ACTIVATION OF BIOLUMINESCENCE BY STRUCTURAL COMPLEMENTATION

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. Provisional Patent Application Serial No. 61/791,549 filed March 15, 2013, which is hereby incorporated by reference in its entirety.

FIELD

Provided herein are compositions and methods for the assembly of a bioluminescent complex from two or more non-luminescent (e.g., substantially non-luminescent) peptide and/or polypeptide units. In particular, bioluminescent activity is conferred upon a non-luminescent polypeptide via structural complementation with another, complementary non-luminescent peptide.

BACKGROUND

Biological processes rely on covalent and non-covalent interactions between molecules, macromolecules and molecular complexes. In order to understand such processes, and to develop techniques and compounds to manipulate them for research, clinical and other practical applications, it is necessary to have tools available to detect and monitor these interactions. The study of these interactions, particularly under physiological conditions (e.g. at normal expression levels for monitoring protein interactions), requires high sensitivity.

SUMMARY

The present disclosure relates to compositions comprising complementary non-luminescent amino acid chains (e.g., substantially non-luminescent peptides and/or polypeptides that are not fragments of a preexisting protein), complexes thereof, and methods of generating an optically detectable bioluminescent signal upon association of the non-luminescent amino acid chains (e.g., peptides and/or polypeptides). In some embodiments, the present disclosure provides two or more non-luminescent, or substantially non-luminescent peptides and/or polypeptides, that, when brought together, assemble into a bioluminescent complex. In some embodiments, a pair of substantially non-luminescent peptide and/or polypeptide units assembles into a bioluminescent complex. In other embodiments, three or more substantially non-luminescent peptide and/or polypeptide units assemble into a bioluminescent complex (e.g., ternary complex, tertiary complex, etc.). Provided herein are technologies for detecting interactions between molecular entities (e.g., proteins, nucleic acids, carbohydrates, small molecules (e.g., small

molecule libraries)) by correlating such interactions to the formation of a bioluminescent complex of otherwise non-luminescent (e.g., substantially non-luminescent) amino acid chains.

In one aspect, the present disclosure provides a polypeptide comprising an amino acid sequence having less than 100% and greater than 90% sequence identity with SEQ ID NO: 440, wherein a bioluminescent signal produced in the presence of a peptide consisting of SEQ ID NO: 2 and a furmiazine substrate is enhanced relative to the polypeptide and the furmiazine substrate in the absence of the peptide consisting of SEQ ID NO: 2.

In another aspect, the present disclosure provides a nucleic acid comprising a sequence coding for a polypeptide as disclosed herein.

In a further aspect, the present disclosure provides a fusion polypeptide comprising the polypeptide as disclosed herein.

In another aspect, the present disclosure provides a bioluminescent complex comprising:

(a) the fusion polypeptide as disclosed herein; and

(b) a second fusion polypeptide comprising a complement peptide comprising an amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 2; wherein a detectable bioluminescent signal is produced by the bioluminescent complex in the presence of a furimazine substrate.

In some embodiments, the present disclosure provides a bioluminescent complex comprising:

(a) a peptide comprising a peptide amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 2; and

(b) a polypeptide comprising a polypeptide amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 440, wherein the bioluminescent complex exhibits detectable luminescence.

In some embodiments, the present disclosure provides a bioluminescent complex comprising:

(a) a first amino acid sequence that is not a fragment of a preexisting protein; and

(b) a second amino acid sequence that is not a fragment of a preexisting protein, wherein the bioluminescent complex exhibits detectable luminescence, wherein the first amino acid sequence and the second amino acid sequence are associated, and wherein the bioluminescent complex emits a detectable bioluminescent signal in the presence of a substrate when the first amino acid sequence and the second amino acid sequence are associated.

In some embodiments, the present disclosure provides a bioluminescent complex comprising:

(a) a non-luminescent pair, wherein each non-luminescent element of the non-luminescent pair is not a fragment of a preexisting protein;

(b) an interaction pair, wherein each interaction element of the interaction pair is covalently attached to one of the non-luminescent elements of the non-luminescent pair.

In some embodiments, the present disclosure provides a method of detecting a stable interaction between a first amino acid sequence and a second amino acid sequence comprising:

(a) attaching the first amino acid sequence to a third amino acid sequence, and attaching the second amino acid sequence to a fourth amino acid sequence, wherein the third and fourth amino acid sequences are not fragments of a preexisting protein, wherein a stable complex of the third and fourth amino acid sequences emits a detectable bioluminescent signal in the presence of a substrate, wherein the non-covalent interactions between the third and fourth amino acid sequences are insufficient to form a complex of the third and fourth amino acid sequences in the absence of additional stabilizing and/or aggregating forces, and wherein a stable interaction between the first amino acid sequence and the second amino acid sequence provides the additional stabilizing and/or aggregating forces to produce a stable complex of the third and fourth amino acid sequences;

(b) placing the first, second, third, and fourth amino acid sequences of step (a) in conditions to allow for stable interactions between the first amino acid sequence and the second amino acid sequence to occur; and

(c) detecting the bioluminescent signal emitted, in the presence of a substrate, by the stable complex of the third and fourth amino acid sequences in the presence of a substrate, wherein detection of the bioluminescent signal indicates a stable interaction between the first amino acid sequence and the second amino acid sequence.

In some embodiments, the present disclosure provides a method of optimizing a non-luminescent pair comprising:

(a) aligning the sequences of three or more related proteins;

(b) determining a consensus sequence for the related proteins;

(c) providing first and second fragments of a protein related to three or more proteins, wherein the fragments are individually substantially non-luminescent but exhibit luminescence upon stable interaction of the fragments;

(d) mutating the first and second fragments at one or more positions each, wherein said mutations alter the sequences of the fragments to be more similar to a corresponding portion of the consensus sequence, wherein the mutating results in a non-luminescent pair that are not fragments of a preexisting protein,

(e) testing the non-luminescent pair for the absence of luminescence when unassociated and luminescence upon stable association of the non-luminescent pair.

In some embodiments, the present disclosure provides a detection reagent comprising: (a) a polypeptide comprising an amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 440, wherein a detectable bioluminescent signal is produced, in the presence of a substrate, when the polypeptide contacts a peptide consisting of SEQ ID NO: 2, and (b) a substrate for a bioluminescent complex produced by said polypeptide and a peptide consisting of SEQ ID NO: 2.

In some embodiments, the assembled pair catalyzes a chemical reaction of an appropriate substrate into a high energy state, and light is emitted. In some embodiments, a bioluminescent complex exhibits luminescence in the presence of substrate (e.g., coelenterazine, furimazine, etc.).

Although the embodiments described herein primarily describe and refer to complementary, non-luminescent amino acid chains that form bioluminescent complexes, it is noted that the present technology can equally be applied to other detectable attributes (e.g., other enzymatic activities, generation of a fluorophore, generation of a chromophore, etc.). The embodiments described herein relating to luminescence should be viewed as applying to complementary, substantially non-enzymatically active amino acid chains (e.g., peptides and/or polypeptides that are not fragments of a preexisting protein) that separately lack a specified detectable activity (e.g., enzymatic activity) or substantially non-enzymatically active subunits of a polypeptide, complexes thereof, and methods of generating the detectable activity (e.g., an enzymatic activity) upon association of the complementary, substantially non-enzymatically active amino acid chains (e.g., peptides and/or polypeptides). Further, embodiments described herein that refer to non-luminescent peptides and/or polypeptides are applied, in some embodiments, to substantially non-luminescent peptides and/or polypeptides.

The present disclosure is further directed to assays for the detection of molecular interactions between molecules of interest by linking the interaction of a pair of non-luminescent peptides/polypeptides to the interaction molecules of interest (e.g., transient association, stable association, complex formation, etc.). In such embodiments, a pair of a non-luminescent elements are tethered (e.g., fused) to molecules of interest and assembly of the bioluminescent complex is operated by the molecular interaction of the molecules of interest. If the molecules of interest engage in a sufficiently stable interaction, the bioluminescent complex forms, and a bioluminescent signal is generated. If the molecules of interest fail to engage in a sufficiently stable interaction, the bioluminescent complex will not form or only form weakly, and a bioluminescent signal is not detectable or is substantially reduced (e.g., substantially

undetectable, essentially not detectable, etc.). In some embodiments, the magnitude of the detectable bioluminescent signal is proportional (e.g., directly proportional) to the amount, strength, favorability, and/or stability of the molecular interactions between the molecules of interest.

In some embodiments, the present disclosure provides peptides comprising an amino acid sequence having less than 100% (e.g., 20%... 30%... 40%... 50%... 60%... 70%... 80%, or more)

sequence identity with SEQ ID NO: 2, wherein a detectable bioluminescent signal is produced when the peptide contacts a polypeptide consisting of SEQ ID NO: 440. In some embodiments, the present disclosure provides peptides comprising an amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 2, wherein a detectable bioluminescent signal is produced when the peptide contacts a polypeptide consisting of SEQ ID NO: 440. In some embodiments, a detectable bioluminescent signal is produced when the peptide contacts a polypeptide having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 440. In certain embodiments, the detectable bioluminescent signal is produced, or is substantially increased, when the peptide associates with the polypeptide comprising or consisting of SEQ ID NO: 440, or a portion thereof. In preferred embodiments, the peptide exhibits alteration (e.g., enhancement) of one or more traits compared to a peptide of SEQ ID NO: 2, wherein the traits are selected from: affinity for the polypeptide consisting of SEQ ID NO: 440, expression, intracellular solubility, intracellular stability and bioluminescent activity when combined with the polypeptide consisting of SEQ ID NO: 440. Although not limited to these sequences, the peptide amino acid sequence may be selected from amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365. In some embodiments, fusion polypeptides are provided that comprise: (a) an above described peptide, and (b) a first interaction polypeptide that forms a complex with a second interaction polypeptide upon contact of the first interaction polypeptide and the second interaction polypeptide. In certain embodiments, bioluminescent complexes are provided that comprise: (a) a first fusion polypeptide described above and (b) a second fusion polypeptide comprising: (i) the second interaction polypeptide and (ii) a complement polypeptide that emits a detectable bioluminescent signal when associated with the peptide comprising an amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 2; wherein the first fusion polypeptide and second fusion polypeptide are associated; and wherein the peptide comprising an amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 2 and the complement polypeptide are associated.

In some embodiments, the present disclosure provides polypeptides comprising an amino acid sequence having less than 100% sequence identity with SEQ ID NO: 440, wherein a detectable bioluminescent signal is produced when the polypeptide contacts a peptide consisting of SEQ ID NO: 2. In some embodiments, the present disclosure provides polypeptides comprising an amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity

with SEQ ID NO: 440, wherein a detectable bioluminescent signal is produced when the polypeptide contacts a peptide consisting of SEQ ID NO: 2. In some embodiments, a detectable bioluminescent signal is produced when the polypeptide contacts a peptide having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 2. In some embodiments, the polypeptide exhibits alteration (e.g., enhancement) of one or more traits compared to a peptide of SEQ ID NO: 440, wherein the traits are selected from: affinity for the peptide consisting of SEQ ID NO: 2, expression, intracellular solubility, intracellular stability, and bioluminescent activity when combined with the peptide consisting of SEQ ID NO: 2. Although not limited to such sequences, the polypeptide amino acid sequence may be selected from one of the amino acid sequences of SEQ ID NOS: 441-2156. In some embodiments, the detectable bioluminescent signal is produced when the polypeptide associates with the peptide consisting of SEQ ID NO: 2. In some embodiments, a fusion polypeptide is provided that comprises: (a) a polypeptide described above and (b) a first interaction polypeptide that forms a complex with a second interaction polypeptide upon contact of the first interaction polypeptide and the second interaction polypeptide. In certain embodiments, a bioluminescent complex is provided that comprises: (a) a first fusion polypeptide described above; and (b) a second fusion polypeptide comprising: (i) the second interaction polypeptide and (ii) a complement peptide that causes the polypeptide comprising an amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 440 to emit a detectable bioluminescent signal when an association is formed between the two; wherein the first fusion polypeptide and second fusion polypeptide are associated; and wherein the polypeptide comprising an amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 440 and the complement peptide are associated.

In some embodiments, the present disclosure provides nucleic acids (e.g., DNA, RNA, etc.), oligonucleotides, vectors, etc., that code for any of the peptides, polypeptides, fusion proteins, etc., described herein. In some embodiments, a nucleic acid comprising or consisting of one of the nucleic acid sequences of SEQ ID NOS: 3-438 and 2162-2365 (coding for non-luminescent peptides) and/or SEQ ID NOS 441-2156 (coding for non-luminescent polypeptides) are provided. In some embodiments, other nucleic acid sequences coding for amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365 and/or SEQ ID NOS 441-2156 are provided.

In certain embodiments, the present disclosure provides bioluminescent complexes comprising: (a) a peptide comprising a peptide amino acid sequence having less than 100%

sequence identity (e.g., >99%, <95%, <90%, <80%, <70%, <60%, <50%, etc.) with SEQ ID NO: 2; and (b) a polypeptide comprising a polypeptide amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 440, wherein the bioluminescent complex exhibits detectable luminescence. In certain embodiments, the present disclosure provides bioluminescent complexes comprising: (a) a peptide comprising a peptide amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 2; and (b) a polypeptide comprising a polypeptide amino acid sequence having less than 100% and greater than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with SEQ ID NO: 440, wherein the bioluminescent complex exhibits detectable luminescence. Although not limited to particular sequences, in some embodiments, the peptide amino acid sequence is selected from one of the amino acid sequences provided in SEQ ID NOS: 3-438 and 2162-2365.

In various embodiments, bioluminescent complexes are provided that comprise: (a) a first amino acid sequence that is not a fragment of a preexisting protein; and (b) a second amino acid sequence that is not a fragment of a preexisting protein, wherein the bioluminescent complex exhibits detectable luminescence, wherein the first amino acid sequence and the second amino acid sequence are associated. Some such bioluminescent complexes further comprise: (c) a third amino acid sequence comprising a first member of an interaction pair, wherein the third amino acid sequence is covalently attached to the first amino acid sequence; and (d) a fourth amino acid sequence comprising a second member of an interaction pair, wherein the fourth amino acid sequence is covalently attached to the second amino acid sequence. In certain embodiments, interactions (e.g., non-covalent interactions (e.g., hydrogen bonds, ionic bonds, van der Waals forces, hydrophobic interactions, etc.) covalent interactions (e.g., disulfide bonds), etc.) between the first amino acid sequence and the second amino acid sequence do not significantly associate the first amino acid sequence and the second amino acid sequence in the absence of the interactions between the first member and the second member of the interaction pair. In some embodiments, a first polypeptide chain comprises the first amino acid sequence and the third amino acid sequence, and wherein a second polypeptide chain comprises the second amino acid sequence and the fourth amino acid sequence. In some embodiments, the first polypeptide chain and the second polypeptide chain are expressed within a cell.

In some embodiments, the present disclosure provides a bioluminescent complex comprising: (a) a pair of non-luminescent elements, wherein each non-luminescent element is not

a fragment of a preexisting protein; (b) an interaction pair, wherein each interaction element of the interaction pair is covalently attached to one of the non-luminescent elements.

Various embodiments described herein provide methods of detecting an interaction between a first amino acid sequence and a second amino acid sequence comprising, for example, the steps of: (a) attaching the first amino acid sequence to a third amino acid sequence and attaching the second amino acid sequence to a fourth amino acid sequence, wherein the third and fourth amino acid sequences are not fragments of a preexisting protein, wherein a complex of the third and fourth amino acid sequences emits a detectable bioluminescent signal (e.g., substantially increased bioluminescence relative to the polypeptide chains separately), wherein the interactions (e.g., non-covalent) between the third and fourth amino acid sequences are insufficient to form, or only weakly form, a complex of the third and fourth amino acid sequences in the absence of additional stabilizing and/or aggregating conditions, and wherein a interaction between the first amino acid sequence and the second amino acid sequence provides the additional stabilizing and/or aggregating forces to produce a complex of the third and fourth amino acid sequences; (b) placing the first, second, third, and fourth amino acid sequences of step (a) in conditions to allow for interactions between the first amino acid sequence and the second amino acid sequence to occur; and (c) detecting the bioluminescent signal emitted by the complex of the third and fourth amino acid sequences, wherein detection of the bioluminescent signal indicates an interaction between the first amino acid sequence and the second amino acid sequence. In some embodiments, attaching the first amino acid sequence to the third amino acid sequence and the second amino acid sequence to the fourth amino acid sequence comprises forming a first fusion protein comprising the first amino acid sequence and the third amino acid sequence and forming a second fusion protein comprising the second amino acid sequence and the fourth amino acid sequence. In some embodiments, the first fusion protein and the second fusion protein further comprise linkers between said first and third amino acid sequences and said second and fourth amino acid sequences, respectively. In certain embodiments, the first fusion protein is expressed from a first nucleic acid sequence coding for the first and third amino acid sequences, and the second fusion protein is expressed from a second nucleic acid sequence coding for the second and fourth amino acid sequences. In some embodiments, a single vector comprises the first nucleic acid sequence and the second nucleic acid sequence. In other embodiments, the first nucleic acid sequence and the second nucleic acid sequence are on separate vectors. In certain embodiments, the steps of (a) “attaching” and (b) “placing” comprise expressing the first and second fusion proteins within a cell.

Provided herein are methods of creating, producing, generating, and/or optimizing a pair of non-luminescent elements comprising: (a) aligning the sequences of three or more related

proteins; (b) determining a consensus sequence for the related proteins; (c) providing first and second fragments of a protein related to three or more proteins (or providing first and second fragments of one of the three or more proteins), wherein the fragments are individually substantially non-luminescent but exhibit luminescence upon interaction of the fragments; (d) mutating the first and second fragments at one or more positions each, wherein the mutations alter the sequences of the fragments to be more similar to a corresponding portion of the consensus sequence (e.g., wherein the mutating results in a pair of non-luminescent elements that are not fragments of a preexisting protein), and (e) testing the pair of non-luminescent elements for the absence (e.g., essential absence, substantial absence, etc.) of luminescence when unassociated, and luminescence upon association of the non-luminescent pair into a bioluminescent complex. Examples of such a process are described in Examples 1-5. In some embodiments, the non-luminescent elements exhibit enhancement of one or more traits compared to the first and second fragments, wherein the traits are selected from: increased reconstitution affinity, decreased reconstitution affinity, enhanced expression, increased intracellular solubility, increased intracellular stability, and increased intensity of reconstituted luminescence.

In some embodiments, the present disclosure provides detection reagents comprising: (a) a polypeptide comprising an amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 440, wherein a detectable bioluminescent signal is produced when the polypeptide contacts a peptide consisting of SEQ ID NO: 2, and (b) a substrate for a bioluminescent complex produced by the polypeptide and a peptide consisting of SEQ ID NO: 2. In some embodiments, the present disclosure provides detection reagents comprising: (a) a peptide comprising an amino acid sequence having less than 100% sequence identity with SEQ ID NO: 2, wherein a detectable bioluminescent signal is produced when the peptide contacts a polypeptide consisting of SEQ ID NO: 440, and (b) a substrate for a bioluminescent complex produced by the peptide and a polypeptide consisting of SEQ ID NO: 440. In some embodiments, the present disclosure provides detection reagents comprising: (a) a peptide comprising an amino acid sequence having less than 100% and greater than 40% sequence identity with SEQ ID NO: 2, wherein a detectable bioluminescent signal is produced when the peptide contacts a polypeptide consisting of SEQ ID NO: 440, and (b) a substrate for a bioluminescent complex produced by the peptide and a polypeptide consisting of SEQ ID NO: 440.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a graph depicting the effect of various mutations of the GVTGWRLCKRISA (SEQ ID NO: 236) peptide on luminescence resulting from complementation with SEQ ID NO: 440.

Figure 2 shows a graph depicting the effect of various mutations of the SEQ ID NO: 440 polypeptide on luminescence resulting from complementation with GVTGWRLCKRISA (SEQ ID NO: 236) or GVTGWRLFKRISA (SEQ ID NO: 108) peptides.

Figure 3A shows the luminescence (RLUs) detected in each non-luminescent polypeptide (NLpoly) mutant containing a single glycine to alanine substitution. Figure 3B shows the fold increase in luminescence over wild-type.

Figure 4A show the luminescence (RLUs) detected in each NLpoly mutant containing a composite of glycine to alanine substitutions. Figure 4B shows the fold increase in luminescence over wild-type.

