptavidin subunit of any one of claims 1 to 28.

30. The mutant streptavidin protein of claim 29 which comprises four mutant streptavidin subunits of any one of claims 1 to 19, 21 or 22 to 28, preferably wherein at least one of said mutant streptavidin subunits is conjugated to a fluorescent label as defined in claims 18 or 19.

31. A mutant streptavidin subunit of any one of claims 1 to 19, 21 or 22 to 28, wherein said subunit is in monomeric form.

32. A nucleic acid molecule comprising a nucleotide sequence which encodes the mutant streptavidin subunit of any one of claims 1 to 28.

33. A vector comprising the nucleic acid molecule of claim 32.

34. A cell comprising the nucleic acid of claim 32 or the vector of claim 33.

35. A process for producing the mutant streptavidin subunit of any one of claims 1 to 28 comprising the steps of:

a) transforming or transfecting a host cell with a vector which comprises a nucleotide sequence encoding the mutant streptavidin subunit;

b) culturing the host cell under conditions which allow the expression of the streptavidin mutant subunit; and

c) isolating the mutant streptavidin subunit.

36. A kit comprising the mutant streptavidin subunit of any one of claims 1 to 28, the mutant streptavidin protein of claim 29 or 30, the mutant streptavidin in

monomeric form of claim 31, the nucleic acid molecule of claim 32, the vector of claim 33 and/or the cell of claim 34.

37. A method of labelling a biotinylated entity comprising the step of contacting said biotinylated entity with a fluorescently labelled mutant streptavidin of claim 29 or 30 under conditions that enable said mutant streptavidin to bind to said biotinylated entity.