Figure 5 shows a graph depicting the luminescence (RLUs) detected in HT-NLpeptide fusions.

Figure 6 shows a graph depicting the luminescence (RLUs) detected in HT-NLpep fusions.

Figure 7 shows a graph depicting the luminescence (RLUs) detected in NLpeptide-HT fusions.

Figure 8 shows the luminescence (RLUs) generated by a luminescent complex after freeze-thaw cycles of non-luminescent peptide (NLpep).

Figure 9 shows concentration normalized activity of peptides, and the TMR gel used to determine the relative concentrations.

Figure 10 shows a graph of the luminescence of various mutations of residue R11 of NLpoly-5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 11 shows a graph of the luminescence of various mutations of residue A15 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 12 shows a graph of the luminescence of various mutations of residue L18 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 13 shows a graph of the luminescence of various mutations of residue F31 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 14 shows a graph of the luminescence of various mutations of residue V58 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 15 shows a graph of the luminescence of various mutations of residue A67 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 16 shows a graph of the luminescence of various mutations of residue M106 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 17 shows a graph of the luminescence of various mutations of residue L149 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 18 shows a graph of the luminescence of various mutations of residue V157 of NLpoly 5A2 in the presence of NLpep53 (top) and in the absence of complimentary peptide (bottom).

Figure 19 shows a graph of the luminescence of NLpep-HT fusions.

Figure 20 shows a graph of the luminescence of NLpep-HT fusions, and a TMR gel indicating their relative expression levels.

Figure 21 shows a graph of the luminescence of NLpep-HT fusions.

Figure 22 shows a graph of the luminescence of NLpoly 5A2 (top) and NLpoly5A2+R11E in the presence of various NLpeps (bottom) .

Figure 23 shows a graph of the luminescence of NLpep-HT fusions.

Figure 24 shows a graph of the luminescence of NLpolys 1-13 with NLpep53 (top) and without complimentary peptide (bottom).

Figure 25 shows a graph of the luminescence of various NLpolys with NLpep53 with NANOGLO or DMEM buffer and furimazine or coelenterazine substrate.

Figure 26 shows a graph comparing luminescence in the presence of a ratio of furimazine with coelenterazine for various NLpolys and NLpep53.

Figure 27 shows a graph comparing luminescence in the presence of a ratio of furimazine to coelenterazine for various NLpolys and NLpep53.

Figure 28 shows a graph comparing luminescence in the presence of furimazine with coelenterazine for various NLpolys and NLpep53 in HEK293 cell lysate.

Figure 29 shows a graph of the luminescence of various combinations of NLpoly and NLpep pairs in DMEM buffer with furimazine.

Figure 30 shows a graph of the signal/background luminescence of various combinations of NLpoly and NLpep pairs in DMEM buffer with furimazine.

Figure 31 shows a graph of luminescence and substrate specificity of various NLpoly mutants with NLpep69 using either furimazine or coelenterazine as a substrate.

Figure 32 shows a comparison of luminescence and substrate specificity of various NLpoly mutants with NLpep69 using either furimazine or coelenterazine as a substrate, and under either lytic (bottom graph) or live cell (top graph) conditions.

Figure 33 shows a comparison of luminescence and substrate specificity of NLpoly mutants with NLpep78 using either furimazine or coelenterazine as a substrate, and under either lytic (bottom graph) or live cell (top graph) conditions.

Figure 34 shows a comparison of luminescence and substrate specificity of various NLpoly mutants with NLpep79 using either furimazine or coelenterazine as a substrate, and under either lytic (bottom graph) or live cell (top graph) conditions.

Figure 35 shows graphs of the luminescence of NLpep78-HT (top) and NLpep79-HT (bottom) fusions in the presence of various NLpolys.

Figure 36 shows a graph of the luminescence of various NLpolys in the absence of NLpep.

Figure 37 shows graphs of the luminescence of NLpep78-HT (top) and NLpep79-HT (bottom) fusions in the presence of various NLpolys with either furimazine or coelenterazine substrates.

Figure 38 shows a graph of the luminescence of NLpep78-HT with various NLpolys expressed in CHO and HeLa cells.

Figure 39 shows graphs of raw and normalized luminescence from NLpoly fused to firefly luciferase expressed in HEK293, Hela, and CHO cell lysates.

Figure 40 shows graphs of raw and normalized luminescence from NLpoly fused to click beetle red luciferase expressed in HEK293, Hela, and CHO cell lysates.

Figure 41 shows a graphs of luminescence of complementation in live cells using either NLpoly wild-type or 5P.

Figure 42 shows graphs of luminescence of cell-free complementation of NLpep78-HT fusion (top) and NLpep79-HT fusion (bottom) with various NLpolys.

Figure 43 shows a graph of binding affinities for various combinations of NLpeps and NLpolys expressed in HeLa, HEK293 and CHO cell lysate.

Figure 44 shows a graph of binding affinities for various combinations of NLpeps and NLpolys in PBS or NANOGLO buffer.

Figure 45 shows a graph of binding affinities for NLpoly 5P with NLpep9 or NLpep53 expressed in HeLa, HEK293 or CHO cell lysate.

Figure 46 shows a graph of luminescence of varying amounts of NLpolys in the absence of NLpep.

Figure 47 shows a graph of background luminescence of various NLpoly variants.

Figure 48 shows a graph of background luminescence of various NLpoly variants.

Figure 49 shows a SDS-PAGE gel of total lysate and soluble fraction of several NLpoly variants

Figure 50 shows (a) a SDS-PAGE gel of the total lysate and soluble fraction of NLpoly variants and (b) background luminescence of NLpoly variants.

Figure 51 shows graphs of the luminescence generated with several NLpoly variants when complemented with 10nm (right) or 100nM (left) of NLpep78.

Figure 52 shows graphs depicting background luminescence in *E. coli* lysate of various NLpoly variants.

Figure 53 shows graphs depicting luminescence in *E. coli* lysate of various NLpoly variants complemented with NLpep78.

Figure 54 shows graphs depicting luminescence in E. coli lysate of various NLpoly variants complemented with NLpep79.

Figure 55 shows a graph of signal to background of various NLPolys variants complemented with NLpep78 or NLpep79 and normalized to NLpoly 5P.

Figure 56 shows a graph depicting background, luminescence with NLpep79 (right) or NLpep78 (left) and signal-to-noise or various NLpoly variants.

Figure 57 shows a SDS-PAGE gel of the total lysate and soluble fraction in various NLpoly 5P variants.

Figure 58 shows (A) the amount of total lysate and soluble fraction of NLpoly 5P and NLpoly I107L, (B) luminescence generated by NLpoly 5P or NLpoly I107L without NLpep or with NLpep78 or NLpep79 and (C) the improved signal-to-background of NLpoly I107L over NLpoly 5P.

Figure 59 shows graphs of luminescence for various NLpoly variants (A) without complementary peptide, (B) with NLpep78-HT and (C) with NLpep79-HT.

Figure 60 shows graphs of luminescence for various NLpoly variants (A) without complementary peptide, (B) with NLpep78-HT and (C) with NLpep79-HT.

Figure 61 shows graphs of luminescence for various NLpoly variants (A) without complementary peptide, (B) with NLpep78-HT and (C) with NLpep79-HT.

Figure 62 shows graphs of luminescence for various NLpoly variants (A) without complementary peptide, (B) with NLpep78-HT and (C) with NLpep79-HT.

Figure 63 shows binding affinity between an elongated NLpoly variant (additional amino acids at the C-terminus) and a shortened NLpep (deleted amino acids at the N-terminus).

Figure 64 shows a graph of binding affinity of various NLpoly variants with NLpep78.

Figure 65 shows the binding and Vmax of NLpep80 and NLpep87 to 5P expressed in mammalian cells (CHO, HEK293T and HeLa).

Figure 66 shows the binding and Vmax of NLpep80 and NLpep87 to NLpoly 5P expressed in *E. coli*.

Figure 67 shows a graph of luminescence of shortened NLpolys with elongated NLpeps.

Figure 68 shows graphs of Kd and Vmax of NLpoly 5P in HeLa lysate with various complementary NLpeps.

Figure 69 shows a graph of binding affinities for several NLpoly variants with NLpep81.

Figure 70 shows a graph of binding affinities for several NLpoly variants with NLpep82.

Figure 71 shows a graph of binding affinities for several NLpoly mutants with NLpep78.

Figure 72 shows a graph of Michaelis constants for several NLpoly mutants with NLpep78.

Figure 73 shows graphs of luminescence from a tertiary complementation of two NLpeps and NLpoly 5P-B9.

Figure 74 shows a graph of luminescence of titration of NLpoly 5P with NLpep88-HT.

Figure 75 shows images of intracellular localization of various NLpep fusions with HaloTag (HT).

Figure 76 shows images of intracellular localization of NLpoly(wt) and NLpoly(5P).

Figure 77 demonstrates the ability to detect via complementation an NLPep-conjugated protein of interest following separation by SDS-PAGE and transfer to a PVDF membrane.

Figure 78 shows a graph of relative luminescent signal from various NLpoly variants compared to NLpoly 5P (in the absence of NLpep).

Figure 79 shows a graph of relative luminescent signal over background from various NLpolys compared to NLpoly 5P (in the absence of NLpep).

Figure 80 compares the dissociation constants for NLpeps consisting of either 1 or 2 repeat units of NLpep78.

Figure 81 shows the affinity between NLpoly 5A2 and NLpep86.

Figure 82 shows graphs of the luminescence from NLpoly variants without NLpep, with NLpep78, and NLpep79.

Figure 83-90 show the dissociation constants as well as the Vmax values for NLpoly 5A2, 5P, 8S and 11S with 96 variants of NLpeps.

Figure 91 shows an image of a protein gel of total lysates and the soluble fraction of the same lysate for NLpoly variants.

Figure 92 shows an image of a protein gel of total lysates and the soluble fraction of the same lysate for NLpoly variants as well as a table containing the dissociation constants for the same variants.

Figure 93 shows the substrate specificity for NLpoly 5P and 11S with NLpep79 and demonstrates that NLpoly 11S has superior specificity for furimazine than 5P.

Figure 94 shows an image of a protein gel that follows the affinity purification of NLpoly 8S through binding NLpep78.

Figure 95 contains a table of the association and dissociation rate constants for the binding of NLpoly WT or 11S to NLpepWT, 78 or 79.

Figure 96 shows the Km values for various pairs of NLpoly/NLpep.

Figure 97 compares the dissociation constant for NLpoly 11S/NLpep79 at sub-saturating and saturating concentrations of furimazine.

Figure 98 compares the Km values for NLpoly 5A2 with NLpepWT, 78 and 79.

Figure 99 shows the luminescence of NLpolys from various steps in the evolution process in the absence of NLpep.

Figure 100 shows the improvement in luminescence from *E. coli*-derived NLpoly over the course of the evolution process with an overall ~10^5 improvement (from NLpolyWT:NLpepWT to NLpoly11S:NLpep80).

Figure 101 shows the improvement in luminescence from HeLa-expressed NLpoly over the course of the evolution process with an overall ~10^5 improvement (from NLpolyWT:NLpepWT to NLpoly11S:NLpep80).

Figure 102 shows the improvement in luminescence from HEK293 cell-expressed NLpoly over the course of the evolution process with an overall ~10^4 improvement (from NLpolyWT:NLpepWT to NLpoly11S:NLpep80).

Figure 103 shows dissociation constants and demonstrates a ~10^4 fold improvement in binding affinity from NLpolyWT:NLpepWT to NLpoly11S:NLpep86.

Figure 104 shows an image of a protein gel of total lysates and the soluble fraction of the same lysate for NLpoly variants from various steps of the evolution process.

Figure 105 shows luminescence of various NLpolys in the absence of NLpep and in the presence of NLpep78 and NLpep79.

Figure 106 shows luminescence of various NLpolys in the absence of NLpep and in the presence of NLpep78 and NLpep79.

Figure 107 shows luminescence of various NLpolys in the absence of NLpep and in the presence of NLpep78 and NLpep79.

Figure 108 shows a comparison of luminescence generated by cells expressing different combinations of FRB and FKBP fused to NLpoly5P and NLpep80/87 after 15 min treatment with rapamycin or vehicle. Fold induction refers to signal generated in the presence of rapamycin compared to signal generated with vehicle.

Figure 109 shows a comparison of luminescence generated by cells expressing different combinations of FRB and FKBP fused to NLpoly5P and NLpep80/87 after 60 min treatment with rapamycin or vehicle.

Figure 110 shows a comparison of luminescence generated by cells expressing different combinations of FRB and FKBP fused to NLpoly5P and NLpep80/87 after 120 min treatment with rapamycin or vehicle.

Figure 111 shows a comparison of luminescence generated by cells expressing different combinations of FRB and FKBP fused to NLpoly5P and NLpep80/87 after 120 min treatment with rapamycin or vehicle. All 8 possible combinations of FRB and FKBP fused to NLpoly/NLpep were tested and less total DNA was used.

Figure 112 shows a comparison of luminescence generated by FRB or FKBP fusions expressed in the absence of binding partner.

Figure 113 shows a comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep80/87 DNA.

Figure 114 shows a comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P or FKBP-NLpep80/87 DNA in the absence of binding partner.

Figure 115 shows a comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep80/87 DNA. This example differs from Figure 113 in that lower levels of DNA were used.

Figure 116 shows a comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P or FKBP-NLpep80/87 DNA in the absence of binding partner. This differs from Figure 114 in that lower levels of DNA were used.

Figure 117 shows a comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep80 DNA after treatment with rapamycin for different lengths of time.

Figure 118 shows a comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep87 DNA after treatment with rapamycin for different lengths of time.

Figure 119 shows a comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly5P with FKBP-NLpep80/87/95/96/97. Assay was performed in both a two-day and three-day format.

Figure 120 shows a comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly5A2 with FKBP-NLpep80/87/95/96/97. Assay was performed in both a two-day and three-day format.

Figure 121shows a comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly5A2 or FRB-NLpoly11S with FKBP-NLpep101/104/105/106/107/108/109/110.

Figure 122 shows a comparison of luminescence generated by cells transfected with different combinations of FRB-NLpoly5A2 or FRB-NLpoly11S with FKBP-NLpep87/96/98/99/100/101/102/103.

Figure 123 shows a comparison of luminescence generated by cells transfected with different levels of FRB-NLpoly11S and FKBP-NLpep87/101/102/107 DNA.

Figure 124 shows a comparison of luminescence generated by cells transfected with different levels of FRB-NLpoly5A2 and FKBP-NLpep87/101/102/107 DNA.

Figure 125 shows a rapamycin dose response curve showing luminescence of cells expressing FRB-NLpoly5P and FKBP-NLpep80/87 DNA.

Figure 126 shows a rapamycin dose response curve showing luminescence of cells expressing FRB-NLpoly5A2 or FRB-NLpoly11S and FKBP-NLpep87/101 DNA.

Figure 127 shows a comparison of luminescence generated by cells expressing FRB-11S and FKBP-101 and treated with substrate PBI-4377 or furimazine.

Figure 128 shows a rapamycin time course of cells expressing FRB-NLpoly11S/5A2 and FKBP-NLpep87/101 conducted in the presence or absence of rapamycin wherein the rapamycin was added manually.

Figure 129 shows a rapamycin time course of cells expressing FRB-NLpoly11S/5A2 and FKBP-NLpep87/101 conducted in the presence or absence of rapamycin wherein the rapamycin was added via instrument injector.

Figure 130 shows luminescence generated by FRB-NLpoly11S and FKBP-NLpep101 as measured on two different luminescence-reading instruments.

Figure 131 provides images showing luminescence of cells expressing FRB-NLpoly11S and FKBP-NLpep101 at various times after treatment with rapamycin.

Figure 132 provides a graph showing Image J quantitation of the signal generated by individual cells expressing FRB-NLpoly11S and FKBP-NLpep101 at various times after treatment with rapamycin.

Figure 133 shows a comparison of luminescence in different cell lines expressing FRB-NLpoly11S and FKBP-NLpep101.

Figure 134 shows a comparison of luminescence generated by cells expressing FRB-NLpoly11S and FKBP-NLpep101 after treatment with the rapamycin competitive inhibitor FK506.

Figure 135 shows (left side) luminescence generated by cells expressing FRB-NLpoly11S and FKBP-NLpep101 after treatment with the rapamycin competitive inhibitor FK506, and (right side) the percent of luminescence remaining after treatment with FK506.

Figure 136 shows luminescence generated by cells transfected with different combinations of V2R-NLpoly5A2 or V2R-NLpoly11S with NLpep87/101-ARRB2 in the presence or absence of the V2R agonist AVP.

Figure 137 shows an AVP treatment time course showing luminescence generated by cells transfected with V2R-NLpoly11S and NLpep87/101-ARRB2 after treatment with AVP wherein AVP was added manually.

Figure 138 shows an AVP treatment time course showing luminescence generated by cells transfected with different combinations of V2R-NLpoly5A2 or V2R-NLpoly11S with NLpep87/101-ARRB2 after treatment with AVP wherein AVP was added via instrument injector.

Figure 139 shows an AVP treatment time course at 37^0C showing luminescence generated by cells expressing different configurations of V2R and ARRB2 fused to NLpoly11S and NLpep101 after treatment with AVP.

Figure 140 shows a comparison of luminescence in different cell lines expressing V2R-NLpep11S and NLpep101-ARRB2.

Figure 141 shows 60X images showing luminescence of cells expressing V2R-NLpoly11S and NLpep101-ARRB2 at various times after treatment with AVP.

Figure 142 shows 150X images showing luminescence of cells expressing V2R-NLpoly11S and NLpep101-ARRB2 at various times after treatment with AVP.

Figure 143 shows a protein gel of total lysates and the soluble fraction of the same lysate for NLpoly variants.

Figure 144 shows the dissociation constants for NLpoly 5P and combinations of mutations at positions 31, 46, 75, 76, and 93 in NLpoly 5P.

Figure 145 shows a transferase example of post translational modification enzyme activity detection using an NLpep and aminopeptidase.

Figure 146 shows a hydrolase example of post translational modification enzyme activity detection using an NLpep and methyl-specific antibody.

Figure 147 contains wavelength scans for NLpoly WT complemented with either NLpepWT or NLpepWT conjugated to TMR.

Figure 148 contains wavelength scans for NanoLuc fused to HaloTag (NL-HT) and NLpoly 5A2 complemented with NLPepWT with 4 additional amino acids (DEVD) and conjugated to Non-chloroTOM (NCT).

Figure 149 shows a schematic a tertiary interaction wherein the energy transfer with an NLpoly and NLpep can also be used to measure three molecules interacting. In the schematic, a GPCR labeled with an NLpoly and a GPCR interacting protein labeled with an NLpep form a bioluminescent complex when they interact. This allows measurement of the binary interaction. If a small molecule GPCR ligand bearing an appropriate fluorescent moiety for energy transfer interacts with this system, energy transfer will occur. Therefore, the binary protein-protein interaction and the ternary drug-protein-protein interaction can be measured in the same experiment.

Figure 150 shows a graph and table of binding affinities of NLpoly11S to synthetic NLPep78 and NLPep78 at the N- or C-terminus of a fusion partner (HaloTag).

Figure 151 shows a graph and table of binding affinities of NLpoly11S to synthetic NLPep79 and NLPep79 at the N- or C-terminus of a fusion partner (HaloTag).

Figure 152 shows a graph depicting normalized fluorescence intensity of NLpoly11S with NLPep86 or PBI-4877.

Figure 153 shows a graph depicting normalized fluorescence intensity of NLpoly11S with NLPep86 or PBI-5434.

Figure 154 shows a graph depicting normalized fluorescence intensity of NLpoly11S with NLPep86 or PBI-5436.

Figure 155 shows a graph demonstrating furimazine binding affinity in affinity buffer of complexes between NLpoly11S and NLpep86, 78, 99, 101, 104, 128 and 114.

Figure 156 shows a graph demonstrating furimazine binding affinity in NanoGlo assay buffer of complexes between NLpoly11S and NLpep86, 78, 99, 101, 104, 128 and 114.

Figure 157 shows graphs depicting the change in affinity (NLpoly156/NLPep1 and NLpoly11S/NLPep1) with increasing concentrations of furimazine substrate.