Figure 1

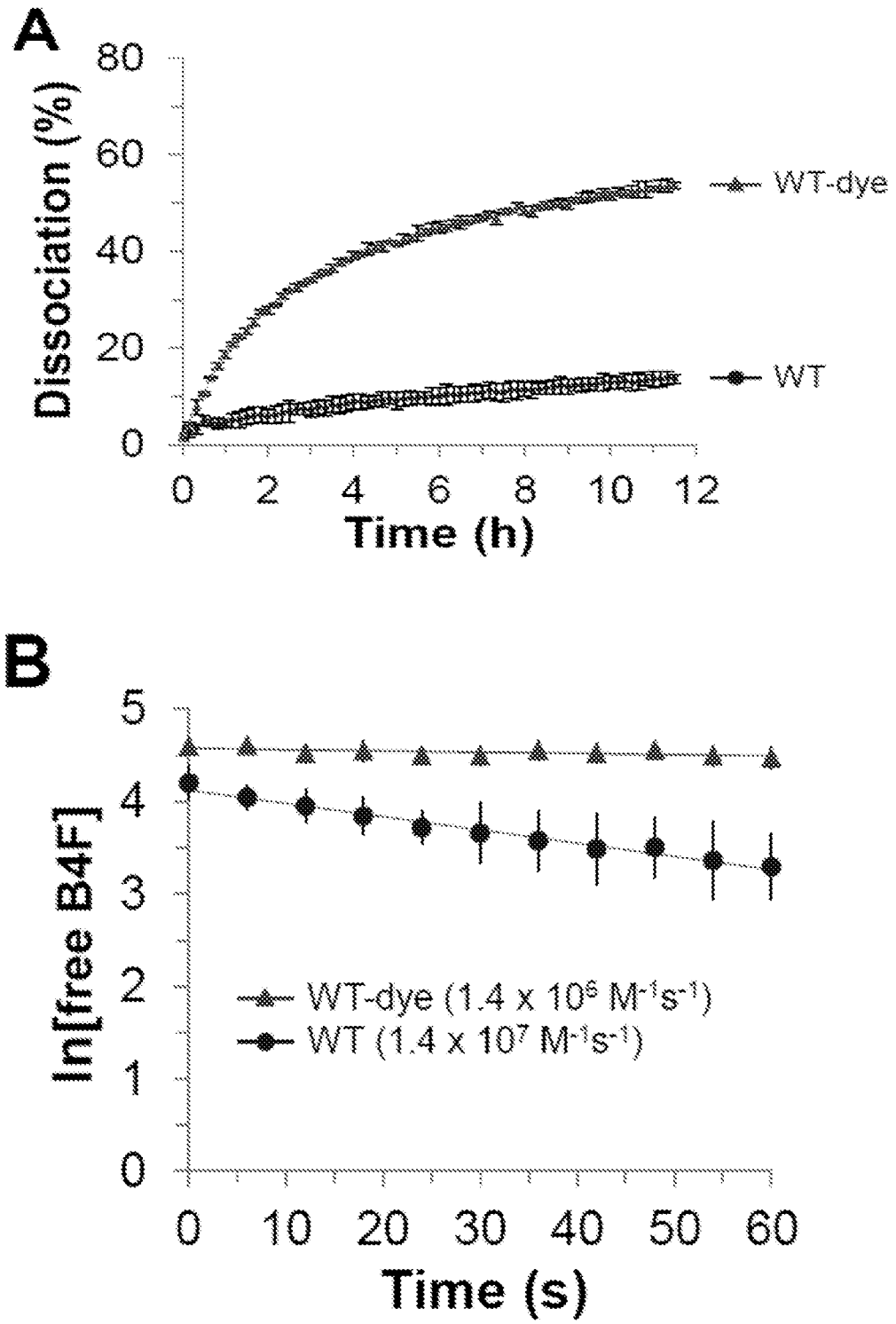

A
Dissociation (%)
80
60
40
20
0
0
2
4
6
8
10
12
Time (h)
WT-dye
WT
B
ln[free B4F]
5
4
3
2
1
0
0
10
20
30
40
50
60
Time (s)
WT-dye (1.4 x 10⁶ M⁻¹s⁻¹)
WT (1.4 x 10⁷ M⁻¹s⁻¹)

Figure 2

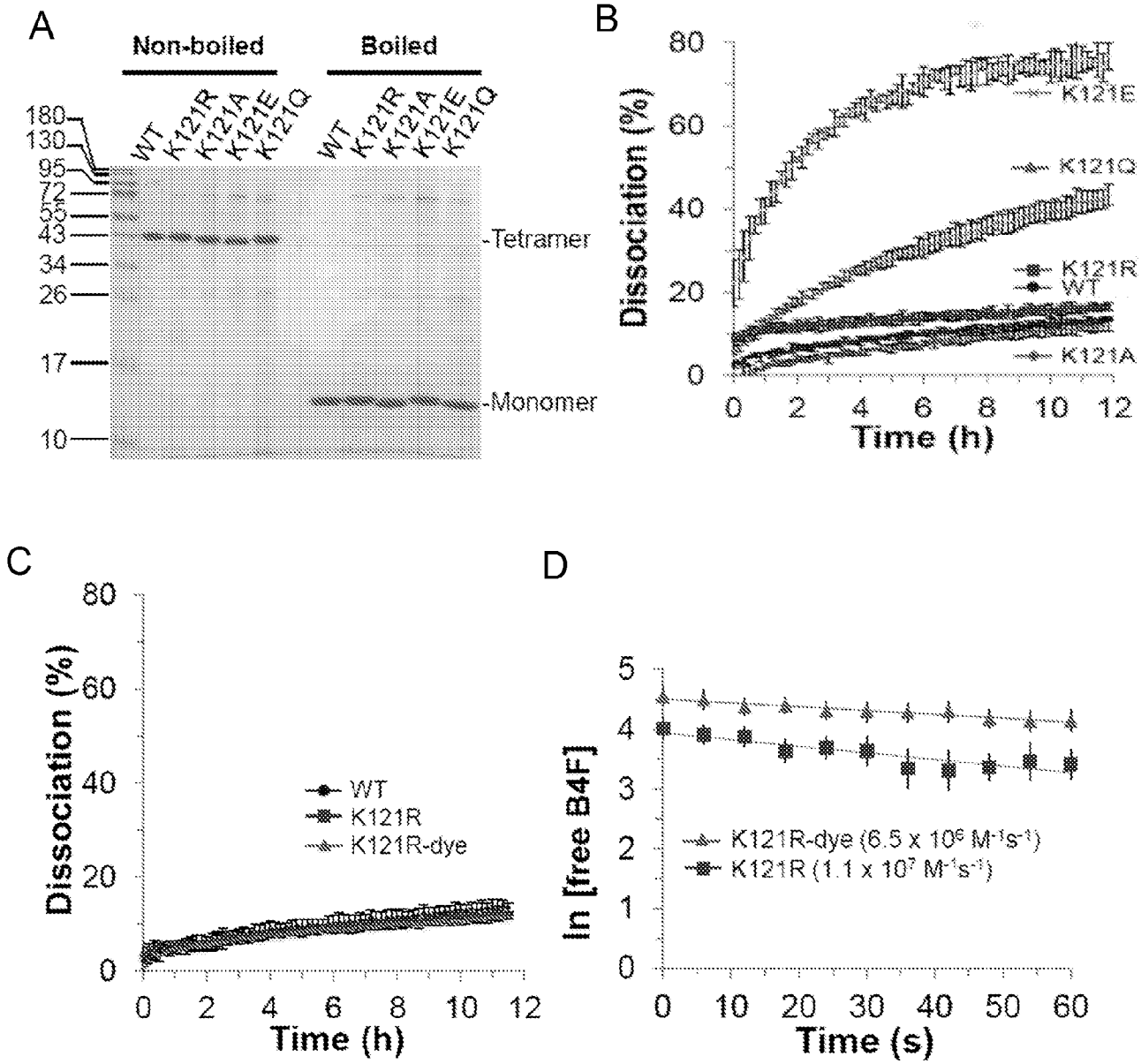
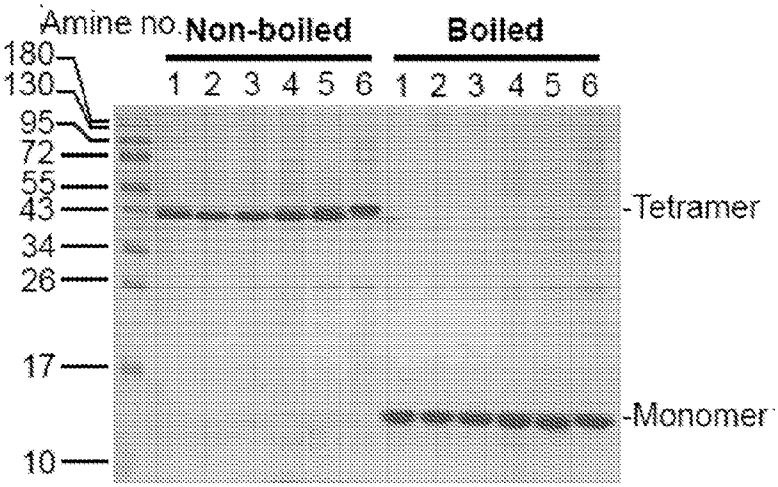