Figure 158 shows graphs depicting the change in affinity (NLpoly156/NLPep1 and NLpoly11S/NLPep1) with increasing concentrations of NLPep1.

Figure 159 shows a graph depicting Vmax and Bmax NLPoly156, NLPoly11S, and NanoLuc® luciferase (Nluc) with NLPep1.

Figure 160 shows a graph depicting RLU as a function of NLPep concentration for NLPoly11S and NLPep86, 78, 79, 99, 101, 104, 114, 128 and wt.

Figure 161 shows a Western blot depicting expression level in HEK293T cells of NLPoly156 and NLPoly11S compared to full-length NanoLuc® luciferase.

Figure 162 shows graphs depicting a comparison of the affinity of the β-lactamase SME and its inhibitor BLIPY50A as unfused proteins or when fused to NLPoly11S and NLPep114.

Figure 163 shows a comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly11S with FKBP-NLpep101/111-136

Figure 164 shows a comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly11S with FKBP-NLpep114 and 137-143.

Figure 165 shows rapamycin dose response curves of cells expressing FRB-NLpoly11S and FKBP-NLpep78/79/99/101/104/114/128

Figure 166 shows response of cells expressing FRB-NLpoly11S and FKBP-78/79/99/101/104/114/128 to the rapamycin competitive inhibitor FK506

Figure 167 shows a comparison of luminescence generated by cells transfected with different ratios of FRB-NLpoly11S and FKBP-NLpep114.

Figure 168 shows a comparison of luminescence generated by cells expressing NLpoly11S/NLpep114 fusions of FRB/FKBP in different orientations and with different linker lengths.

Figure 169 shows graphs depicting rapamycin (A) dose-specific and (B) time-specific induction of FRB-NLpoly11S/FKBP-NLpep114 or split firefly complementation signals.

Figure 170 shows graphs depicting FK506(A) dose-specific and (B) time-specific inhibition of FRB-NLpoly11S/FKBP-NLpep114 or split firefly complementation signals.

Figure 171 shows Western blots depicting similar expression levels of FKBP-NLpep114 and FKBP-Fluc(394-544) at equal levels of transfected DNA.

Figure 172 shows graphs depicting (A) dose-specific and (B) time-specific inhibition of NLpoly11S-BRD4 and Histone H3.3-NLpep114 interaction by IBET-151.

Figure 173 shows a graph depicting dose dependent increases in RAS/CRAF, BRAF/BRAF and CRAF/BRAF dimerization in response to BRAF inhibitor GDC0879.

Figure 174 shows a graph depicting RLU as a function of NLPep concentration for NLpoly11S and NLpep86, wt, and NLpep114.

Figure 175 shows a schematic of an assay utilizing a high affinity peptide of a luminescent pair as an intracellular protein tag and the polypeptide of the luminescent pair as a detection reagent.

Figure 176 shows a graph demonstrating the linear range of the affinity of NLpoly11S and MLpep86.

Figure 177 shows images demonstrating the sensitivity of detecting proteins tagged with a high affinity NLPep using 11S. This figure also compares the detection using NLPep/NLPoly to the detection using fluorescently labeled HaloTag.

Figure 178 shows a graph demonstating the stability of NLpoly11S.

Figure 179 shows a graph demonstrating the linear range of the affinity of NLpoly11S and NLpep78.

Figure 180 shows a summary of NLpep sequences. High affinity (spontaneous) peptides are those peptides (NLpep) which bind to NLpoly11S with high affinity. Dark/Quencher peptides are those peptides (NLpep) which can reduce the levels of light being produced or detected from NLpoly11S.

Figure 181 shows a schematic for the concept of structural complementation where the LSP and SSP (i.e., NLpoly and NLpep) are brought together to produce a bioluminescent signal (panels A, B). Upon disruption of a protein interaction (i.e. X and Y), LSP and SSP come apart resulting in a decrease in luminescence (Panel C).

Figure 182 A shows two options (A, B) for engineering structural complementation to be a loss of signal upon protein interaction between X and Y and a gain of signal upon disruption of the interaction between X and Y. Option A represents intermolecular structural complementation.

Option B represents intramolecular structural complementation. Figure 182B shows a list of genetic constructs that could be suitable for intramolecular structural complementation.

Figure 183 shows (A) inhibition of NLpoly11S and NLpep114 binding by various dark peptides, and (B) dose-dependent inhibition by Lys-162 and Gln-162 peptides.

Figure 184 A shows that inhibition by Q-162 and A-162 is dose-dependent. Panel B shows that Q-162 produces a signal on its own in a dose-dependent manner, while the dose-dependency of A-162 is subtle at best.

Figure 185 shows graphs demonstrating dose-response of the dark peptides with CP Nluc.

Figure 186 shows graphs depicting a time course of dark peptide with CP Nluc.

Figure 187 shows the dark peptide dose-dependent inhibition of luminescence generated from FRB-NLpoly11S alone and also between FRB-NLpoly11S and FKBP-NLpep114 in the presence and absence of rapamycin.

Figure 188 shows the dark peptide dose-dependent inhibition of luminescence generated from either FRB-NanoLuc (311) or NanoLuc-FRB (307) in the presence and absence of rapamycin (RLU).

Figure 189 shows the dark peptide dose-dependent inhibition of luminescence generated from either FRB-NanoLuc (311) or NanoLuc-FRB (307) in the presence and absence of rapamycin (normalized to no dark peptide control; 100%).

Figure 190 shows that the dark peptides, when fused to FKBP, can compete with both low (114) and high (80) affinity peptides (also FKBP fusions) and as a result reduce the total luminescence being produced and detected in live cells.

Figure 191 shows the signal comparison between Fluc and NLpep86-based assays for intracellular levels of Fluc.

Figure 192 shows graphs demonstrating the utility of tandem linked NLpeps in complementing Npoly11S.

Figure 193 shows a graph demonstrating that NLpoly and NLpep components do not interfere with intracellular degradation of reporter protein FlucP.

Figure 194 shows a schematic demonstrating and extracellular protease activity assay.

Figure 195 shows a schematic of an assay for measuring the activity of an enzyme using a ProNLpep.

Figure 196 shows a schematic of an assay for screening antibodies, proteins, peptides or transporters that mediate cellular internalization.

Figure 197 shows a schematic of a post-translational modification transferase assay.

Figure 198 shows a schematic of a post-translational modification hydrolase assay.

Figure 199 shows graphs correlating Tyrosine Kinase SRC activity with luminescence over background in a post-translational modification assay.

Figure 200 shows a graph depicting spontaneous complementation of three different versions of NLpoly11S with twelve synthetic peptides.

Figure 201 shows a schematic of a homogeneous immunoassay format utilizing fusions of NLpep and NLpoly with separate binding moieties A and B.

Figure 202 shows graphs demonstrating: (A) reduction in background luminescence from NLpoly11S upon complex formation with GWALFKK and Dabcyl-GWALFKK, and (B) NLpep86 forms a complex with NLpoly11S in the presence of GWALFKK and Dabcyl-GWALFKK.

Figure 203 shows graphs demonstrating: (A) VTGWALFEEIL (Trp 11mer) and VTGYALFEEIL (Tyr 11mer) induce luminescence over background (NLpoly11S alone; no peptide control), and that the N-terminal Dabcyl versions of each provide significant quenching of this signal, and (B) that NLpep86 forms a complex with NLpoly11S in the presence of Dabcyl versions of Trp 11mer and Tyr 11mer.

DEFINITIONS

As used herein, the term "substantially" means that the recited characteristic, parameter, and/or value need not be achieved exactly, but that deviations or variations, including for example, tolerances, measurement error, measurement accuracy limitations and other factors known to skill in the art, may occur in amounts that do not preclude the effect the characteristic was intended to provide. A characteristic or feature that is substantially absent (e.g., substantially non-luminescent) may be one that is within the noise, beneath background, below the detection capabilities of the assay being used, or a small fraction (e.g., <1%, <0.1%, <0.01%, <0.001%, <0.00001%, <0.000001%, <0.0000001%) of the significant characteristic (e.g., luminescent intensity of a bioluminescent protein or bioluminescent complex).

As used herein, the term "bioluminescence" refers to production and emission of light by a chemical reaction catalyzed by, or enabled by, an enzyme, protein, protein complex, or other biomolecule (e.g., bioluminescent complex). In typical embodiments, a substrate for a bioluminescent entity (e.g., bioluminescent protein or bioluminescent complex) is converted into an unstable form by the bioluminescent entity; the substrate subsequently emits light.

As used herein the term "complementary" refers to the characteristic of two or more structural elements (e.g., peptide, polypeptide, nucleic acid, small molecule, etc.) of being able to hybridize, dimerize, or otherwise form a complex with each other. For example, a "complementary peptide and polypeptide" are capable of coming together to form a complex.

Complementary elements may require assistance to form a complex (e.g., from interaction elements), for example, to place the elements in the proper conformation for complementarity, to co-localize complementary elements, to lower interaction energy for complementary, etc.

As used herein, the term "complex" refers to an assemblage or aggregate of molecules (e.g., peptides, polypeptides, etc.) in direct and/or indirect contact with one another. In one aspect, "contact," or more particularly, "direct contact" means two or more molecules are close enough so that attractive noncovalent interactions, such as Van der Waal forces, hydrogen bonding, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules. In such an aspect, a complex of molecules (e.g., a peptide and polypeptide) is formed under assay conditions such that the complex is thermodynamically favored (e.g., compared to a non-aggregated, or non-complexed, state of its component molecules). As used herein the term "complex," unless described as otherwise, refers to the assemblage of two or more molecules (e.g., peptides, polypeptides or a combination thereof).

As used herein, the term "non-luminescent" refers to an entity (e.g., peptide, polypeptide, complex, protein, etc.) that exhibits the characteristic of not emitting a detectable amount of light in the visible spectrum (e.g., in the presence of a substrate). For example, an entity may be referred to as non-luminescent if it does not exhibit detectable luminescence in a given assay. As used herein, the term "non-luminescent" is synonymous with the term "substantially non-luminescent. For example, a non-luminescent polypeptide (NLpoly) is substantially non-luminescent, exhibiting, for example, a 10-fold or more (e.g., 100-fold, 200-fold, 500-fold, $1x10^3$-fold, $1x10^4$-fold, $1x10^5$-fold, $1x10^6$-fold, $1x10^7$-fold, etc.) reduction in luminescence compared to a complex of the NLpoly with its non-luminescent complement peptide. In some embodiments, an entity is "non-luminescent" if any light emission is sufficiently minimal so as not to create interfering background for a particular assay.

As used herein, the terms "non-luminescent peptide" (e.g., NLpep) and "non-luminescent polypeptide" (e.g., NLpoly) refer to peptides and polypeptides that exhibit substantially no luminescence (e.g., in the presence of a substrate), or an amount that is beneath the noise, or a 10-fold or more (e.g., 100-fold, 200-fold, 500-fold, $1x10^3$-fold, $1x10^4$-fold, $1x10^5$-fold, $1x10^6$-fold, $1x10^7$-fold, etc.) when compared to a significant signal (e.g., luminescent complex) under standard conditions (e.g., physiological conditions, assay conditions, etc.) and with typical instrumentation (e.g., luminometer, etc.). In some embodiments, such non-luminescent peptides and polypeptides assemble, according to the criteria described herein, to form a bioluminescent complex. As used herein, a "non-luminescent element" is a non-luminescent peptide or non-luminescent polypeptide. The term "bioluminescent complex" refers to the assembled complex of two or more non-luminescent peptides and/or non-luminescent polypeptides. The

bioluminescent complex catalyzes or enables the conversion of a substrate for the bioluminescent complex into an unstable form; the substrate subsequently emits light. When uncomplexed, two non-luminescent elements that form a bioluminescent complex may be referred to as a "non-luminescent pair." If a bioluminescent complex is formed by three or more non-luminescent peptides and/or non-luminescent polypeptides, the uncomplexed constituents of the bioluminescent complex may be referred to as a "non-luminescent group."

As used herein, the term "interaction element" refers to a moiety that assists in bringing together a pair of non-luminescent elements or a non-luminescent group to form a bioluminescent complex. In a typical embodiment, a pair of interaction elements (a.k.a. "interaction pair") is attached to a pair of non-luminescent elements (e.g., non-luminescent peptide/polypeptide pair), and the attractive interaction between the two interaction elements facilitates formation of the bioluminescent complex; although the present invention is not limited to such a mechanism, and an understanding of the mechanism is not required to practice the invention. Interaction elements may facilitate formation of the bioluminescent complex by any suitable mechanism (e.g., bringing non-luminescent pair/group into close proximity, placing a non-luminescent pair/group in proper conformation for stable interaction, reducing activation energy for complex formation, combinations thereof, etc.). An interaction element may be a protein, polypeptide, peptide, small molecule, cofactor, nucleic acid, lipid, carbohydrate, antibody, etc. An interaction pair may be made of two of the same interaction elements (i.e. homopair) or two different interaction elements (i.e. heteropair). In the case of a heteropair, the interaction elements may be the same type of moiety (e.g., polypeptides) or may be two different types of moieties (e.g., polypeptide and small molecule). In some embodiments, in which complex formation by the interaction pair is studied, an interaction pair may be referred to as a "target pair" or a "pair of interest," and the individual interaction elements are referred to as "target elements" (e.g., "target peptide," "target polypeptide," etc.) or "elements of interest" (e.g., "peptide of interest," "polypeptide or interest," etc.).

As used herein, the term "preexisting protein" refers to an amino acid sequence that was in physical existence prior to a certain event or date. A "peptide that is not a fragment of a preexisting protein" is a short amino acid chain that is not a fragment or sub-sequence of a protein (e.g., synthetic or naturally-occurring) that was in physical existence prior to the design and/or synthesis of the peptide.

As used herein, the term "fragment" refers to a peptide or polypeptide that results from dissection or "fragmentation" of a larger whole entity (e.g., protein, polypeptide, enzyme, etc.), or a peptide or polypeptide prepared to have the same sequence as such. Therefore, a fragment is a subsequence of the whole entity (e.g., protein, polypeptide, enzyme, etc.) from which it is made

and/or designed. A peptide or polypeptide that is not a subsequence of a preexisting whole protein is not a fragment (e.g., not a fragment of a preexisting protein). A peptide or polypeptide that is "not a fragment of a preexisting bioluminescent protein" is an amino acid chain that is not a subsequence of a protein (e.g., natural or synthetic) that: (1) was in physical existence prior to design and/or synthesis of the peptide or polypeptide, and (2) exhibits substantial bioluminescent activity.

As used herein, the term "subsequence" refers to peptide or polypeptide that has 100% sequence identify with another, larger peptide or polypeptide. The subsequence is a perfect sequence match for a portion of the larger amino acid chain.

As used herein, the term "sequence identity" refers to the degree two polymer sequences (e.g., peptide, polypeptide, nucleic acid, etc.) have the same sequential composition of monomer subunits. The term "sequence similarity" refers to the degree with which two polymer sequences (e.g., peptide, polypeptide, nucleic acid, etc.) have similar polymer sequences. For example, similar amino acids are those that share the same biophysical characteristics and can be grouped into the families, e.g., acidic (e.g., aspartate, glutamate), basic (e.g., lysine, arginine, histidine), non-polar (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan) and uncharged polar (e.g., glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). The "percent sequence identity" (or "percent sequence similarity") is calculated by: (1) comparing two optimally aligned sequences over a window of comparison (e.g., the length of the longer sequence, the length of the shorter sequence, a specified window), (2) determining the number of positions containing identical (or similar) monomers (e.g., same amino acids occurs in both sequences, similar amino acid occurs in both sequences) to yield the number of matched positions, (3) dividing the number of matched positions by the total number of positions in the comparison window (e.g., the length of the longer sequence, the length of the shorter sequence, a specified window), and (4) multiplying the result by 100 to yield the percent sequence identity or percent sequence similarity. For example, if peptides A and B are both 20 amino acids in length and have identical amino acids at all but 1 position, then peptide A and peptide B have 95% sequence identity. If the amino acids at the non-identical position shared the same biophysical characteristics (e.g., both were acidic), then peptide A and peptide B would have 100% sequence similarity. As another example, if peptide C is 20 amino acids in length and peptide D is 15 amino acids in length, and 14 out of 15 amino acids in peptide D are identical to those of a portion of peptide C, then peptides C and D have 70% sequence identity, but peptide D has 93.3% sequence identity to an optimal comparison window of peptide C. For the purpose of calculating "percent sequence identity" (or "percent sequence similarity") herein, any gaps in aligned sequences are treated as mismatches at that position.

As used herein, the term “physiological conditions” encompasses any conditions compatible with living cells, e.g., predominantly aqueous conditions of a temperature, pH, salinity, chemical makeup, etc. that are compatible with living cells.

As used herein, the term "sample" is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum and the like. Sample may also refer to cell lysates or purified forms of the peptides and/or polypeptides described herein. Cell lysates may include cells that have been lysed with a lysing agent or lysates such as rabbit reticulocyte or wheat germ lysates. Sample may also include cell-free expression systems. Environmental samples include environmental material such as surface matter, soil, water, crystals and industrial samples. Such examples are not however to be construed as limiting the sample types applicable to the present invention.

As used herein, unless otherwise specified, the terms “peptide” and “polypeptide” refer to polymer compounds of two or more amino acids joined through the main chain by peptide amide bonds (--C(O)NH--). The term “peptide” typically refers to short amino acid polymers (e.g., chains having fewer than 25 amino acids), whereas the term “polypeptide” typically refers to longer amino acid polymers (e.g., chains having more than 25 amino acids).

DETAILED DESCRIPTION

The study of protein interactions, particularly under physiological conditions and/or at physiologic expression levels, requires high sensitivity. In particular embodiments described herein, protein interactions with small molecules, nucleic acids, other proteins, etc. are detected based on the association of two non-luminescent elements that come together to from a bioluminescent complex capable of producing a detectable signal (e.g., luminescence). The formation of the bioluminescent complex is dependent upon the protein interaction that is being monitored.

Provided herein are compositions and methods for the assembly of a bioluminescent complex from two or more non-luminescent peptide and/or polypeptide units (e.g., non-luminescent pair). In some embodiments, the non-luminescent peptide and/or polypeptide units are not fragments of a preexisting protein (e.g., are not complementary subsequences of a known polypeptide sequence). In particular, bioluminescent activity is conferred upon a non-luminescent polypeptide via structural complementation with a non-luminescent peptide.

In some embodiments, provided herein are non-luminescent pairs for use in detecting and monitoring molecular interactions (e.g., protein-protein, protein-DNA, protein-RNA interactions, RNA-DNA, protein-small molecule, RNA-small-molecule, etc.). Also provided herein are

complementary panels of interchangeable non-luminescent elements (e.g., peptides and polypeptides) that have variable affinities and luminescence upon formation of the various bioluminescent complexes (e.g., a high-affinity/high-luminescence pair, a moderate-affinity/high-luminescence pair, a low-affinity/moderate-luminescence pair, etc.). Utilizing different combinations of non-luminescent elements provides an adaptable system comprising various pairs ranging from lower to higher affinities, luminescence and other variable characteristics. This adaptability allows the detection/monitoring of molecular interactions to be fine-tuned to the specific molecule(s) of interest and expands the range of molecular interactions that can be monitored to include interactions with very high or low affinities. Further provided herein are methods by which non-luminescent pairs (or groups) and panels of non-luminescent pairs (or groups) are developed and tested.

In some embodiments, the interaction between the peptide/polypeptide members of the non-luminescent pair alone is insufficient to form the bioluminescent complex and produce the resulting bioluminescent signal. However, if an interaction element is attached to each peptide/polypeptide member of the non-luminescent pair, then the interactions of the interaction pair (e.g., to form an interaction complex) facilitate formation of the bioluminescent complex. In such embodiments, the bioluminescent signal from the bioluminescent complex (or the capacity to produce such a signal in the presence of substrate) serves as a reporter for the formation of the interaction complex. If an interaction complex is formed, then a bioluminescent complex is formed, and a bioluminescent signal is detected/measured/monitored (e.g., in the presence of substrate). If an interaction complex fails to form (e.g., due to unfavorable conditions, due to unstable interaction between the interaction elements, due to incompatible interaction elements), then a bioluminescent complex does not form, and a bioluminescent signal is not produced.