A
Non-boiled
Boiled
WT
K121R
K121A
K121E
K121Q
180
130
95
72
55
43
34
26
17
10
-Tetramer
-Monomer
B
Dissociation (%)
Time (h)
K121E
K121Q
K121R
WT
K121A
C
Dissociation (%)
Time (h)
WT
K121R
K121R-dye
D
ln [free B4F]
Time (s)
K121R-dye (6.5 x 10⁶ M⁻¹s⁻¹)
K121R (1.1 x 10⁷ M⁻¹s⁻¹)

Figure 3

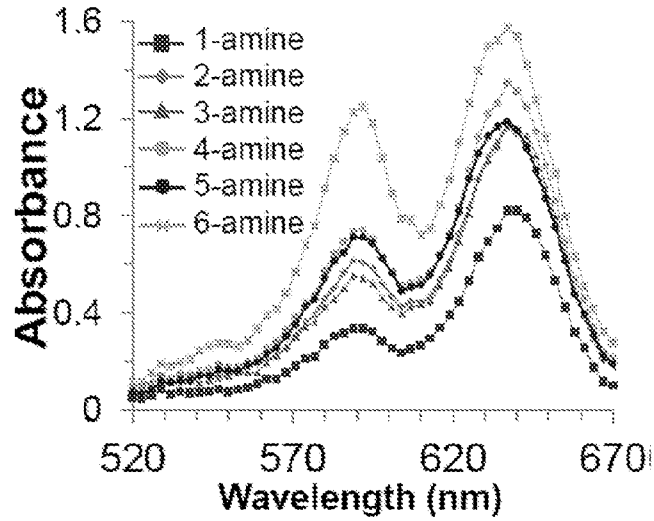
A

Amine position	Number of amines in streptavidin variant					
	1	2	3	4	5	6
α	√	√	√	√	√	√
ε-K80		√		√	√	√
ε-K121					√	
ε-K132			√	√	√	√
ε-K134			√	√	√	√
ε-N82K						√
ε-R103K						√