In certain embodiments, the interaction pair comprises two molecules of interest (e.g., proteins of interest). For example, assays can be performed to detect the interaction of two molecules of interest by tethering each one to a separate member of a non-luminescent pair. If the molecules of interest interact (e.g., transiently interact, stably interact, etc.), the non-luminescent pair is brought into close proximity in a suitable conformation and a bioluminescent complex is formed (and bioluminescent signal is produced/detected (in the presence of substrate)). In the absence of an interaction between the molecules of interest (e.g., no complex formation, not even transient interaction, etc.), the non-luminescent pair does not interact in a sufficient manner, and a bioluminescent signal is not produced or only weakly produced. Such embodiments can be used to study the effect of inhibitors on complex formation, the effect of mutations on complex formation, the effect of conditions (e.g., temperature, pH, etc.) on complex

formation, the interaction of a small molecule (e.g., potential therapeutic) with a target molecule, etc.

Different non-luminescent pairs may require different strength, duration and/or stability of the interaction complex to result in bioluminescent complex formation. In some embodiments, a stable interaction complex is required to produce a detectable bioluminescent signal. In other embodiments, even a weak or transient interaction complex results in bioluminescent complex formation. In some embodiments, the strength or extent of an interaction complex is directly proportional to the strength of the resulting bioluminescent signal. Some non-luminescent pairs produce a detectable signal when combined with an interaction complex with a high millimolar dissociation constant (e.g., K_d>100 mM). Other non-luminescent pairs require an interaction pair with a low millimolar (e.g., K_d<100 mM), micromolar (e.g., K_d<1 mM), nanomolar (e.g., K_d<1 μM), or even picomolar (e.g., K_d<1 nM) dissociation constant in order to produce a bioluminescent complex with a detectable signal.

In some embodiments, one or more of the non-luminescent peptides/polypeptides are not fragments of a pre-existing protein. In some embodiments, one or more of the non-luminescent peptides/polypeptides are not fragments of a pre-existing bioluminescent protein. In some embodiments, neither/none of the non-luminescent peptides/polypeptides are fragments of a pre-existing protein. In some embodiments, neither/none of the non-luminescent peptides/polypeptides are fragments of a pre-existing bioluminescent protein. In some embodiments, neither the non-luminescent peptide nor non-luminescent polypeptide that assemble together to form a bioluminescent complex are fragments of a pre-existing protein. In some embodiments, a non-luminescent element for use in embodiments of the present invention is not a subsequence of a preexisting protein. In some embodiments, a non-luminescent pair for use in embodiments described herein does not comprise complementary subsequences of a preexisting protein.

In some embodiments, non-luminescent peptides/polypeptides are substantially non-luminescent in isolation. In certain embodiments, when placed in suitable conditions (e.g., physiological conditions), non-luminescent peptides/polypeptides interact to form a bioluminescent complex and produce a bioluminescent signal in the presence of substrate. In other embodiments, without the addition of one or more interaction elements (e.g., complementary interaction elements attached to the component non-luminescent peptide and non-luminescent polypeptide), non-luminescent peptides/polypeptides are unable to form a bioluminescent complex or only weakly form a complex. In such embodiments, non-luminescent peptides/polypeptides are substantially non-luminescent in each other's presence alone, but produce significant detectable luminescence when aggregated, associated, oriented, or

otherwise brought together by interaction elements. In some embodiments, without the addition of one or more interaction elements (e.g., complementary interaction elements attached to the component peptide and polypeptide), peptides and/or polypeptides that assemble into the bioluminescent complex produce a low level of luminescence in each other's presence, but undergo a significant increase in detectable luminescence when aggregated, associated, oriented, or otherwise brought together by interaction elements.

In some embodiments, compositions and methods described herein comprise one or more interaction elements. In a typical embodiment, an interaction element is a moiety (e.g., peptide, polypeptide, protein, small molecule, nucleic acid, lipid, carbohydrate, etc.) that is attached to a peptide and/or polypeptide to assemble into the bioluminescent complex. The interaction element facilitates the formation of a bioluminescent complex by any suitable mechanism, including: interacting with one or both non-luminescent elements, inducing a conformational change in a non-luminescent element, interacting with another interaction element (e.g., an interaction element attached to the other non-luminescent element), bringing non-luminescent elements into close proximity, orienting non-luminescent elements for proper interaction, etc.

In some embodiments, one or more interaction elements are added to a solution containing the non-luminescent elements, but are not attached to the non-luminescent elements. In such embodiments, the interaction element(s) interact with the non-luminescent elements to induce formation of the bioluminescent complex or create conditions suitable for formation of the bioluminescent complex. In other embodiments, a single interaction element is attached to one member of a non-luminescent pair. In such embodiments, the lone interaction element interacts with one or both of the non-luminescent elements to create favorable interactions for formation of the bioluminescent complex. In typical embodiments of the present invention, one interaction element is attached to each member of a non-luminescent pair. Favorable interactions between the interaction elements facilitate interactions between the non-luminescent elements. The interaction pair may stably interact, transiently interact, form a complex, etc. The interaction of the interaction pair facilitates interaction of the non-luminescent elements (and formation of a bioluminescent complex) by any suitable mechanism, including, but not limited to: bringing the non-luminescent pair members into close proximity, properly orienting the non-luminescent pair members from interaction, reducing non-covalent forces acting against non-luminescent pair interaction, etc.

In some embodiments, an interaction pair comprises any two chemical moieties that facilitate interaction of an associated non-luminescent pair. An interaction pair may consist of, for example: two complementary nucleic acids, two polypeptides capable of dimerization (e.g., homodimer, heterodimer, etc.), a protein and ligand, protein and small molecule, an antibody and

epitope, a reactive pair of small molecules, etc. Any suitable pair of interacting molecules may find use as an interaction pair.

In some embodiments, an interaction pair comprises two molecules of interest (e.g., proteins of interest) or target molecules. In some embodiments, compositions and methods herein provide useful assays (e.g., in vitro, in vivo, in situ, whole animal, etc.) for studying the interactions between a pair of target molecules.

In certain embodiments, a pair off interaction elements, each attached to one of the non-luminescent elements, interact with each other and thereby facilitate formation of the bioluminescent complex. In some embodiments, the presence of a ligand, substrate, co-factor or addition interaction element (e.g., not attached to non-luminescent element) is necessary to induce the interaction between the interaction elements and facilitate bioluminescent complex formation. In some embodiments, detecting a signal from the bioluminescent complex indicates the presence of the ligand, substrate, co-factor or addition interaction element or conditions that allow for interaction with the interaction elements.

In some embodiments, a pair off interaction elements, and a pair of non-luminescent elements are all present in a single amino acid chain (e.g., (interaction element 1)-NLpep-(interaction element 2)-NLpoly, NLpoly-(interaction element 1)-NLpep--(interaction element 2), NLpoly-(interaction element 1)-(interaction element 2)-NLpep, etc.). In some embodiments in which a pair off interaction elements, and a pair of non-luminescent elements are all present in a single amino acid chain, a ligand, substrate, co-factor or addition interaction element is required for the interaction pair to form an interaction complex and facilitate formation of the bioluminescent complex.

In certain embodiments, an interaction element and a non-luminescent element are attached, fused, linked, connected, etc. In typical embodiments, a first non-luminescent element and a first interaction element are attached to each other, and a second non-luminescent element and a second interaction element are attached to each other. Attachment of signal and interaction elements may be achieved by any suitable mechanism, chemistry, linker, etc. The interaction and non-luminescent elements are typically attached through covalent connection, but non-covalent linking of the two elements is also provided. In some embodiments, the signal and interaction elements are directly connected and, in other embodiments, they are connected by a linker.

In some embodiments, in which the interaction element is a peptide or polypeptide, the signal and interaction elements are contained within a single amino acid chain. In some embodiments, a single amino acid chain comprises, consists of, or consists essentially of a non-luminescent element and interaction element. In some embodiments, a single amino acid chain

comprises, consists of, or consists essentially of a non-luminescent element, an interaction element, and optionally one or more an N-terminal sequence, a C-terminal sequence, regulatory elements (e.g., promoter, translational start site, etc.), and a linker sequence. In some embodiments, the signal and interaction elements are contained within a fusion polypeptide. The signal and interaction elements (and any other amino acid segments to be included in the fusion) may be expressed separately; however, in other embodiments, a fusion protein is expressed that comprises or consist of both the interaction and signal sequences.

In some embodiments, a first fusion protein comprising a first non-luminescent element and first interaction element as well as a second fusion protein comprising a second non-luminescent element and second interaction element are expressed within the same cells. In such embodiments, the first and second fusion proteins are purified and/or isolated from the cells, or the interaction of the fusion proteins is assayed within the cells. In other embodiments, first and second fusion proteins are expressed in separate cells and combined (e.g., following purification and/or isolation, or following fusion of the cells or portions of the cells, or by transfer of a fusion protein from one cell to another, or by secretion of one or more fusion proteins into the extracellular medium) for signal detection. In some embodiments, one or more fusion proteins are expressed in cell lysate (e.g., rabbit reticulocyte lysate) or in a cell-free system. In some embodiments, one or more fusion proteins are expressed from the genome of a virus or other cellular pathogen.

In certain embodiments, nucleic acids, DNA, RNA, vectors, etc. are provided that encode peptide, polypeptides, fusion polypeptide, fusion proteins, etc. of the present invention. Such nucleic acids and vectors may be used for expression, transformation, transfection, injection, etc.

In some embodiments, a non-luminescent element and interaction element are connected by a linker. In some embodiments, a linker connects the signal and interaction elements while providing a desired amount of space/distance between the elements. In some embodiments, a linker allows both the signal and interaction elements to form their respective pairs (e.g., non-luminescent pair and interaction pair) simultaneously. In some embodiments, a linker assists the interaction element in facilitating the formation of a non-luminescent pair interaction. In some embodiments, when an interaction pair is formed, the linkers that connect each non-luminescent element to their respective interaction elements position the non-luminescent elements at the proper distance and conformation to form a bioluminescent complex. In some embodiments, an interaction element and non-luminescent element are held in close proximity (e.g., <4 monomer units) by a linker. In some embodiments, a linker provides a desired amount of distance (e.g., 1, 2, 3, 4, 5, 6…10… 20, or more monomer units) between signal and interaction elements (e.g., to prevent undesirable interactions between signal and interaction elements, for steric

considerations, to allow proper orientation of non-luminescent element upon formation of interaction complex, to allow propagation of a complex-formation from interaction complex to non-luminescent elements, etc.). In certain embodiments, a linker provides appropriate attachment chemistry between the signal and interaction elements. A linker may also improve the synthetic process of making the signal and interaction element (e.g., allowing them to be synthesized as a single unit, allowing post synthesis connection of the two elements, etc.).

In some embodiments, a linker is any suitable chemical moiety capable of linking, connecting, or tethering a non-luminescent element to an interaction element. In some embodiments, a linker is a polymer of one or more repeating or non-repeating monomer units (e.g., nucleic acid, amino acid, carbon-containing polymer, carbon chain, etc.). When a non-luminescent element and interaction element are part of a fusion protein, a linker (when present) is typically an amino acid chain. When a non-luminescent element and interaction element are tethered together after the expression of the individual elements, a linker may comprise any chemical moiety with functional (or reactive) groups at either end that are reactive with functional groups on the signal and interaction elements, respectively. Any suitable moiety capable of tethering the signal and interaction elements may find use as a linker.

A wide variety of linkers may be used. In some embodiments, the linker is a single covalent bond. In some embodiments, the linker comprises a linear or branched, cyclic or heterocyclic, saturated or unsaturated, structure having 1-20 nonhydrogen atoms (e.g., C, N, P, O and S) and is composed of any combination of alkyl, ether, thioether, imine, carboxylic, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. In some embodiments, linkers are longer than 20 nonhydrogen atoms (e.g. 21 non-hydrogen atoms, 25 non-hydrogen atoms, 30 non-hydrogen atoms, 40 non-hydrogen atoms, 50 non-hydrogen atoms, 100 non-hydrogen atoms, etc.) In some embodiments, the linker comprises 1-50 non-hydrogen atoms (in addition to hydrogen atoms) selected from the group of C, N, P, O and S (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40,41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 non-hydrogen atoms).

The present invention is not limited by the types of linkers available. The signal and interaction elements are linked, either directly (e.g. linker consists of a single covalent bond) or linked via a suitable linker. The present invention is not limited to any particular linker group. A variety of linker groups are contemplated, and suitable linkers could comprise, but are not limited to, alkyl groups, methylene carbon chains, ether, polyether, alkyl amide linker, a peptide linker, a modified peptide linker, a Poly(ethylene glycol) (PEG) linker, a streptavidin-biotin or avidin-biotin linker, polyaminoacids (e.g. polylysine), functionalised PEG, polysaccharides,

glycosaminoglycans, dendritic polymers (WO93/06868 and by Tomalia et al. in Angew. Chem. Int. Ed. Engl. 29:138-175 (1990), herein incorporated by reference in their entireties), PEG-chelant polymers (W94/08629, WO94/09056 and WO96/26754, herein incorporated by reference in their entireties), oligonucleotide linker, phospholipid derivatives, alkenyl chains, alkynyl chains, disulfide, or a combination thereof. In some embodiments, the linker is cleavable (e.g., enzymatically (e.g., TEV protease site), chemically, photoinduced, etc.

In some embodiments, substantially non-luminescent peptides and polypeptides are provided with less than 100% sequence identity and/or similarity to any portion of an existing luciferase (e.g., a firefly luciferase, a Renilla luciferase, an Oplophorus luciferase, enhanced Oplophorus luciferases as described in U.S. Pat. App. 2010/0281552 and U.S. Pat. App. 2012/0174242, herein incorporated by reference in their entireties). Certain embodiments of the present invention involve the formation of bioluminescent complexes of non-luminescent peptides and polypeptides with less than 100% sequence identity with all or a portion (e.g., 8 or more amino acids, less than about 25 amino acids for peptides) of SEQ ID NO: 2157 (e.g., complete NANOLUC sequence). Certain embodiments of the present invention involve the formation of bioluminescent complexes of non-luminescent peptides and polypeptides with less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity with all or a portion (e.g., 8 or more amino acids, less than about 25 amino acids for peptides) of SEQ ID NO: 2157 (e.g., complete NANOLUC sequence). In some embodiments, non-luminescent peptides and polypeptides are provided with less than 100% sequence similarity with a portion (e.g., 8 or more amino acids, less than about 25 amino acids for peptides) of SEQ ID NO: 2157 (e.g., peptides and polypeptides that interact to form bioluminescent complexes). In some embodiments, non-luminescent peptides and polypeptides are provided with less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence similarity with a portion (e.g., 8 or more amino acids, less than about 25 amino acids for peptides) of SEQ ID NO: 2157 (e.g., peptides and polypeptides that interact to form bioluminescent complexes). Non-luminescent peptides are provided that have less than 100% sequence identity and/or similarity with about a 25 amino acid or less portion of SEQ ID NO: 2157, wherein such peptides form a bioluminescent complex when combined under appropriate conditions (e.g., stabilized by an interaction pair) with a polypeptide having less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity and/or similarity with another portion SEQ ID NO: 2157. Non-luminescent peptides are provided that have less than 100%sequence identity and/or similarity with about a 25 amino acid or less portion of SEQ ID NO: 2157, wherein such

peptides form a bioluminescent complex when combined under appropriate conditions (e.g., stabilized by an interaction pair) with a polypeptide having less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity and/or similarity with another portion SEQ ID NO: 2157. Non-luminescent peptides are provided that have less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity and/or similarity with about a 25 amino acid or less portion of SEQ ID NO: 2157, wherein such peptides form a bioluminescent complex when combined under appropriate conditions (e.g., stabilized by an interaction pair) with a polypeptide having less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity and/or similarity with another portion SEQ ID NO: 2157. Similarly, non-luminescent polypeptides are provided that have less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity or similarity with a portion of SEQ ID NO: 2157, wherein such polypeptides form a bioluminescent complex when combined under appropriate conditions (e.g., stabilized by an interaction pair) with a peptide having less than 100%, but optionally more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity and/or similarity with another portion SEQ ID NO: 2157. In some embodiments, non-luminescent peptides with less than 100sequence identity or similarity with SEQ ID NO: 2 are provided. In some embodiments, non-luminescent peptides with less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity or similarity with SEQ ID NO: 2 are provided. In some embodiments, non-luminescent polypeptides with less than 100 sequence identity or similarity with SEQ ID NO: 440 are provided. In some embodiments, non-luminescent polypeptides with less than 100%, but more than 40% (e.g., >40%, >45%, >50%, >55%, >60%, >65%, >70%, >75%, >80%, >85%, >90%, >95%, >98%, >99%) sequence identity or similarity with SEQ ID NO: 440 are provided.

In some embodiments, non-luminescent peptides that find use in embodiments of the present invention include peptides with one or more amino acid substitutions, deletions, or additions from GVTGWRLCKRISA (SEQ ID NO: 236). In some embodiments, the present invention provides peptides comprising one or more of amino acid sequences of Table 1, and/or nucleic acids comprising the nucleic acid sequences of Table 1 (which code for the peptide sequences of Table 1).

Table 1. Peptide sequences

SEQ ID NO.	PEPTIDE NO.	POLYMER	SEQUENCE
3	NLpep2 (w/ Met)	N.A.	ATGGACGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCG
4	NLpep2 (w/ Met)	A.A.	MDVTGWRLCERILA
5	NLpep3 (w/ Met)	N.A.	ATGGGAGTGACCGCCTGGCGGCTGTGCGAACGCATTCTGGCG
6	NLpep3 (w/ Met)	A.A.	MGVTAWRLCERILA
7	NLpep4 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTCTGGCG
8	NLpep4 (w/ Met)	A.A.	MGVTGWRLCKRILA
9	NLpep5 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTAGCGCG
10	NLpep5 (w/ Met)	A.A.	MGVTGWRLCERISA
11	NLpep6 (w/ Met)	N.A.	ATGGACGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
12	NLpep6 (w/ Met)	A.A.	MDVTGWRLCKRISA
13	NLpep7 (w/ Met)	N.A.	ATGGACGTGACCGGCTGGCGGCTGTGCAAGCGCATTCTGGCG
14	NLpep7 (w/ Met)	A.A.	MDVTGWRLCKRILA
15	NLpep8 (w/ Met)	N.A.	ATGGACGTGACCGGCTGGCGGCTGTGCGAACGCATTAGCGCG
16	NLpep8 (w/ Met)	A.A.	MDVTGWRLCERISA
17	NLpep9 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
18	NLpep9 (w/ Met)	A.A.	MGVTGWRLCKRISA
19	NLpep10 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGAACGAACGCATTCTGGCG
20	NLpep10 (w/ Met)	A.A.	MGVTGWRLNERILA
21	NLpep11 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGCAGGAACGCATTCTGGCG
22	NLpep11 (w/ Met)	A.A.	MGVTGWRLQERILA
23	NLpep12 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGAAGAAGCGCCGGAGCCGG
24	NLpep12 (w/ Met)	A.A.	MGVTGWRLKKRRSR
25	NLpep13 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
26	NLpep13 (w/ Met)	A.A.	MNVTGWRLCKRISA
27	NLpep14 (w/ Met)	N.A.	ATGAGCGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
28	NLpep14 (w/ Met)	A.A.	MSVTGWRLCKRISA
29	NLpep15 (w/ Met)	N.A.	ATGGAGGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
30	NLpep15 (w/ Met)	A.A.	MEVTGWRLCKRISA
31	NLpep16 (w/ Met)	N.A.	ATGGGCGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
32	NLpep16 (w/ Met)	A.A.	MHVTGWRLCKRISA
33	NLpep17 (w/ Met)	N.A.	ATGGGACACACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
34	NLpep17 (w/ Met)	A.A.	MGITGWRLCKRISA
35	NLpep18 (w/ Met)	N.A.	ATGGGAGCCACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
36	NLpep18 (w/ Met)	A.A.	MGATGWRLCKRISA
37	NLpep19 (w/ Met)	N.A.	ATGGGAAAGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
38	NLpep19 (w/ Met)	A.A.	MGKTGWRLCKRISA

39	NLpep20 (w/ Met)	N.A.	ATGGGACAGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
40	NLpep20 (w/ Met)	A.A.	MGQTGWRLCKRISA
41	NLpep21 (w/ Met)	N.A.	ATGGGAAGCACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
42	NLpep21 (w/ Met)	A.A.	MGSTGWRLCKRISA
43	NLpep22 (w/ Met)	N.A.	ATGGGAGTGGTGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
44	NLpep22 (w/ Met)	A.A.	MGVVGWRLCKRISA
45	NLpep23 (w/ Met)	N.A.	ATGGGAGTGAAGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
46	NLpep23 (w/ Met)	A.A.	MGVKGWRLCKRISA
47	NLpep24 (w/ Met)	N.A.	ATGGGAGTGCAGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
48	NLpep24 (w/ Met)	A.A.	MGVQGWRLCKRISA
49	NLpep25 (w/ Met)	N.A.	ATGGGAGTGACCGGCACCCGGCTGTGCAAGCGCATTAGCGCG
50	NLpep25 (w/ Met)	A.A.	MGVTGTRLCKRISA
51	NLpep26 (w/ Met)	N.A.	ATGGGAGTGACCGGCAAGCGGCTGTGCAAGCGCATTAGCGCG
52	NLpep26 (w/ Met)	A.A.	MGVTGKRLCKRISA
53	NLpep27 (w/ Met)	N.A.	ATGGGAGTGACCGGCGTGCGGCTGTGCAAGCGCATTAGCGCG
54	NLpep27 (w/ Met)	A.A.	MGVTGVRLCKRISA
55	NLpep28 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCACTGCAAGCGCATTAGCGCG
56	NLpep28 (w/ Met)	A.A.	MGVTGWRICKRISA
57	NLpep29 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGGTGTGCAAGCGCATTAGCGCG
58	NLpep29 (w/ Met)	A.A.	MGVTGWRVCKRISA
59	NLpep30 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGACCTGCAAGCGCATTAGCGCG
60	NLpep30 (w/ Met)	A.A.	MGVTGWRTCKRISA
61	NLpep31 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGTACTGCAAGCGCATTAGCGCG
62	NLpep31 (w/ Met)	A.A.	MGVTGWRYCKRISA
63	NLpep32 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGAAGTGCAAGCGCATTAGCGCG
64	NLpep32 (w/ Met)	A.A.	MGVTGWRKCKRISA
65	NLpep33 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGAACAAGCGCATTAGCGCG
66	NLpep33 (w/ Met)	A.A.	MGVTGWRLNKRISA
67	NLpep34 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGACCAAGCGCATTAGCGCG
68	NLpep34 (w/ Met)	A.A.	MGVTGWRLTKRISA
69	NLpep35 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGAAGATTAGCGCG
70	NLpep35 (w/ Met)	A.A.	MGVTGWRLCKKISA
71	NLpep36 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGAACATTAGCGCG
72	NLpep36 (w/ Met)	A.A.	MGVTGWRLCKNISA
73	NLpep37 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCGTGAGCGCG
74	NLpep37 (w/ Met)	A.A.	MGVTGWRLCKRVSA
75	NLpep38 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCCAGAGCGCG
76	NLpep38 (w/ Met)	A.A.	MGVTGWRLCKRQSA
77	NLpep39 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCGAGAGCGCG
78	NLpep39 (w/ Met)	A.A.	MGVTGWRLCKRESA
79	NLpep40 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCCGGAGCGCG

80	NLpep40 (w/ Met)	A.A.	MGVTGWRLCKRRSA
81	NLpep41 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCTTCAGCGCG
82	NLpep41 (w/ Met)	A.A.	MGVTGWRLCKRFSA
83	NLpep42 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCAAC
84	NLpep42 (w/ Met)	A.A.	MGVTGWRLCKRISN
85	NLpep43 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCACC
86	NLpep43 (w/ Met)	A.A.	MGVTGWRLCKRIST
87	NLpep44 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCCGG
88	NLpep44 (w/ Met)	A.A.	MGVTGWRLCKRISR
89	NLpep45 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCCTG
90	NLpep45 (w/ Met)	A.A.	MGVTGWRLCKRISL
91	NLpep46 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGAG
92	NLpep46 (w/ Met)	A.A.	MGVTGWRLCKRISE
93	NLpep47 (w/ Met)	N.A.	ATGGGAGTGACCGGCTTCCGGCTGTGCAAGCGCATTAGCGCG
94	NLpep47 (w/ Met)	A.A.	MGVTGFRLCKRISA
95	NLpep48 (w/ Met)	N.A.	ATGGGAGTGACCGGCTACCGGCTGTGCAAGCGCATTAGCGCG
96	NLpep48 (w/ Met)	A.A.	MGVTGYRLCKRISA
97	NLpep49(w/ Met)	N.A.	ATGGGAGTGACCGGCGAGCGGCTGTGCAAGCGCATTAGCGCG
98	NLpep49(w/ Met)	A.A.	MGVTGERLCKRISA
99	NLpep50 (w/ Met)	N.A.	ATGCAGGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
100	NLpep50 (w/ Met)	A.A.	MQVTGWRLCKRISA
101	NLpep51 (w/ Met)	N.A.	ATGACCGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
102	NLpep51 (w/ Met)	A.A.	MTVTGWRLCKRISA
103	NLpep52 (w/ Met)	N.A.	ATGGGAGTGGAGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
104	NLpep52 (w/ Met)	A.A.	MGVEGWRLCKRISA
105	NLpep53 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
106	NLpep53 (w/ Met)	A.A.	MGVTGWRLFKRISA
107	NLpep54 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTACAAGCGCATTAGCGCG
108	NLpep54 (w/ Met)	A.A.	MGVTGWRLYKRISA
109	NLpep55 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGAGCAAGCGCATTAGCGCG
110	NLpep55 (w/ Met)	A.A.	MGVTGWRLSKRISA
111	NLpep56 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGGGCAAGCGCATTAGCGCG
112	NLpep56 (w/ Met)	A.A.	MGVTGWRLHKRISA
113	NLpep57 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGATGAAGCGCATTAGCGCG
114	NLpep57 (w/ Met)	A.A.	MGVTGWRLMKRISA
115	NLpep58 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGGCCAAGCGCATTAGCGCG
116	NLpep58 (w/ Met)	A.A.	MGVTGWRLAKRISA
117	NLpep59 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGCAGAAGCGCATTAGCGCG
118	NLpep59 (w/ Met)	A.A.	MGVTGWRLQKRISA
119	NLpep60 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGCTGAAGCGCATTAGCGCG
120	NLpep60 (w/ Met)	A.A.	MGVTGWRLLKRISA

121	NLpep61 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGAAGAAGCGCATTAGCGCG
122	NLpep61 (w/ Met)	A.A.	MGVTGWRLKKRISA
123	NLpep62 (w/ Met)	N.A.	ATGAACCACACCGGCTGGCGGCTGAACAAGAAGGTGAGCAAC
124	NLpep62 (w/ Met)	A.A.	MNITGWRLNKKVSN
125	NLpep63 (w/ Met)	N.A.	ATGAACCACACCGGCTACCGGCTGAACAAGAAGGTGAGCAAC
126	NLpep63 (w/ Met)	A.A.	MNITGYRLNKKVSN
127	NLpep64 (w/ Met)	N.A.	ATGTGCGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
128	NLpep64 (w/ Met)	A.A.	MCVTGWRLFKRISA
129	NLpep65 (w/ Met)	N.A.	ATGCCCGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
130	NLpep65 (w/ Met)	A.A.	MPVTGWRLFKRISA
131	NLpep66 (w/ Met)	N.A.	ATGAACCACACCGGCTACCGGCTGTTCAAGAAGGTGAGCAAC
132	NLpep66 (w/ Met)	A.A.	MNITGYRLFKKVSN
133	NLpep67 (w/ Met)	N.A.	ATGAACGTGACCGGCTACCGGCTGTTCAAGAAGGTGAGCAAC
134	NLpep67 (w/ Met)	A.A.	MNVTGYRLFKKVSN
135	NLpep68 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCAAGAAGGTGAGCAAC
136	NLpep68 (w/ Met)	A.A.	MNVTGWRLFKKVSN
137	NLpep69 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
138	NLpep69 (w/ Met)	A.A.	MNVTGWRLFKKISN
139	NLpep70 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCAAC
140	NLpep70 (w/ Met)	A.A.	MNVTGWRLFKRISN
141	NLpep71 (w/ Met)	N.A.	ATGGGAGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCAAC
142	NLpep71 (w/ Met)	A.A.	MGVTGWRLFKRISN
143	NLpep72 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCGAACGCATTAGCAAC
144	NLpep72 (w/ Met)	A.A.	MNVTGWRLFERISN
145	NLpep73 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCAAGCGCATTCTGAAC
146	NLpep73 (w/ Met)	A.A.	MNVTGWRLFKRILN
147	NLpep74 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
148	NLpep74 (w/ Met)	A.A.	MNVTGWRLFKRISA
149	NLpep75 (w/ Met)	N.A.	ATGAACGTGACCGGCTGGCGGCTGTTCGAAAAGATTAGCAAC
150	NLpep75 (w/ Met)	A.A.	MNVTGWRLFEKISN
151	NLpep76 (w/ Met)	N.A.	ATGAACGTGAGCGGCTGGCGGCTGTTCGAAAAGATTAGCAAC
152	NLpep76 (w/ Met)	A.A.	MNVSGWRLFEKISN
153	NLpep77 (w/ Met)	N.A.	ATG-GTGACCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
154	NLpep77 (w/ Met)	A.A.	M-VTGWRLFKKISN
155	NLpep78 (w/ Met)	N.A.	ATGAACGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
156	NLpep78 (w/ Met)	A.A.	MNVSGWRLFKKISN
157	NLpep79 (w/ Met)	N.A.	ATGAACGTGACCGGCTACCGGCTGTTCAAGAAGATTAGCAAC
158	NLpep79 (w/ Met)	A.A.	MNVTGYRLFKKISN
159	NLpep80(w/ Met)	N.A.	ATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
160	NLpep80(w/ Met)	A.A.	MVSGWRLFKKISN
161	NLpep81 (w/ Met)	N.A.	ATGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC

440	NLpep81 (w/ Met)	A.A.	MSGWRLFKKISN
163	NLpep82 (w/ Met)	N.A.	ATGGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
164	NLpep82 (w/ Met)	A.A.	MGWRLFKKISN
165	NLpep83 (w/ Met)	N.A.	ATGAACGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGC
166	NLpep83 (w/ Met)	A.A.	MNVSGWRLFKKIS
167	NLpep84 (w/ Met)	N.A.	ATGAACGTGAGCGGCTGGCGGCTGTTCAAGAAGATT
168	NLpep84 (w/ Met)	A.A.	MNVSGWRLFKKI
169	NLpep85 (w/ Met)	N.A.	ATGAACGTGAGCGGCTGGCGGCTGTTCAAGAAG
170	NLpep85 (w/ Met)	A.A.	MNVSGWRLFKK
171	NLpep86 (w/ Met)	N.A.	ATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGC
172	NLpep86 (w/ Met)	A.A.	MVSGWRLFKKIS
173	NLpep87 (w/ Met)	N.A.	ATGAGCGGCTGGCGGCTGTTCAAGAAGATT
174	NLpep87 (w/ Met)	A.A.	MSGWRLFKKI
175	NLpep88 (w/ Met)	N.A.	ATGAACGTGAGCGGCTGGGGCCTGTTCAAGAAGATTAGCAAC
176	NLpep88 (w/ Met)	A.A.	MNVSGWGLFKKISN
177	NLpep89 (w/ Met)	N.A.	ATGCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
178	NLpep89 (w/ Met)	A.A.	MPVSGWRLFKKISN
179	NLpep90 (w/ Met)	N.A.	ATGAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
180	NLpep90 (w/ Met)	A.A.	MNPVSGWRLFKKISN
181	NLpep91 (w/ Met)	N.A.	ATGATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
182	NLpep91 (w/ Met)	A.A.	MINPVSGWRLFKKISN
183	NLpep92 (w/ Met)	N.A.	ATGACCATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
184	NLpep92 (w/ Met)	A.A.	MTINPVSGWRLFKKISN
185	NLpep93 (w/ Met)	N.A.	ATGGTGACCATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
186	NLpep93 (w/ Met)	A.A.	MVTINPVSGWRLFKKISN
187	NLpep94 (w/ Met)	N.A.	ATGCGGGTGACCATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
188	NLpep94 (w/ Met)	A.A.	MRVTINPVSGWRLFKKISN
189	NLpep95 (w/ Met)	N.A.	ATGAGCGGCTGGCGGCTGCTGAAGAAGATT
190	NLpep95 (w/ Met)	A.A.	MSGWRLLKKI
191	NLpep96 (w/ Met)	N.A.	ATGACCGGCTACCGGCTGCTGAAGAAGATT
192	NLpep96 (w/ Met)	A.A.	MTGYRLLKKI
193	NLpep97 (w/ Met)	N.A.	ATGAGCGGCTGGCGGCTGTTCAAGAAG
194	NLpep97 (w/ Met)	A.A.	MSGWRLFKK
195	NLpep98 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCAAGAAGATTAGC
196	NLpep98 (w/ Met)	A.A.	MVTGYRLFKKIS
197	NLpep99 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGAAGATTAGC
198	NLpep99 (w/ Met)	A.A.	MVTGYRLFEKIS

199	NLpep100 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGCAGATTAGC
200	NLpep100 (w/ Met)	A.A.	MVTGYRLFEQIS
201	NLpep101 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGAAGGAGAGC
202	NLpep101 (w/ Met)	A.A.	MVTGYRLFEKES
203	NLpep102 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGCAGGAGAGC
204	NLpep102 (w/ Met)	A.A.	MVTGYRLFEQES
205	NLpep103 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGCAGGAGCTG
206	NLpep103 (w/ Met)	A.A.	MVTGYRLFEQEL
207	NLpep104 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGAAGATTAGC
208	NLpep104 (w/ Met)	A.A.	MVEGYRLFEKIS
209	NLpep105 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGCAGATTAGC
210	NLpep105 (w/ Met)	A.A.	MVEGYRLFEQIS
211	NLpep106 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGAAGGAGAGC
212	NLpep106 (w/ Met)	A.A.	MVEGYRLFEKES
213	NLpep107 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGCAGGAGAGC
214	NLpep107 (w/ Met)	A.A.	MVEGYRLFEQES
215	NLpep108 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGCAGGAGCTG
216	NLpep108 (w/ Met)	A.A.	MVEGYRLFEQEL
217	NLpep109 (w/ Met)	N.A.	ATGATTAGCGGCTGGCGGCTGATGAAGAACATTAGC
218	NLpep109 (w/ Met)	A.A.	MISGWRLMKNIS
219	NLpep110 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCAAGAAGATTAGC
220	NLpep110 (w/ Met)	A.A.	MVEGYRLFKKIS
221	NLpep2 (w/o Met)	N.A.	GACGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCG
222	NLpep2 (w/o Met)	A.A.	DVTGWRLCERILA
223	NLpep3 (w/o Met)	N.A.	GGAGTGACCGCCTGGCGGCTGTGCGAACGCATTCTGGCG
224	NLpep3 (w/o Met)	A.A.	GVTAWRLCERILA
225	NLpep4 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTCTGGCG
226	NLpep4 (w/o Met)	A.A.	GVTGWRLCKRILA
227	NLpep5 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCGAACGCATTAGCGCG
228	NLpep5 (w/o Met)	A.A.	GVTGWRLCERISA
229	NLpep6 (w/o Met)	N.A.	GACGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
230	NLpep6 (w/o Met)	A.A.	DVTGWRLCKRISA
231	NLpep7 (w/o Met)	N.A.	GACGTGACCGGCTGGCGGCTGTGCAAGCGCATTCTGGCG
232	NLpep7 (w/o Met)	A.A.	DVTGWRLCKRILA
233	NLpep8 (w/o Met)	N.A.	GACGTGACCGGCTGGCGGCTGTGCGAACGCATTAGCGCG
234	NLpep8 (w/o Met)	A.A.	DVTGWRLCERISA
235	NLpep9 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
236	NLpep9 (w/o Met)	A.A.	GVTGWRLCKRISA
237	NLpep10 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGAACGAACGCATTCTGGCG
238	NLpep10 (w/o Met)	A.A.	GVTGWRLNERILA
239	NLpep11 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGCAGGAACGCATTCTGGCG

240	NLpep11 (w/o Met)	A.A.	GVTGWRLQERILA
241	NLpep12 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGAAGAAGCGCCGGAGCCGG
242	NLpep12 (w/o Met)	A.A.	GVTGWRLKKRRSR
243	NLpep13 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
244	NLpep13 (w/o Met)	A.A.	NVTGWRLCKRISA
245	NLpep14 (w/o Met)	N.A.	AGCGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
246	NLpep14 (w/o Met)	A.A.	SVTGWRLCKRISA
247	NLpep15 (w/o Met)	N.A.	GAGGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
248	NLpep15 (w/o Met)	A.A.	EVTGWRLCKRISA
249	NLpep16 (w/o Met)	N.A.	GGCGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
250	NLpep16 (w/o Met)	A.A.	HVTGWRLCKRISA
251	NLpep17 (w/o Met)	N.A.	GGACACACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
252	NLpep17 (w/o Met)	A.A.	GITGWRLCKRISA
253	NLpep18 (w/o Met)	N.A.	GGAGCCACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
254	NLpep18 (w/o Met)	A.A.	GATGWRLCKRISA
255	NLpep19 (w/o Met)	N.A.	GGAAAGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
256	NLpep19 (w/o Met)	A.A.	GKTGWRLCKRISA
257	NLpep20 (w/o Met)	N.A.	GGACAGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
258	NLpep20 (w/o Met)	A.A.	GQTGWRLCKRISA
259	NLpep21 (w/o Met)	N.A.	GGAAGCACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
260	NLpep21 (w/o Met)	A.A.	GSTGWRLCKRISA
261	NLpep22 (w/o Met)	N.A.	GGAGTGGTGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
262	NLpep22 (w/o Met)	A.A.	GVVGWRLCKRISA
263	NLpep23 (w/o Met)	N.A.	GGAGTGAAGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
264	NLpep23 (w/o Met)	A.A.	GVKGWRLCKRISA
265	NLpep24 (w/o Met)	N.A.	GGAGTGCAGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
266	NLpep24 (w/o Met)	A.A.	GVQGWRLCKRISA
267	NLpep25 (w/o Met)	N.A.	GGAGTGACCGGCACCCGGCTGTGCAAGCGCATTAGCGCG
268	NLpep25 (w/o Met)	A.A.	GVTGTRLCKRISA
269	NLpep26 (w/o Met)	N.A.	GGAGTGACCGGCAAGCGGCTGTGCAAGCGCATTAGCGCG
270	NLpep26 (w/o Met)	A.A.	GVTGKRLCKRISA
271	NLpep27 (w/o Met)	N.A.	GGAGTGACCGGCGTGCGGCTGTGCAAGCGCATTAGCGCG
272	NLpep27 (w/o Met)	A.A.	GVTGVRLCKRISA
273	NLpep28 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCACTGCAAGCGCATTAGCGCG
274	NLpep28 (w/o Met)	A.A.	GVTGWRICKRISA
275	NLpep29 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGGTGTGCAAGCGCATTAGCGCG
276	NLpep29 (w/o Met)	A.A.	GVTGWRVCKRISA
277	NLpep30 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGACCTGCAAGCGCATTAGCGCG
278	NLpep30 (w/o Met)	A.A.	GVTGWRTCKRISA
279	NLpep31 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGTACTGCAAGCGCATTAGCGCG
280	NLpep31 (w/o Met)	A.A.	GVTGWRYCKRISA