B



C



D

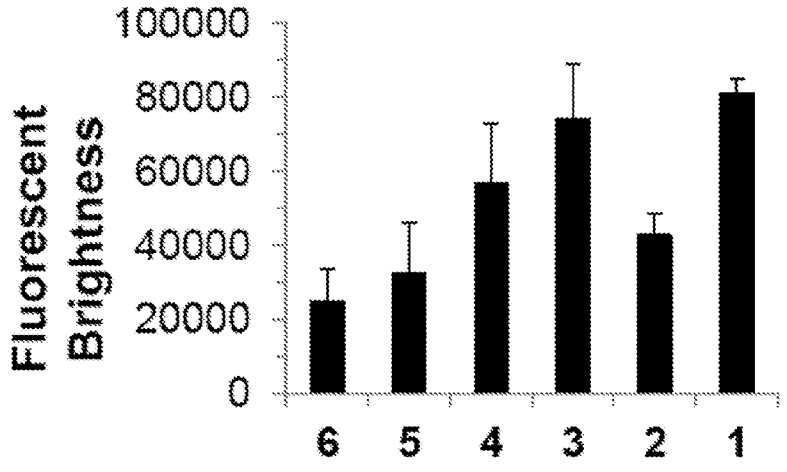


Figure 4

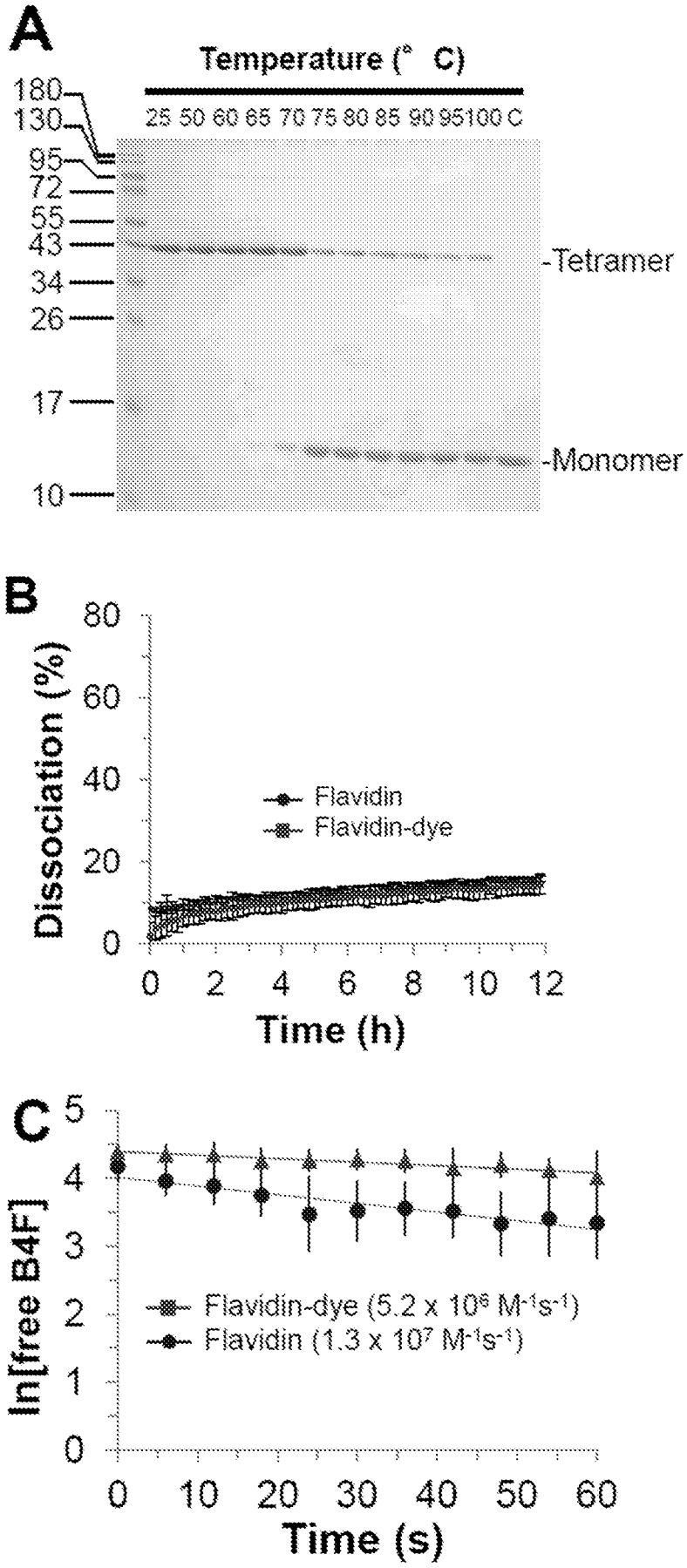

A
Temperature (° C)
25 50 60 65 70 75 80 85 90 95100 C
180
130
95
72
55
43
34
26
17
10
-Tetramer
-Monomer
B
Dissociation (%)
80
60
40
20
0
Flavidin
Flavidin-dye
0 2 4 6 8 10 12
Time (h)
C
ln[free B4F]
5
4
3
2
1
0
Flavidin-dye (5.2 x 10⁶ M⁻¹s⁻¹)
Flavidin (1.3 x 10⁷ M⁻¹s⁻¹)
0 10 20 30 40 50 60
Time (s)