281	NLpep32 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGAAGTGCAAGCGCATTAGCGCG
282	NLpep32 (w/o Met)	A.A.	GVTGWRKCKRISA
283	NLpep33 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGAACAAGCGCATTAGCGCG
284	NLpep33 (w/o Met)	A.A.	GVTGWRLNKRISA
285	NLpep34 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGACCAAGCGCATTAGCGCG
286	NLpep34 (w/o Met)	A.A.	GVTGWRLTKRISA
287	NLpep35 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGAAGATTAGCGCG
288	NLpep35 (w/o Met)	A.A.	GVTGWRLCKKISA
289	NLpep36 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGAACATTAGCGCG
290	NLpep36 (w/o Met)	A.A.	GVTGWRLCKNISA
291	NLpep37 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCGTGAGCGCG
292	NLpep37 (w/o Met)	A.A.	GVTGWRLCKRVSA
293	NLpep38 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCCAGAGCGCG
294	NLpep38 (w/o Met)	A.A.	GVTGWRLCKRQSA
295	NLpep39 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCGAGAGCGCG
296	NLpep39 (w/o Met)	A.A.	GVTGWRLCKRESA
297	NLpep40 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCCGGAGCGCG
298	NLpep40 (w/o Met)	A.A.	GVTGWRLCKRRSA
299	NLpep41 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCTTCAGCGCG
300	NLpep41 (w/o Met)	A.A.	GVTGWRLCKRFSA
301	NLpep42 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCAAC
302	NLpep42 (w/o Met)	A.A.	GVTGWRLCKRISN
303	NLpep43 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCACC
304	NLpep43 (w/o Met)	A.A.	GVTGWRLCKRIST
305	NLpep44 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCCGG
306	NLpep44 (w/o Met)	A.A.	GVTGWRLCKRISR
307	NLpep45 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCCTG
308	NLpep45 (w/o Met)	A.A.	GVTGWRLCKRISL
309	NLpep46 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGAG
310	NLpep46 (w/o Met)	A.A.	GVTGWRLCKRISE
311	NLpep47 (w/o Met)	N.A.	GGAGTGACCGGCTTCCGGCTGTGCAAGCGCATTAGCGCG
312	NLpep47 (w/o Met)	A.A.	GVTGFRLCKRISA
313	NLpep48 (w/o Met)	N.A.	GGAGTGACCGGCTACCGGCTGTGCAAGCGCATTAGCGCG
314	NLpep48 (w/o Met)	A.A.	GVTGYRLCKRISA
315	NLpep49(w/o Met)	N.A.	GGAGTGACCGGCGAGCGGCTGTGCAAGCGCATTAGCGCG
316	NLpep49(w/o Met)	A.A.	GVTGERLCKRISA
317	NLpep50 (w/o Met)	N.A.	CAGGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
318	NLpep50 (w/o Met)	A.A.	QVTGWRLCKRISA
319	NLpep51 (w/o Met)	N.A.	ACCGTGACCGGCTGGCGGCTGTGCAAGCGCATTAGCGCG
320	NLpep51 (w/o Met)	A.A.	TVTGWRLCKRISA
321	NLpep52 (w/o Met)	N.A.	GGAGTGGAGGGCTGGCGGCTGTGCAAGCGCATTAGCGCG

322	NLpep52 (w/o Met)	A.A.	GVEGWRLCKRISA
323	NLpep53 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
324	NLpep53 (w/o Met)	A.A.	GVTGWRLFKRISA
325	NLpep54 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTACAAGCGCATTAGCGCG
326	NLpep54 (w/o Met)	A.A.	GVTGWRLYKRISA
327	NLpep55 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGAGCAAGCGCATTAGCGCG
328	NLpep55 (w/o Met)	A.A.	GVTGWRLSKRISA
329	NLpep56 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGGGCAAGCGCATTAGCGCG
330	NLpep56 (w/o Met)	A.A.	GVTGWRLHKRISA
331	NLpep57 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGATGAAGCGCATTAGCGCG
332	NLpep57 (w/o Met)	A.A.	GVTGWRLMKRISA
333	NLpep58 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGGCCAAGCGCATTAGCGCG
334	NLpep58 (w/o Met)	A.A.	GVTGWRLAKRISA
335	NLpep59 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGCAGAAGCGCATTAGCGCG
336	NLpep59 (w/o Met)	A.A.	GVTGWRLQKRISA
337	NLpep60 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGCTGAAGCGCATTAGCGCG
338	NLpep60 (w/o Met)	A.A.	GVTGWRLLKRISA
339	NLpep61 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGAAGAAGCGCATTAGCGCG
340	NLpep61 (w/o Met)	A.A.	GVTGWRLKKRISA
341	NLpep62 (w/o Met)	N.A.	AACCACACCGGCTGGCGGCTGAACAAGAAGGTGAGCAAC
342	NLpep62 (w/o Met)	A.A.	NITGWRLNKKVSN
343	NLpep63 (w/o Met)	N.A.	AACCACACCGGCTACCGGCTGAACAAGAAGGTGAGCAAC
344	NLpep63 (w/o Met)	A.A.	NITGYRLNKKVSN
345	NLpep64 (w/o Met)	N.A.	TGCGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
346	NLpep64 (w/o Met)	A.A.	CVTGWRLFKRISA
347	NLpep65 (w/o Met)	N.A.	CCCGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
348	NLpep65 (w/o Met)	A.A.	PVTGWRLFKRISA
349	NLpep66 (w/o Met)	N.A.	AACCACACCGGCTACCGGCTGTTCAAGAAGGTGAGCAAC
350	NLpep66 (w/o Met)	A.A.	NITGYRLFKKVSN
351	NLpep67 (w/o Met)	N.A.	AACGTGACCGGCTACCGGCTGTTCAAGAAGGTGAGCAAC
352	NLpep67 (w/o Met)	A.A.	NVTGYRLFKKVSN
353	NLpep68 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCAAGAAGGTGAGCAAC
354	NLpep68 (w/o Met)	A.A.	NVTGWRLFKKVSN
355	NLpep69 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
356	NLpep69 (w/o Met)	A.A.	NVTGWRLFKKISN
357	NLpep70 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCAAC
358	NLpep70 (w/o Met)	A.A.	NVTGWRLFKRISN
359	NLpep71 (w/o Met)	N.A.	GGAGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCAAC
360	NLpep71 (w/o Met)	A.A.	GVTGWRLFKRISN
361	NLpep72 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCGAACGCATTAGCAAC
362	NLpep72 (w/o Met)	A.A.	NVTGWRLFERISN

363	NLpep73 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCAAGCGCATTCTGAAC
364	NLpep73 (w/o Met)	A.A.	NVTGWRLFKRILN
365	NLpep74 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCAAGCGCATTAGCGCG
366	NLpep74 (w/o Met)	A.A.	NVTGWRLFKRISA
367	NLpep75 (w/o Met)	N.A.	AACGTGACCGGCTGGCGGCTGTTCGAAAAGATTAGCAAC
368	NLpep75 (w/o Met)	A.A.	NVTGWRLFEKISN
369	NLpep76 (w/o Met)	N.A.	AACGTGAGCGGCTGGCGGCTGTTCGAAAAGATTAGCAAC
370	NLpep76 (w/o Met)	A.A.	NVSGWRLFEKISN
371	NLpep77 (w/o Met)	N.A.	GTGACCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
372	NLpep77 (w/o Met)	A.A.	VTGWRLFKKISN
373	NLpep78 (w/o Met)	N.A.	AACGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
374	NLpep78 (w/o Met)	A.A.	NVSGWRLFKKISN
375	NLpep79 (w/o Met)	N.A.	AACGTGACCGGCTACCGGCTGTTCAAGAAGATTAGCAAC
376	NLpep79 (w/o Met)	A.A.	NVTGYRLFKKISN
377	NLpep80(w/o Met)	N.A.	GTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
378	NLpep80(w/o Met)	A.A.	VSGWRLFKKISN
379	NLpep81 (w/o Met)	N.A.	AGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
380	NLpep81 (w/o Met)	A.A.	SGWRLFKKISN
381	NLpep82 (w/o Met)	N.A.	GGCTGGCGGCTGTTCAAGAAGATTAGCAAC
382	NLpep82 (w/o Met)	A.A.	GWRLFKKISN
383	NLpep83 (w/o Met)	N.A.	AACGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGC
384	NLpep83 (w/o Met)	A.A.	NVSGWRLFKKIS
385	NLpep84 (w/o Met)	N.A.	AACGTGAGCGGCTGGCGGCTGTTCAAGAAGATT
386	NLpep84 (w/o Met)	A.A.	NVSGWRLFKKI
387	NLpep85 (w/o Met)	N.A.	AACGTGAGCGGCTGGCGGCTGTTCAAGAAG
388	NLpep85 (w/o Met)	A.A.	NVSGWRLFKK
389	NLpep86 (w/o Met)	N.A.	GTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGC
390	NLpep86 (w/o Met)	A.A.	VSGWRLFKKIS
391	NLpep87 (w/o Met)	N.A.	AGCGGCTGGCGGCTGTTCAAGAAGATT
392	NLpep87 (w/o Met)	A.A.	SGWRLFKKI
393	NLpep88 (w/o Met)	N.A.	AACGTGAGCGGCTGGGGCCTGTTCAAGAAGATTAGCAAC
394	NLpep88 (w/o Met)	A.A.	NVSGWGLFKKISN
395	NLpep89 (w/o Met)	N.A.	CCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
396	NLpep89 (w/o Met)	A.A.	PVSGWRLFKKISN
397	NLpep90 (w/o Met)	N.A.	AACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
398	NLpep90 (w/o Met)	A.A.	NPVSGWRLFKKISN
399	NLpep91 (w/o Met)	N.A.	ATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
400	NLpep91 (w/o Met)	A.A.	INPVSGWRLFKKISN
401	NLpep92 (w/o Met)	N.A.	ACCATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
402	NLpep92 (w/o Met)	A.A.	TINPVSGWRLFKKISN

403	NLpep93 (w/o Met)	N.A.	GTGACCATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
404	NLpep93 (w/o Met)	A.A.	VTINPVSGWRLFKKISN
405	NLpep94 (w/o Met)	N.A.	CGGGTGACCATCAACCCCGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCAAC
406	NLpep94 (w/o Met)	A.A.	RVTINPVSGWRLFKKISN
407	NLpep95 (w/o Met)	N.A.	AGCGGCTGGCGGCTGCTGAAGAAGATT
408	NLpep95 (w/o Met)	A.A.	SGWRLLKKI
409	NLpep96 (w/o Met)	N.A.	ACCGGCTACCGGCTGCTGAAGAAGATT
410	NLpep96 (w/o Met)	A.A.	TGYRLLKKI
411	NLpep97 (w/o Met)	N.A.	AGCGGCTGGCGGCTGTTCAAGAAG
412	NLpep97 (w/o Met)	A.A.	SGWRLFKK
413	NLpep98 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCAAGAAGATTAGC
414	NLpep98 (w/o Met)	A.A.	VTGYRLFKKIS
415	NLpep99 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGAAGATTAGC
416	NLpep99 (w/o Met)	A.A.	VTGYRLFEKIS
417	NLpep100 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGCAGATTAGC
418	NLpep100 (w/o Met)	A.A.	VTGYRLFEQIS
419	NLpep101 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGAAGGAGAGC
420	NLpep101 (w/o Met)	A.A.	VTGYRLFEKES
421	NLpep102 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGCAGGAGAGC
422	NLpep102 (w/o Met)	A.A.	VTGYRLFEQES
423	NLpep103 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGCAGGAGCTG
424	NLpep103 (w/o Met)	A.A.	VTGYRLFEQEL
425	NLpep104 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGAAGATTAGC
426	NLpep104 (w/o Met)	A.A.	VEGYRLFEKIS
427	NLpep105 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGCAGATTAGC
428	NLpep105 (w/o Met)	A.A.	VEGYRLFEQIS
429	NLpep106 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGAAGGAGAGC
430	NLpep106 (w/o Met)	A.A.	VEGYRLFEKES

431	NLpep107 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGCAGGAGAGC
432	NLpep107 (w/o Met)	A.A.	VEGYRLFEQES
433	NLpep108 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGCAGGAGCTG
434	NLpep108 (w/o Met)	A.A.	VEGYRLFEQEL
435	NLpep109 (w/o Met)	N.A.	ATTAGCGGCTGGCGGCTGATGAAGAACATTAGC
436	NLpep109 (w/o Met)	A.A.	ISGWRLMKNIS
437	NLpep110 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCAAGAAGATTAGC
438	NLpep110 (w/o Met)	A.A.	VEGYRLFKKIS
2162	NLpep111 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGATCAGC
2163	NLpep111 (w/ Met)	A.A.	MVTGYRLFEEIS
2164	NLpep112 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGGCCAGC
2165	NLpep112 (w/ Met)	A.A.	MVTGYRLFEEAS
2166	NLpep113 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGGAGAGC
2167	NLpep113 (w/ Met)	A.A.	MVTGYRLFEEES
2168	NLpep114 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGATCCTG
2169	NLpep114 (w/ Met)	A.A.	MVTGYRLFEEIL
2170	NLpep115 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGGCCCTG
2171	NLpep115 (w/ Met)	A.A.	MVTGYRLFEEAL
2172	NLpep116 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGGAGCTG
2173	NLpep116 (w/ Met)	A.A.	MVTGYRLFEEEL
2174	NLpep117 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGGAGATCAGC
2175	NLpep117 (w/ Met)	A.A.	MVEGYRLFEEIS
2176	NLpep118 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGGAGGCCAGC

2177	NLpep118 (w/ Met)	A.A.	MVEGYRLFEEAS
2178	NLpep119 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGGAGGAGAGC
2179	NLpep119 (w/ Met)	A.A.	MVEGYRLFEEES
2180	NLpep120 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGGAGATCCTG
2181	NLpep120 (w/ Met)	A.A.	MVEGYRLFEEIL
2182	NLpep121 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGGAGGCCCTG
2183	NLpep121 (w/ Met)	A.A.	MVEGYRLFEEAL
2184	NLpep122 (w/ Met)	N.A.	ATGGTGGAGGGCTACCGGCTGTTCGAGGAGGAGCTG
2185	NLpep122 (w/ Met)	A.A.	MVEGYRLFEEEL
2186	NLpep123 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCAAGAAGATCCTG
2187	NLpep123 (w/ Met)	A.A.	MVTGYRLFKKIL
2188	NLpep124 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGATGAAGAAGATCCTG
2189	NLpep124 (w/ Met)	A.A.	MVTGYRLMKKIL
2190	NLpep125 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGCACAAGAAGATCCTG
2191	NLpep125 (w/ Met)	A.A.	MVTGYRLHKKIL
2192	NLpep126 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGCTGAAGAAGATCCTG
2193	NLpep126 (w/ Met)	A.A.	MVTGYRLLKKIL
2194	NLpep127 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGAGCAAGAAGATCCTG
2195	NLpep127 (w/ Met)	A.A.	MVTGYRLSKKIL
2196	NLpep128 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGAAGATCCTG
2197	NLpep128 (w/ Met)	A.A.	MVTGYRLFEKIL
2198	NLpep129(w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGATGGAGAAGATCCTG
2199	NLpep129(w/ Met)	A.A.	MVTGYRLMEKIL
2200	NLpep130 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGCACGAGAAGATCCTG

2201	NLpep130 (w/ Met)	A.A.	MVTGYRLHEKIL
2202	NLpep131 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGCTGGAGAAGATCCTG
2203	NLpep131 (w/ Met)	A.A.	MVTGYRLLEKIL
2204	NLpep132 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGAGCGAGAAGATCCTG
2205	NLpep132 (w/ Met)	A.A.	MVTGYRLSEKIL
2206	NLpep133 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGATGGAGGAGATCCTG
2207	NLpep133 (w/ Met)	A.A.	MVTGYRLMEEIL
2208	NLpep134(w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGCACGAGGAGATCCTG
2209	NLpep134(w/ Met)	A.A.	MVTGYRLHEEIL
2210	NLpep135 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGCTGGAGGAGATCCTG
2211	NLpep135 (w/ Met)	A.A.	MVTGYRLLEEIL
2212	NLpep136 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGAGCGAGGAGATCCTG
2213	NLpep136 (w/ Met)	A.A.	MVTGYRLSEEIL
2214	NLpep137(w/ Met)	N.A.	ATGGTGAGCGGCTACCGGCTGTTCGAGGAGATCCTG
2215	NLpep137(w/ Met)	A.A.	MVSGYRLFEEIL
2216	NLpep138(w/ Met)	N.A.	ATGGTGACCGGCTGGCGGCTGTTCGAGGAGATCCTG
2217	NLpep138(w/ Met)	A.A.	MVTGWRLFEEIL
2218	NLpep139 (w/ Met)	N.A.	ATGGTGAGCGGCTGGCGGCTGTTCGAGGAGATCCTG
2219	NLpep139 (w/ Met)	A.A.	MVSGWRLFEEIL
2220	NLpep140 (w/ Met)	N.A.	ATGAACGTGACCGGCTACCGGCTGTTCGAGGAGATCCTG
2221	NLpep140 (w/ Met)	A.A.	MNVTGYRLFEEIL
2222	NLpep141 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGATCCTGAAC
2223	NLpep141 (w/ Met)	A.A.	MVTGYRLFEEILN
2224	NLpep142 (w/ Met)	N.A.	ATGAACGTGACCGGCTACCGGCTGTTCGAGGAGATCCTGAAC

2225	NLpep142 (w/ Met)	A.A.	MNVTGYRLFEEILN
2226	NLpep143 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCGAGGAGATC
2227	NLpep143 (w/ Met)	A.A.	MVTGYRLFEEI
2228	NLpep144 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCCAGAAGATCAGC
2229	NLpep144 (w/ Met)	A.A.	MVTGYRLFQKIS
2230	NLpep145 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCAAGAAGATCAGCAAC
2231	NLpep145 (w/ Met)	A.A.	MVTGYRLFKKISN
2232	NLpep146 (w/ Met)	N.A.	ATGGTGACCGGCTACCGGCTGTTCAAGAAGATCAGC
2233	NLpep146 (w/ Met)	A.A.	MVTGYRLFKKIS
2234	NLpep147 (w/ Met)	A.A.	MVSGWRLFKKISA
2235	NLpep148 (w/ Met)	A.A.	MGVSGWRLFKKIS
2236	NLpep149 (w/ Met)	A.A.	MSVSGWRLFKKISN
2237	NLpep150 (w/ Met)	A.A.	MSVSGWRLFKKISA
2238	NLpep151 (w/ Met)	A.A.	MNSVSGWRLFKKISA
2239	NLpep152 (w/ Met)	A.A.	MNSVSGWRLFKKISN
2240	NLpep153 (w/ Met)	A.A.	MSNVSGWRLFKKIS
2241	NLpep154 (w/ Met)	A.A.	MSGVSGWRLFKKIS
2242	NLpep155 (w/ Met)	A.A.	MNSNVSGWRLFKKIS
2243	NLpep156 (w/ Met)	A.A.	MNSGVSGWRLFKKIS
2244	NLpep157 (w/ Met)	A.A.	MSVSGWRLFKKIS
2245	NLpep158 (w/ Met)	A.A.	MNSVSGWRLFKKIS
2246	NLpep159 (w/ Met)	A.A.	MSNVSGWRLFKKISN
2247	NLpep160 (w/ Met)	A.A.	MNSNVSGWRLFKKISN
2248	NLpep161 (w/ Met)	A.A.	MGWRLFKK

2249	NLpep162(w/ Met)	A.A.	MGWALFKK
2250	NLpep163 (w/ Met)	A.A.	MVTGWALFEEIL
2251	NLpep164 (w/ Met)	A.A.	MVTGYALFQEIL
2252	NLpep165 (w/ Met)	A.A.	MVTGYALFEQIL
2253	NLpep166 (w/ Met)	A.A.	MVTGYALFEEIL
2254	NLpep167 (w/ Met)	N.A.	ATGGTGTCCGGCTGGGCACTGTTCAAGAAAATTTCC
2255	NLpep167 (w/ Met)	A.A.	MVSGWALFKKIS
2256	NLpep168 (w/ Met)	A.A.	MVSGWKLFKKIS
2257	NLpep169 (w/ Met)	N.A.	ATGGTGTCCGGCTGGCAGCTGTTCAAGAAAATTTCC
2258	NLpep169 (w/ Met)	A.A.	MVSGWQLFKKIS
2259	NLpep170 (w/ Met)	A.A.	MVSGWELFKKIS
2260	NLpep171 (w/ Met)	N.A.	ATGGTGTCCGGCTGGCTGCTGTTCAAGAAAATTTCC
2261	NLpep171 (w/ Met)	A.A.	MVSGWLLFKKIS
2262	NLpep172(w/ Met)	N.A.	ATGGTGTCCGGCTGGGTGCTGTTCAAGAAAATTTCC
2263	NLpep172(w/ Met)	A.A.	MVSGWVLFKKIS
2264	NLpep111 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGATCAGC
2265	NLpep111 (w/o Met)	A.A.	VTGYRLFEEIS
2266	NLpep112 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGGCCAGC
2267	NLpep112 (w/o Met)	A.A.	VTGYRLFEEAS
2268	NLpep113 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGGAGAGC
2269	NLpep113 (w/o Met)	A.A.	VTGYRLFEEES
2270	NLpep114 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGATCCTG
2271	NLpep114 (w/o Met)	A.A.	VTGYRLFEEIL
2272	NLpep115 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGGCCCTG

2273	NLpep115 (w/o Met)	A.A.	VTGYRLFEEAL
2274	NLpep116 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGGAGCTG
2275	NLpep116 (w/o Met)	A.A.	VTGYRLFEEEL
2276	NLpep117 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGGAGATCAGC
2277	NLpep117 (w/o Met)	A.A.	VEGYRLFEEIS
2278	NLpep118 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGGAGGCCAGC
2279	NLpep118 (w/o Met)	A.A.	VEGYRLFEEAS
2280	NLpep119 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGGAGGAGAGC
2281	NLpep119 (w/o Met)	A.A.	VEGYRLFEEES
2282	NLpep120 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGGAGATCCTG
2283	NLpep120 (w/o Met)	A.A.	VEGYRLFEEIL
2284	NLpep121 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGGAGGCCCTG
2285	NLpep121 (w/o Met)	A.A.	VEGYRLFEEAL
2286	NLpep122 (w/o Met)	N.A.	GTGGAGGGCTACCGGCTGTTCGAGGAGGAGCTG
2287	NLpep122 (w/o Met)	A.A.	VEGYRLFEEEL
2288	NLpep123 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCAAGAAGATCCTG
2289	NLpep123 (w/o Met)	A.A.	VTGYRLFKKIL
2290	NLpep124 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGATGAAGAAGATCCTG
2291	NLpep124 (w/o Met)	A.A.	VTGYRLMKKIL
2292	NLpep125 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGCACAAGAAGATCCTG
2293	NLpep125 (w/o Met)	A.A.	VTGYRLHKKIL
2294	NLpep126 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGCTGAAGAAGATCCTG
2295	NLpep126 (w/o Met)	A.A.	VTGYRLLKKIL
2296	NLpep127 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGAGCAAGAAGATCCTG

2297	NLpep127 (w/o Met)	A.A.	VTGYRLSKKIL
2298	NLpep128 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGAAGATCCTG
2299	NLpep128 (w/o Met)	A.A.	VTGYRLFEKIL
2300	NLpep129(w/o Met)	N.A.	GTGACCGGCTACCGGCTGATGGAGAAGATCCTG
2301	NLpep129(w/o Met)	A.A.	VTGYRLMEKIL
2302	NLpep130 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGCACGAGAAGATCCTG
2303	NLpep130 (w/o Met)	A.A.	VTGYRLHEKIL
2304	NLpep131 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGCTGGAGAAGATCCTG
2305	NLpep131 (w/o Met)	A.A.	VTGYRLLEKIL
2306	NLpep132 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGAGCGAGAAGATCCTG
2307	NLpep132 (w/o Met)	A.A.	VTGYRLSEKIL
2308	NLpep133 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGATGGAGGAGATCCTG
2309	NLpep133 (w/o Met)	A.A.	VTGYRLMEEIL
2310	NLpep134(w/o Met)	N.A.	GTGACCGGCTACCGGCTGCACGAGGAGATCCTG
2311	NLpep134(w/o Met)	A.A.	VTGYRLHEEIL
2312	NLpep135 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGCTGGAGGAGATCCTG
2313	NLpep135 (w/o Met)	A.A.	VTGYRLLEEIL
2314	NLpep136 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGAGCGAGGAGATCCTG
2315	NLpep136 (w/o Met)	A.A.	VTGYRLSEEIL
2316	NLpep137(w/o Met)	N.A.	GTGAGCGGCTACCGGCTGTTCGAGGAGATCCTG
2317	NLpep137(w/o Met)	A.A.	VSGYRLFEEIL
2318	NLpep138(w/o Met)	N.A.	GTGACCGGCTGGCGGCTGTTCGAGGAGATCCTG
2319	NLpep138(w/o Met)	A.A.	VTGWRLFEEIL
2320	NLpep139 (w/o Met)	N.A.	GTGAGCGGCTGGCGGCTGTTCGAGGAGATCCTG

2321	NLpep139 (w/o Met)	A.A.	VSGWRLFEEIL
2322	NLpep140 (w/o Met)	N.A.	AACGTGACCGGCTACCGGCTGTTCGAGGAGATCCTG
2323	NLpep140 (w/o Met)	A.A.	NVTGYRLFEEIL
2324	NLpep141 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGATCCTGAAC
2325	NLpep141 (w/o Met)	A.A.	VTGYRLFEEILN
2326	NLpep142 (w/o Met)	N.A.	AACGTGACCGGCTACCGGCTGTTCGAGGAGATCCTGAAC
2327	NLpep142 (w/o Met)	A.A.	NVTGYRLFEEILN
2328	NLpep143 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCGAGGAGATC
2329	NLpep143 (w/o Met)	A.A.	VTGYRLFEEI
2330	NLpep144 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCCAGAAGATCAGC
2331	NLpep144 (w/o Met)	A.A.	VTGYRLFQKIS
2332	NLpep145 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCAAGAAGATCAGCAAC
2333	NLpep145 (w/o Met)	A.A.	VTGYRLFKKISN
2334	NLpep146 (w/o Met)	N.A.	GTGACCGGCTACCGGCTGTTCAAGAAGATCAGC
2335	NLpep146 (w/o Met)	A.A.	VTGYRLFKKIS
2336	NLpep147 (w/o Met)	A.A.	VSGWRLFKKISA
2337	NLpep148 (w/o Met)	A.A.	GVSGWRLFKKIS
2338	NLpep149 (w/o Met)	A.A.	SVSGWRLFKKISN
2339	NLpep150 (w/o Met)	A.A.	SVSGWRLFKKISA
2340	NLpep151 (w/o Met)	A.A.	NSVSGWRLFKKISA
2341	NLpep152 (w/o Met)	A.A.	NSVSGWRLFKKISN
2342	NLpep153 (w/o Met)	A.A.	SNVSGWRLFKKIS
2343	NLpep154 (w/o Met)	A.A.	SGVSGWRLFKKIS
2344	NLpep155 (w/o Met)	A.A.	NSNVSGWRLFKKIS

2345	NLpep156 (w/o Met)	A.A.	NSGVSGWRLFKKIS
2346	NLpep157 (w/o Met)	A.A.	SVSGWRLFKKIS
2347	NLpep158 (w/o Met)	A.A.	NSVSGWRLFKKIS
2348	NLpep159 (w/o Met)	A.A.	SNVSGWRLFKKISN
2349	NLpep160 (w/o Met)	A.A.	NSNVSGWRLFKKISN
2350	NLpep161 (w/o Met)	A.A.	GWRLFKK
2351	NLpep162(w/o Met)	A.A.	GWALFKK
2352	NLpep163 (w/o Met)	A.A.	VTGWALFEEIL
2353	NLpep164 (w/o Met)	A.A.	VTGYALFQEIL
2354	NLpep165 (w/o Met)	A.A.	VTGYALFEQIL
2355	NLpep166 (w/o Met)	A.A.	VTGYALFEEIL
2356	NLpep167 (w/o Met)	N.A.	GTGTCCGGCTGGGCACTGTTCAAGAAAATTTCC
2357	NLpep167 (w/o Met)	A.A.	VSGWALFKKIS
2358	NLpep168 (w/o Met)	A.A.	VSGWKLFKKIS
2359	NLpep169 (w/o Met)	N.A.	GTGTCCGGCTGGCAGCTGTTCAAGAAAATTTCC
2360	NLpep169 (w/o Met)	A.A.	VSGWQLFKKIS
2361	NLpep170 (w/o Met)	A.A.	VSGWELFKKIS
2362	NLpep171 (w/o Met)	N.A.	GTGTCCGGCTGGCTGCTGTTCAAGAAAATTTCC
2363	NLpep171 (w/o Met)	A.A.	VSGWLLFKKIS
2364	NLpep172(w/o Met)	N.A.	GTGTCCGGCTGGGTGCTGTTCAAGAAAATTTCC
2365	NLpep172(w/o Met)	A.A.	VSGWVLFKKIS

In certain embodiments, a peptide from Table 1 is provided. In some embodiments, peptides comprise a single amino acid difference from GVTGWRLCKRISA (SEQ ID NO: 236) and/or any of the peptides listed in Table 1. In some embodiments, peptides comprise two or

more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, etc.) amino acid differences from GVTGWRLCKRISA (SEQ ID NO: 236) and/or any of the peptides listed in Table 1. In some embodiments, peptides are provided comprising one of the amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365. In some embodiments, peptides are provided comprising one of the amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365 with one or more additions, substitutions, and/or deletions. In some embodiments, a peptide or a portion thereof comprises greater than 70% sequence identity (e.g., 71%, 75%, 80%, 85%, 90%, 95%, 99%) with one or more of the amino acid sequence of SEQ ID NOS: 3-438 and 2162-2365. In some embodiments, nucleic acids are provided comprising one of the nucleic acid coding sequences of SEQ ID NOS: 3-438 and 2162-2365. In some embodiments, nucleic acids are provided comprising one of the nucleic acid sequences of SEQ ID NOS: 3-438 and 2162-2365with one or more additions, substitutions, and/or deletions. In some embodiments, a nucleic acid or a portion thereof comprises greater than 70% sequence identity (e.g., 71%, 75%, 80%, 85%, 90%, 95%, 99%) with one or more of the nucleic acid sequence of SEQ ID NOS: 3-438 and 2162-2365. In some embodiments, nucleic acids are provided that code for one of the amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365. In some embodiments, nucleic acids are provided that code for one of the amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365 with one or more additions, substitutions, and/or deletions. In some embodiments, a nucleic acid is provided that codes for an amino acid with greater than 70% sequence identity (e.g., 71%, 75%, 80%, 85%, 90%, 95%, 99%) with one or more of the amino acid sequences of SEQ ID NOS: 3-438 and 2162-2365.

In certain embodiments, a nucleic acid from Table 1 is provided. In some embodiments, a nucleic acid encoding a peptide from Table 1 is provided. In some embodiments, a nucleic acid of the present invention codes for a peptide that comprises a single amino acid difference from MGVTGWRLCERILA (SEQ ID NO: 2) and/or any of the peptides listed in Table 1. In some embodiments, nucleic acids code for peptides comprising two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, etc.) amino acid differences from MGVTGWRLCERILA (SEQ ID NO: 2) and/or any of the peptides listed in Table 1. In some embodiments, nucleic acids are provided comprising the sequence of one of the nucleic acids in Table 1. In some embodiments, nucleic acids are provided comprising one of the nucleic acids of Table 1 with one or more additions, substitutions, and/or deletions. In some embodiments, a nucleic acid or a portion thereof comprises greater than 70% sequence identity (e.g., 71%, 75%, 80%, 85%, 90%, 95%, 99%) with one or more of the nucleic acids of Table 1.

In some embodiments, non-luminescent polypeptides that find use in embodiments of the present invention include polypeptides with one or more amino acid substitutions, deletions, or additions from SEQ ID NO: 440. In some embodiments, the present invention provides

polypeptides comprising one or more of amino acid sequences of Table 2, and/or nucleic acids comprising the nucleic acid sequences of Table 2 (which code for the polypeptide sequences of Table 2).

Table 2. Polypeptide sequences

SEQ ID NO	Polymer	ID	SEQ ID NO	Poly.	ID	SEQ ID NO	Poly.	ID
441	N.A.	R11N	727	N.A.	5A2+V58P	1013	N.A.	5P D6 (-152-157)
442	A.A	R11N	728	A.A	5A2+V58P	1014	A.A	5P D6 (-152-157)
443	N.A.	T13I	729	N.A.	5A2+V58Q	1015	N.A.	5P D7 (-151-157)
444	A.A	T13I	730	A.A	5A2+V58Q	1016	A.A	5P D7 (-151-157)
445	N.A.	G15S	731	N.A.	5A2+V58R	1017	N.A.	5P +F31A
446	A.A	G15S	732	A.A	5A2+V58R	1018	A.A	5P +F31A
447	N.A.	L18Q	733	N.A.	5A2+V58S	1019	N.A.	5P+F31C
448	A.A	L18Q	734	A.A	5A2+V58S	1020	A.A	5P+F31C
449	N.A.	Q20K	735	N.A.	5A2+V58T	1021	N.A.	5P+F31D
450	A.A	Q20K	736	A.A	5A2+V58T	1022	A.A	5P+F31D
451	N.A.	V27M	737	N.A.	5A2+V58W	1023	N.A.	5P+F31E
452	A.A	V27M	738	A.A	5A2+V58W	1024	A.A	5P+F31E
453	N.A.	F31I	739	N.A.	5A2+V58Y	1025	N.A.	5P+F31G
454	A.A	F31I	740	A.A	5A2+V58Y	1026	A.A	5P+F31G
455	N.A.	F31L	741	N.A.	5A2+A67C	1027	N.A.	5P+F31H
456	A.A	F31L	742	A.A	5A2+A67C	1028	A.A	5P+F31H
457	N.A.	F31V	743	N.A.	5A2+A67D	1029	N.A.	5P+F31I
458	A.A	F31V	744	A.A	5A2+A67D	1030	A.A	5P+F31I
459	N.A.	Q32R	745	N.A.	5A2+A67E	1031	N.A.	5P+F31K
460	A.A	Q32R	746	A.A	5A2+A67E	1032	A.A	5P+F31K

461	N.A.	N33K	747	N.A.	5A2+A67F	1033	N.A.	5P+F31L
462	A.A	N33K	748	A.A	5A2+A67F	1034	A.A	5P+F31L
463	N.A.	N33R	749	N.A.	5A2+A67G	1035	N.A.	5P+F31M
464	A.A	N33R	750	A.A	5A2+A67G	1036	A.A	5P+F31M
465	N.A.	I56N	751	N.A.	5A2+A67H	1037	N.A.	5P+F31N
466	A.A	I56N	752	A.A	5A2+A67H	1038	A.A	5P+F31N
467	N.A.	V58A	753	N.A.	5A2+A67I	1039	N.A.	5P+F31P
468	A.A	V58A	754	A.A	5A2+A67I	1040	A.A	5P+F31P
469	N.A.	I59T	755	N.A.	5A2+A67K	1041	N.A.	5P+F31Q
470	A.A	I59T	756	A.A	5A2+A67K	1042	A.A	5P+F31Q
471	N.A.	G67S	757	N.A.	5A2+A67L	1043	N.A.	5P+F31R
472	A.A	G67S	758	A.A	5A2+A67L	1044	A.A	5P+F31R
473	N.A.	G67D	759	N.A.	5A2+A67M	1045	N.A.	5P+F31S
474	A.A	G67D	760	A.A	5A2+A67M	1046	A.A	5P+F31S
475	N.A.	K75E	761	N.A.	5A2+A67N	1047	N.A.	5P+F31T
476	A.A	K75E	762	A.A	5A2+A67N	1048	A.A	5P+F31T
477	N.A.	M106V	763	N.A.	5A2+A67P	1049	N.A.	5P+F31V
478	A.A	M106V	764	A.A	5A2+A67P	1050	A.A	5P+F31V
479	N.A.	M106I	765	N.A.	5A2+A67Q	1051	N.A.	5P+F31W
480	A.A	M106I	766	A.A	5A2+A67Q	1052	A.A	5P+F31W
481	N.A.	D108N	767	N.A.	5A2+A67R	1053	N.A.	5P+F31Y
482	A.A	D108N	768	A.A	5A2+A67R	1054	A.A	5P+F31Y
483	N.A.	R112Q	769	N.A.	5A2+A67S	1055	N.A.	5P +L46A
484	A.A	R112Q	770	A.A	5A2+A67S	1056	A.A	5P +L46A
485	N.A.	N144T	771	N.A.	5A2+A67T	1057	N.A.	5P+L46C

486	A.A	N144T	772	A.A	5A2+A67T	1058	A.A	5P+L46C
487	N.A.	L149M	773	N.A.	5A2+A67V	1059	N.A.	5P+L46D
488	A.A	L149M	774	A.A	5A2+A67V	1060	A.A	5P+L46D
489	N.A.	N156D	775	N.A.	5A2+A67W	1061	N.A.	5P+L46E
490	A.A	N156D	776	A.A	5A2+A67W	1062	A.A	5P+L46E
491	N.A.	N156S	777	N.A.	5A2+A67Y	1063	N.A.	5P+L46F
492	A.A	N156S	778	A.A	5A2+A67Y	1064	A.A	5P+L46F
493	N.A.	V157D	779	N.A.	5A2+M106A	1065	N.A.	5P+L46G
494	A.A	V157D	780	A.A	5A2+M106A	1066	A.A	5P+L46G
495	N.A.	V157S	781	N.A.	5A2+M106C	1067	N.A.	5P+L46H
496	A.A	V157S	782	A.A	5A2+M106C	1068	A.A	5P+L46H
497	N.A.	G8A	783	N.A.	5A2+M106D	1069	N.A.	5P+L46I
498	A.A	G8A	784	A.A	5A2+M106D	1070	A.A	5P+L46I
499	N.A.	G15A	785	N.A.	5A2+M106E	1071	N.A.	5P+L46K
500	A.A	G15A	786	A.A	5A2+M106E	1072	A.A	5P+L46K
501	N.A.	G25A	787	N.A.	5A2+M106F	1073	N.A.	5P+L46M
502	A.A	G25A	788	A.A	5A2+M106F	1074	A.A	5P+L46M
503	N.A.	G26A	789	N.A.	5A2+M106G	1075	N.A.	5P+L46N
504	A.A	G26A	790	A.A	5A2+M106G	1076	A.A	5P+L46N
505	N.A.	G35A	791	N.A.	5A2+M106H	1077	N.A.	5P+L46P
506	A.A	G35A	792	A.A	5A2+M106H	1078	A.A	5P+L46P
507	N.A.	G48A	793	N.A.	5A2+M106I	1079	N.A.	5P+L46Q
508	A.A	G48A	794	A.A	5A2+M106I	1080	A.A	5P+L46Q
509	N.A.	G51A	795	N.A.	5A2+M106K	1081	N.A.	5P+L46R

510	A.A	G51A	796	A.A	5A2+M106K	1082	A.A	5P+L46R
511	N.A.	G64A	797	N.A.	5A2+M106L	1083	N.A.	5P+L46S
512	A.A	G64A	798	A.A	5A2+M106L	1084	A.A	5P+L46S
513	N.A.	G67A	799	N.A.	5A2+M106N	1085	N.A.	5P+L46T
514	A.A	G67A	800	A.A	5A2+M106N	1086	A.A	5P+L46T
515	N.A.	G71A	801	N.A.	5A2+M106P	1087	N.A.	5P+L46V
516	A.A	G71A	802	A.A	5A2+M106P	1088	A.A	5P+L46V
517	N.A.	G95A	803	N.A.	5A2+M106Q	1089	N.A.	5P+L46W
518	A.A	G95A	804	A.A	5A2+M106Q	1090	A.A	5P+L46W
519	N.A.	G101A	805	N.A.	5A2+M106R	1091	N.A.	5P+L46Y
520	A.A	G101A	806	A.A	5A2+M106R	1092	A.A	5P+L46Y
521	N.A.	G111A	807	N.A.	5A2+M106S	1093	N.A.	5P +N108A
522	A.A	G111A	808	A.A	5A2+M106S	1094	A.A	5P +N108A
523	N.A.	G116A	809	N.A.	5A2+M106T	1095	N.A.	5P+N108C
524	A.A	G116A	810	A.A	5A2+M106T	1096	A.A	5P+N108C
525	N.A.	G122A	811	N.A.	5A2+M106V	1097	N.A.	5P+N108D
526	A.A	G122A	812	A.A	5A2+M106V	1098	A.A	5P+N108D
527	N.A.	G129A	813	N.A.	5A2+M106W	1099	N.A.	5P+N108E
528	A.A	G129A	814	A.A	5A2+M106W	1100	A.A	5P+N108E
529	N.A.	G134A	815	N.A.	5A2+M106Y	1101	N.A.	5P+N108F
530	A.A	G134A	816	A.A	5A2+M106Y	1102	A.A	5P+N108F
531	N.A.	G147A	817	N.A.	5A2+L149A	1103	N.A.	5P+N108G
532	A.A	G147A	818	A.A	5A2+L149A	1104	A.A	5P+N108G
533	N.A.	I54A	819	N.A.	5A2+L149C	1105	N.A.	5P+N108H