Figure 5

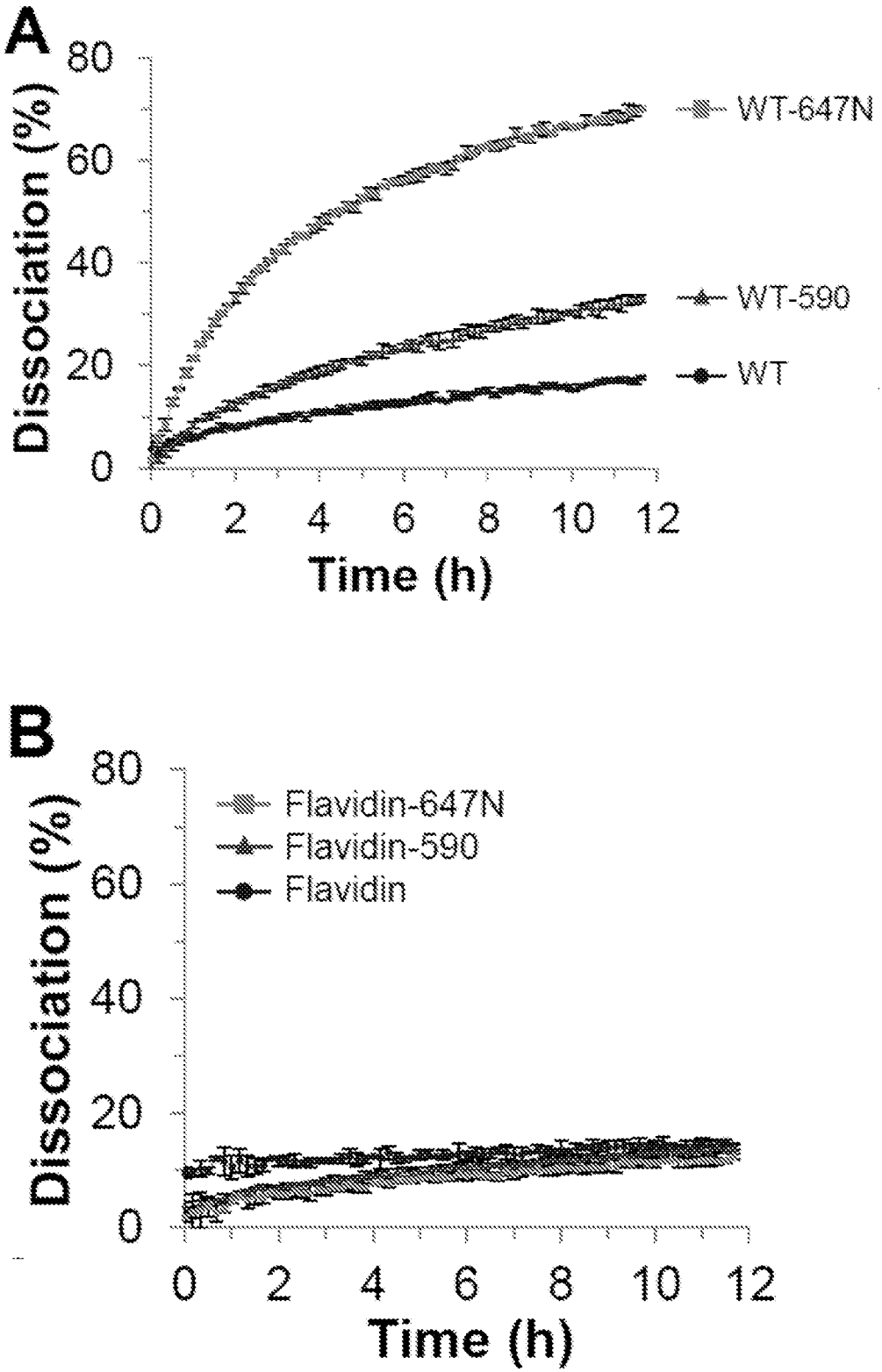

A
Dissociation (%)
80
60
40
20
0
0 2 4 6 8 10 12
Time (h)
WT-647N
WT-590
WT
B
Dissociation (%)
80
60
40
20
0
0 2 4 6 8 10 12
Time (h)
Flavidin-647N
Flavidin-590
Flavidin

Figure 6

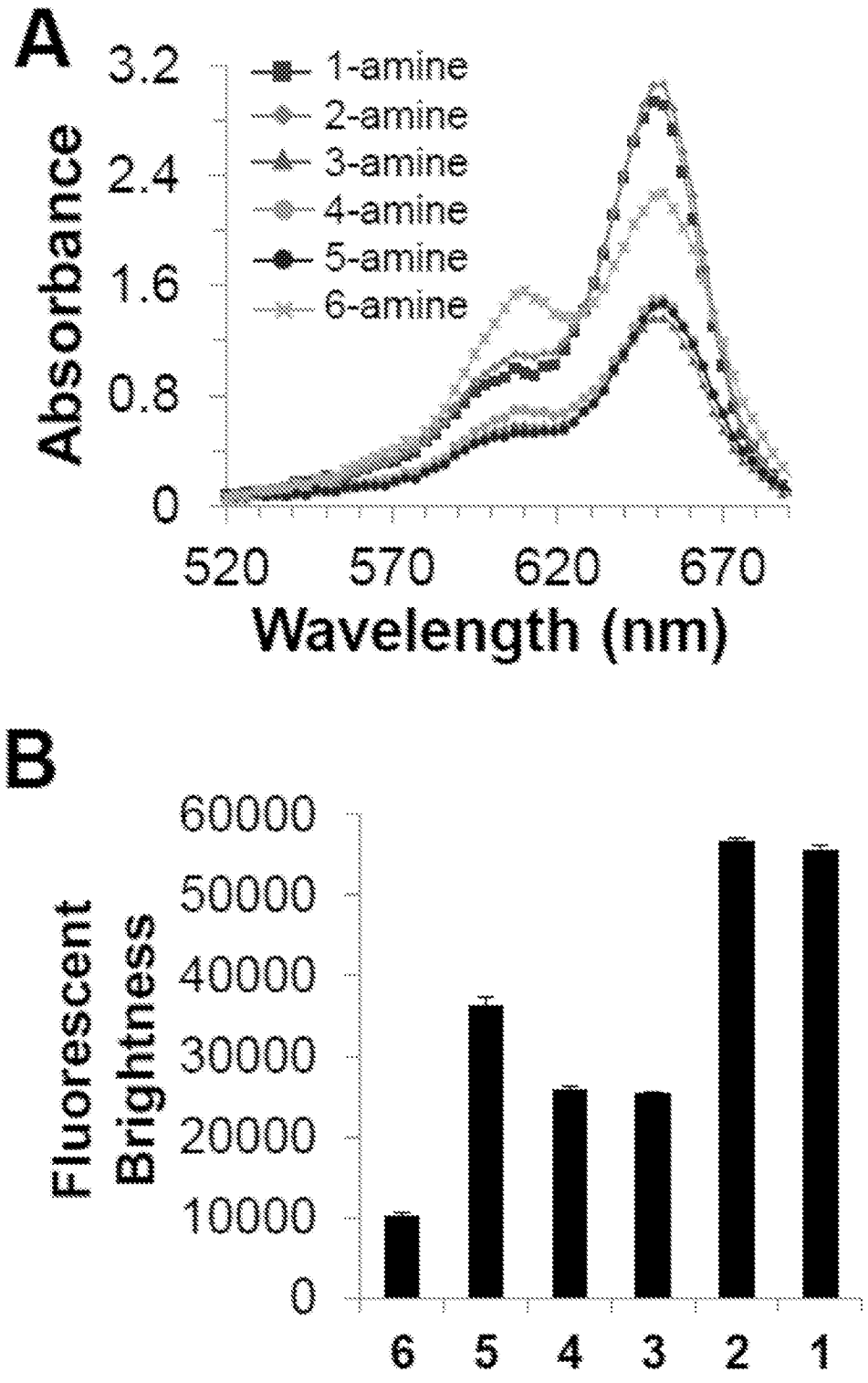
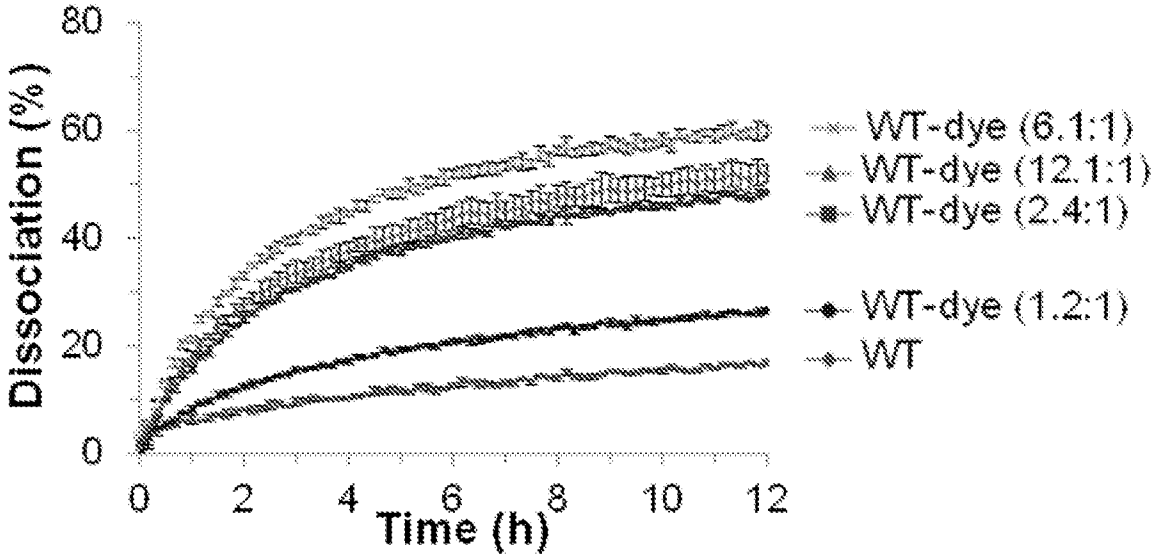

A
Absorbance
3.2
2.4
1.6
0.8
0
1-amine
2-amine
3-amine
4-amine
5-amine
6-amine
520
570
620
670
Wavelength (nm)
B
Fluorescent Brightness
60000
50000
40000
30000
20000
10000
0
6
5
4
3
2
1

Figure 7

A


Dissociation (%)
80
60
40
20
0
0
2
4
6
8
10
12
Time (h)
WT-dye (6.1:1)
WT-dye (12.1:1)
WT-dye (2.4:1)
WT-dye (1.2:1)
WT

B

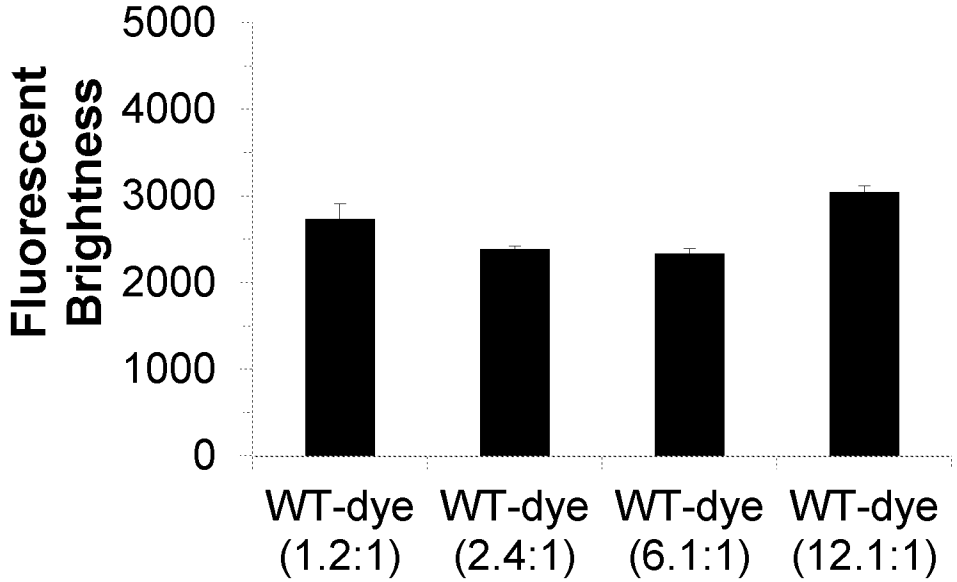
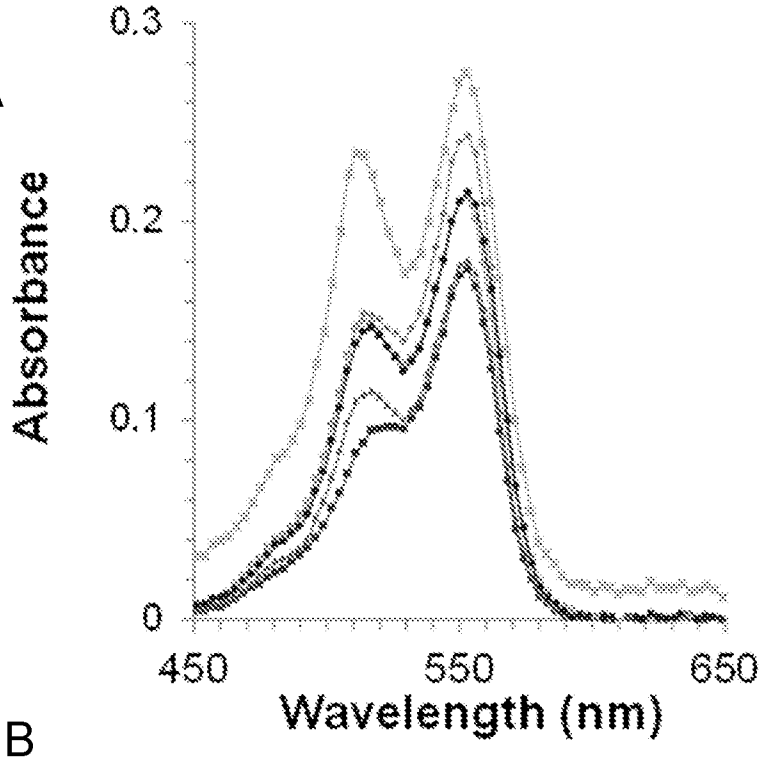

Fluorescent Brightness
5000
4000
3000
2000
1000
0
WT-dye (1.2:1)
WT-dye (2.4:1)
WT-dye (6.1:1)
WT-dye (12.1:1)

Figure 8

A


0.3
0.2
0.1
0
Absorbance
450
550
650
Wavelength (nm)

B

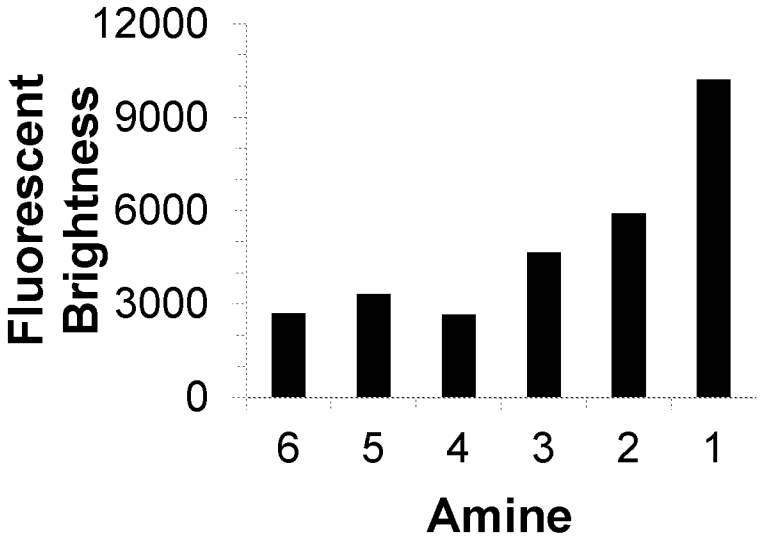
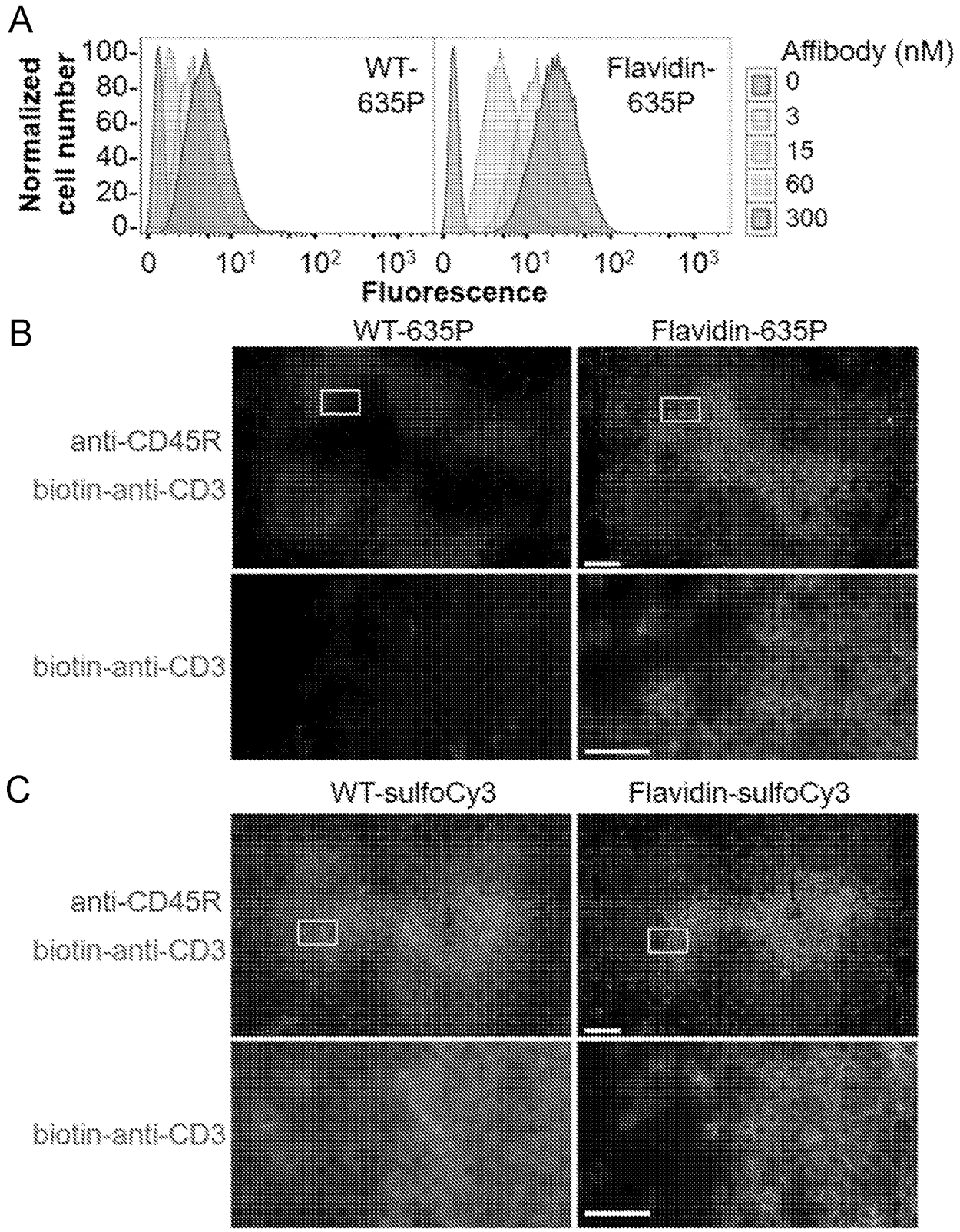

12000
9000
6000
3000
0
Fluorescent Brightness
6
5
4
3
2
1
Amine

Figure 9


A
Normalized cell number
100
80
60
40
20
0
WT-635P
Flavidin-635P
Affibody (nM)
0
3
15
60
300
0
10¹
10²
10³
Fluorescence
B
WT-635P
Flavidin-635P
anti-CD45R
biotin-anti-CD3
biotin-anti-CD3
C
WT-sulfoCy3
Flavidin-sulfoCy3
anti-CD45R
biotin-anti-CD3
biotin-anti-CD3

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/052799

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/36
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)
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, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 2010 063373 A (TOKYO INST TECH) 25 March 2010 (2010-03-25) SEQ ID NO:13 -----	1-13,16, 29,31-36
X	CN 101 899 117 A (WENZHOU MEDICAL COLLEGE) 1 December 2010 (2010-12-01) claims -----	1,2, 31-36
Y	WO 2013/038272 A2 (UTI LIMITED PARTNERSHIP [CA]; WONG SUI-LAM [CA]; WU SAU-CHING [CA]; BA) 21 March 2013 (2013-03-21) claims -----	1-37
Y	WO 2004/018509 A1 (JYVASKYLAEN YLIOPISTO [FI]; KULOMAA MARKUU SAKARI [FI]; NORDLUND HENRI) 4 March 2004 (2004-03-04) claims -----	1-37

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 October 2017	10/11/2017

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

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/052799

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2010063373 A	25-03-2010	NONE	
CN 101899117 A	01-12-2010	NONE	
WO 2013038272 A2	21-03-2013	US 2014378657 A1	25-12-2014
		WO 2013038272 A2	21-03-2013
WO 2004018509 A1	04-03-2004	AT 377024 T	15-11-2007
		AU 2003262585 A1	11-03-2004
		DE 60317220 T2	14-08-2008
		EP 1546195 A1	29-06-2005
		US 2006281899 A1	14-12-2006
		WO 2004018509 A1	04-03-2004