534	A.A	I54A	820	A.A	5A2+L149C	1106	A.A	5P+N108H
535	N.A.	5A1 (G15A/D19A/G35A/G51A/G67A)	821	N.A.	5A2+L149D	1107	N.A.	5P+N108I
536	A.A	5A1 (G15A/D19A/G35A/G51A/G67A)	822	A.A	5A2+L149D	1108	A.A	5P+N108I
537	N.A.	4A1 (G15A/G35A/G67A/G71A)	823	N.A.	5A2+L149E	1109	N.A.	5P+N108K
538	A.A	4A1 (G15A/G35A/G67A/G71A)	824	A.A	5A2+L149E	1110	A.A	5P+N108K
539	N.A.	5A2 (G15A/G35A/G51A/G67A/G71A)	825	N.A.	5A2+L149F	1111	N.A.	5P+N108L
540	A.A	5A2 (G15A/G35A/G51A/G67A/G71A)	826	A.A	5A2+L149F	1112	A.A	5P+N108L
541	N.A.	5A2+A15G	827	N.A.	5A2+L149G	1113	N.A.	5P+N108M
542	A.A	5A2+A15G	828	A.A	5A2+L149G	1114	A.A	5P+N108M
543	N.A.	5A2+A35G	829	N.A.	5A2+L149H	1115	N.A.	5P+N108P
544	A.A	5A2+A35G	830	A.A	5A2+L149H	1116	A.A	5P+N108P
545	N.A.	5A2+A51G	831	N.A.	5A2+L149I	1117	N.A.	5P+N108Q
546	A.A	5A2+A51G	832	A.A	5A2+L149I	1118	A.A	5P+N108Q
547	N.A.	5A2+A67G	833	N.A.	5A2+L149K	1119	N.A.	5P+N108R
548	A.A	5A2+A67G	834	A.A	5A2+L149K	1120	A.A	5P+N108R
549	N.A.	5A2+A71G	835	N.A.	5A2+L149M	1121	N.A.	5P+N108S
550	A.A	5A2+A71G	836	A.A	5A2+L149M	1122	A.A	5P+N108S
551	N.A.	5A2+R11A	837	N.A.	5A2+L149N	1123	N.A.	5P+N108T

552	A.A	5A2+R11A	838	A.A	5A2+L149N	1124	A.A	5P+N108T
553	N.A.	5A2+R11C	839	N.A.	5A2+L149P	1125	N.A.	5P+N108V
554	A.A	5A2+R11C	840	A.A	5A2+L149P	1126	A.A	5P+N108V
555	N.A.	5A2+R11D	841	N.A.	5A2+L149Q	1127	N.A.	5P+N108W
556	A.A	5A2+R11D	842	A.A	5A2+L149Q	1128	A.A	5P+N108W
557	N.A.	5A2+R11E	843	N.A.	5A2+L149R	1129	N.A.	5P+N108Y
558	A.A	5A2+R11E	844	A.A	5A2+L149R	1130	A.A	5P+N108Y
559	N.A.	5A2+R11F	845	N.A.	5A2+L149S	1131	N.A.	5P +T144A
560	A.A	5A2+R11F	846	A.A	5A2+L149S	1132	A.A	5P +T144A
561	N.A.	5A2+R11G	847	N.A.	5A2+L149T	1133	N.A.	5P+T144C
562	A.A	5A2+R11G	848	A.A	5A2+L149T	1134	A.A	5P+T144C
563	N.A.	5A2+R11H	849	N.A.	5A2+L149V	1135	N.A.	5P+T144D
564	A.A	5A2+R11H	850	A.A	5A2+L149V	1136	A.A	5P+T144D
565	N.A.	5A2+R11I	851	N.A.	5A2+L149W	1137	N.A.	5P+T144E
566	A.A	5A2+R11I	852	A.A	5A2+L149W	1138	A.A	5P+T144E
567	N.A.	5A2+R11K	853	N.A.	5A2+L149Y	1139	N.A.	5P+T144F
568	A.A	5A2+R11K	854	A.A	5A2+L149Y	1140	A.A	5P+T144F
569	N.A.	5A2+R11L	855	N.A.	5A2+V157A	1141	N.A.	5P+T144G
570	A.A	5A2+R11L	856	A.A	5A2+V157A	1142	A.A	5P+T144G
571	N.A.	5A2+R11M	857	N.A.	5A2+V157C	1143	N.A.	5P+T144H
572	A.A	5A2+R11M	858	A.A	5A2+V157C	1144	A.A	5P+T144H
573	N.A.	5A2+R11N	859	N.A.	5A2+V157D	1145	N.A.	5P+T144I
574	A.A	5A2+R11N	860	A.A	5A2+V157D	1146	A.A	5P+T144I
575	N.A.	5A2+R11P	861	N.A.	5A2+V157E	1147	N.A.	5P+T144K

576	A.A	5A2+R11P	862	A.A	5A2+V157E	1148	A.A	5P+T144K
577	N.A.	5A2+R11Q	863	N.A.	5A2+V157F	1149	N.A.	5P+T144L
578	A.A	5A2+R11Q	864	A.A	5A2+V157F	1150	A.A	5P+T144L
579	N.A.	5A2+R11S	865	N.A.	5A2+V157G	1151	N.A.	5P+T144M
580	A.A	5A2+R11S	866	A.A	5A2+V157G	1152	A.A	5P+T144M
581	N.A.	5A2+R11T	867	N.A.	5A2+V157H	1153	N.A.	5P+T144N
582	A.A	5A2+R11T	868	A.A	5A2+V157H	1154	A.A	5P+T144N
583	N.A.	5A2+R11V	869	N.A.	5A2+V157I	1155	N.A.	5P+T144P
584	A.A	5A2+R11V	870	A.A	5A2+V157I	1156	A.A	5P+T144P
585	N.A.	5A2+R11W	871	N.A.	5A2+V157K	1157	N.A.	5P+T144Q
586	A.A	5A2+R11W	872	A.A	5A2+V157K	1158	A.A	5P+T144Q
587	N.A.	5A2+R11Y	873	N.A.	5A2+V157L	1159	N.A.	5P+T144R
588	A.A	5A2+R11Y	874	A.A	5A2+V157L	1160	A.A	5P+T144R
589	N.A.	5A2+A15C	875	N.A.	5A2+V157M	1161	N.A.	5P+T144S
590	A.A	5A2+A15C	876	A.A	5A2+V157M	1440	A.A	5P+T144S
591	N.A.	5A2+A15D	877	N.A.	5A2+V157N	1163	N.A.	5P+T144V
592	A.A	5A2+A15D	878	A.A	5A2+V157N	1164	A.A	5P+T144V
593	N.A.	5A2+A15E	879	N.A.	5A2+V157P	1165	N.A.	5P+T144W
594	A.A	5A2+A15E	880	A.A	5A2+V157P	1166	A.A	5P+T144W
595	N.A.	5A2+A15F	881	N.A.	5A2+V157Q	1167	N.A.	5P+T144Y
596	A.A	5A2+A15F	882	A.A	5A2+V157Q	1168	A.A	5P+T144Y
597	N.A.	5A2+A15G	883	N.A.	5A2+V157R	1169	N.A.	5P +P157A
598	A.A	5A2+A15G	884	A.A	5A2+V157R	1170	A.A	5P +P157A
599	N.A.	5A2+A15H	885	N.A.	5A2+V157S	1171	N.A.	5P+P157C

600	A.A	5A2+A15H	886	A.A	5A2+V157S	1172	A.A	5P+P157C
601	N.A.	5A2+A15I	887	N.A.	5A2+V157T	1173	N.A.	5P+P157D
602	A.A	5A2+A15I	888	A.A	5A2+V157T	1174	A.A	5P+P157D
603	N.A.	5A2+A15K	889	N.A.	5A2+V157W	1175	N.A.	5P+P157E
604	A.A	5A2+A15K	890	A.A	5A2+V157W	1176	A.A	5P+P157E
605	N.A.	5A2+A15L	891	N.A.	5A2+V157Y	1177	N.A.	5P+P157F
606	A.A	5A2+A15L	892	A.A	5A2+V157Y	1178	A.A	5P+P157F
607	N.A.	5A2+A15M	893	N.A.	5A2+Q20K	1179	N.A.	5P+P157G
608	A.A	5A2+A15M	894	A.A	5A2+Q20K	1180	A.A	5P+P157G
609	N.A.	5A2+A15N	895	N.A.	5A2+V27M	1181	N.A.	5P+P157H
610	A.A	5A2+A15N	896	A.A	5A2+V27M	1182	A.A	5P+P157H
611	N.A.	5A2+A15P	897	N.A.	5A2+N33K	1183	N.A.	5P+P157I
612	A.A	5A2+A15P	898	A.A	5A2+N33K	1184	A.A	5P+P157I
613	N.A.	5A2+A15Q	899	N.A.	5A2+V38I	1185	N.A.	5P+P157K
614	A.A	5A2+A15Q	900	A.A	5A2+V38I	1186	A.A	5P+P157K
615	N.A.	5A2+A15R	901	N.A.	5A2+I56N	1187	N.A.	5P+P157L
616	A.A	5A2+A15R	902	A.A	5A2+I56N	1188	A.A	5P+P157L
617	N.A.	5A2+A15S	903	N.A.	5A2+D108N	1189	N.A.	5P+P157M
618	A.A	5A2+A15S	904	A.A	5A2+D108N	1190	A.A	5P+P157M
619	N.A.	5A2+A15T	905	N.A.	5A2+N144T	1191	N.A.	5P+P157N
620	A.A	5A2+A15T	906	A.A	5A2+N144T	1192	A.A	5P+P157N
621	N.A.	5A2+A15V	907	N.A.	5A2+V27M+A35G	1193	N.A.	5P+P157Q
622	A.A	5A2+A15V	908	A.A	5A2+V27M+A35G	1194	A.A	5P+P157Q
623	N.A.	5A2+A15W	909	N.A.	5A2+A71G+K75E	1195	N.A.	5P+P157R

624	A.A	5A2+A15W	910	A.A	5A2+A71G+K75E	1196	A.A	5P+P157R
625	N.A.	5A2+A15Y	911	N.A.	5A2+R11E+L149M	1197	N.A.	5P+P157S
626	A.A	5A2+A15Y	912	A.A	5A2+R11E+L149M	1198	A.A	5P+P157S
627	N.A.	5A2+L18A	913	N.A.	5A2+R11E+V157P	1199	N.A.	5P+P157T
628	A.A	5A2+L18A	914	A.A	5A2+R11E+V157P	1200	A.A	5P+P157T
629	N.A.	5A2+L18C	915	N.A.	5A2+D108N+N144T	1201	N.A.	5P+P157V
630	A.A	5A2+L18C	916	A.A	5A2+D108N+N144T	1202	A.A	5P+P157V
631	N.A.	5A2+L18D	917	N.A.	5A2+L149M+V157D	1203	N.A.	5P+P157W
632	A.A	5A2+L18D	918	A.A	5A2+L149M+V157D	1204	A.A	5P+P157W
633	N.A.	5A2+L18E	919	N.A.	5A2+L149M+V157P	1205	N.A.	5P+P157Y
634	A.A	5A2+L18E	920	A.A	5A2+L149M+V157P	1206	A.A	5P+P157Y
635	N.A.	5A2+L18F	921	N.A.	3P (5A2+R11E+L149M+V157P)	1207	N.A.	5P+I107L
636	A.A	5A2+L18F	922	A.A	3P (5A2+R11E+L149M+V157P)	1208	A.A	5P+I107L
637	N.A.	5A2+L18G	923	N.A.	3P+D108N	1209	N.A.	5P +K75E
638	A.A	5A2+L18G	924	A.A	3P+D108N	1210	A.A	5P +K75E
639	N.A.	5A2+L18H	925	N.A.	3P+N144T	1211	N.A.	5P +K123E+N156D
640	A.A	5A2+L18H	926	A.A	3P+N144T	1212	A.A	5P +K123E+N156D
641	N.A.	5A2+L18I	927	N.A.	3E (5A2+R11E+L149M+V157E)	1213	N.A.	5P +I76V

642	A.A	5A2+L18I	928	A.A	3E (5A2+R11E+L149M+V157E)	1214	A.A	5P +I76V
643	N.A.	5A2+L18K	929	N.A.	3E+D108N	1215	N.A.	5P +G48D+H57R+L92M+I99V
644	A.A	5A2+L18K	930	A.A	3E+D108N	1216	A.A	5P +G48D+H57R+L92M+I99V
645	N.A.	5A2+L18M	931	N.A.	3E+N144T	1217	N.A.	5P +F31L+V36A+I99V
646	A.A	5A2+L18M	932	A.A	3E+N144T	1218	A.A	5P +F31L+V36A+I99V
647	N.A.	5A2+L18N	933	N.A.	5P (3P+D108N+N144T)	1219	N.A.	5P+F31L+H93P
648	A.A	5A2+L18N	934	A.A	5P (3P+D108N+N144T)	1220	A.A	5P+F31L+H93P
649	N.A.	5A2+L18P	935	N.A.	6P (5P+I56N)	1221	N.A.	5P+V90A
650	A.A	5A2+L18P	936	A.A	6P (5P+I56N)	1222	A.A	5P+V90A
651	N.A.	5A2+L18Q	937	N.A.	5E (3E+D108N+N144T)	1223	N.A.	5P+I44V
652	A.A	5A2+L18Q	938	A.A	5E (3E+D108N+N144T)	1224	A.A	5P+I44V
653	N.A.	5A2+L18R	939	N.A.	6E (5E+I56N)	1225	N.A.	5P+L46R+H86Q+M106V
654	A.A	5A2+L18R	940	A.A	6E (5E+I56N)	1226	A.A	5P+L46R+H86Q+M106V

655	N.A.	5A2+L18S	941	N.A.	NLpoly1 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+M106V+ L149M+V157D)	1227	N.A.	5P+R141H
656	A.A	5A2+L18S	942	A.A	NLpoly1 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+M106V+ L149M+V157D)	1228	A.A	5P+R141H
657	N.A.	5A2+L18T	943	N.A.	NLpoly2 (5A2+A15S+L18Q +F31I+V58A+A67 D+M106V+L149M +V157D)	1229	N.A.	5P+N33D+V58A
658	A.A	5A2+L18T	944	A.A	NLpoly2 (5A2+A15S+L18Q +F31I+V58A+A67 D+M106V+L149M +V157D)	1230	A.A	5P+N33D+V58A
659	N.A.	5A2+L18V	945	N.A.	NLpoly3 (5A2+R11N+L18Q +F31I+V58A+A67 D+M106V+L149M +V157D)	1231	N.A.	5P+I56N+P157H

660	A.A	5A2+L18V	946	A.A	NLpoly3 (5A2+R11N+L18Q +F31I+V58A+A67 D+M106V+L149M +V157D)	1232	A.A	5P+I56N+P157H
661	N.A.	5A2+L18W	947	N.A.	NLpoly4 (5A2+R11N+A15S +F31I+V58A+A67 D+M106V+L149M +V157D)	1233	N.A.	5P+L46Q+P157H
662	A.A	5A2+L18W	948	A.A	NLpoly4 (5A2+R11N+A15S +F31I+V58A+A67 D+M106V+L149M +V157D)	1234	A.A	5P+L46Q+P157H
663	N.A.	5A2+L18Y	949	N.A.	NLpoly5 (5A2+R11N+A15S +L18Q+V58A+A6 7D+M106V+L149 M+V157D)	1235	N.A.	5P+I59V
664	A.A	5A2+L18Y	950	A.A	NLpoly5 (5A2+R11N+A15S +L18Q+V58A+A6 7D+M106V+L149 M+V157D)	1236	A.A	5P+I59V
665	N.A.	5A2+F31A	951	N.A.	NLpoly6 (5A2+R11N+A15S +L18Q+F31I+A67 D+M106V+L149M +V157D)	1237	N.A.	5P+A51T+E74K+P 113L

666	A.A	5A2+F31A	952	A.A	NLpoly6 (5A2+R11N+A15S +L18Q+F31I+A67 D+M106V+L149M +V157D)	1238	A.A	5P+A51T+E74K+P 113L
667	N.A.	5A2+F31C	953	N.A.	NLpoly7 (5A2+R11N+A15S +L18Q+F31I+V58 A+M106V+L149M +V157D)	1239	N.A.	5P+V36A
668	A.A	5A2+F31C	954	A.A	NLpoly7 (5A2+R11N+A15S +L18Q+F31I+V58 A+M106V+L149M +V157D)	1240	A.A	5P+V36A
669	N.A.	5A2+F31D	955	N.A.	NLpoly8 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+L149M+ V157D)	1241	N.A.	5P+A51T
670	A.A	5A2+F31D	956	A.A	NLpoly8 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+L149M+ V157D)	1242	A.A	5P+A51T
671	N.A.	5A2+F31E	957	N.A.	NLpoly9 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+M106V+ V157D)	1243	N.A.	5P+H57R

672	A.A	5A2+F31E	958	A.A	NLpoly9 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+M106V+ V157D)	1244	A.A	5P+H57R
673	N.A.	5A2+F31G	959	N.A.	NLpoly10 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+M106V+ L149M)	1245	N.A.	5P+V58A
674	A.A	5A2+F31G	960	A.A	NLpoly10 (5A2+R11N+A15S +L18Q+F31I+V58 A+A67D+M106V+ L149M)	1246	A.A	5P+V58A
675	N.A.	5A2+F31H	961	N.A.	NLpoly11 (5A2+A15S+L18Q +M106V+L149M+ V157D)	1247	N.A.	5P+E74K
676	A.A	5A2+F31H	962	A.A	NLpoly11 (5A2+A15S+L18Q +M106V+L149M+ V157D)	1248	A.A	5P+E74K
677	N.A.	5A2+F31I	963	N.A.	NLpoly12 (5A2+A15S+L18Q +A67D+M106V+L 149M+V157D)	1249	N.A.	5P+H86Q

678	A.A	5A2+F31I	964	A.A	NLpoly12 (5A2+A15S+L18Q +A67D+M106V+L 149M+V157D)	1250	A.A	5P+H86Q
679	N.A.	5A2+F31K	965	N.A.	NLpoly13 (5A2+R11N+A15S +L18Q+M106V+L 149M+V157D)	1251	N.A.	5P+H93P
680	A.A	5A2+F31K	966	A.A	NLpoly13 (5A2+R11N+A15S +L18Q+M106V+L 149M+V157D)	1252	A.A	5P+H93P
681	N.A.	5A2+F31L	967	N.A.	5P+V	1253	N.A.	5P+I99V
682	A.A	5A2+F31L	968	A.A	5P+V	1254	A.A	5P+I99V
683	N.A.	5A2+F31M	969	N.A.	5P+A	1255	N.A.	5P+K123E
684	A.A	5A2+F31M	970	A.A	5P+A	1256	A.A	5P+K123E
685	N.A.	5A2+F31N	971	N.A.	5P+VT	1257	N.A.	5P+T128S
686	A.A	5A2+F31N	972	A.A	5P+VT	1258	A.A	5P+T128S
687	N.A.	5A2+F31P	973	N.A.	5P+VA	1259	N.A.	5P+L142Q+T154N
688	A.A	5A2+F31P	974	A.A	5P+VA	1260	A.A	5P+L142Q+T154N
689	N.A.	5A2+F31Q	975	N.A.	5P+AT	1261	N.A.	5P+H57Q
690	A.A	5A2+F31Q	976	A.A	5P+AT	1262	A.A	5P+H57Q
691	N.A.	5A2+F31R	977	N.A.	5P+AA	1263	N.A.	5P+L92M
692	A.A	5A2+F31R	978	A.A	5P+AA	1264	A.A	5P+L92M
693	N.A.	5A2+F31S	979	N.A.	5P+GG	1265	N.A.	5P+P113L
694	A.A	5A2+F31S	980	A.A	5P+GG	1266	A.A	5P+P113L
695	N.A.	5A2+F31T	981	N.A.	5P+AA	1267	N.A.	5P+G48D
696	A.A	5A2+F31T	982	A.A	5P+AA	1268	A.A	5P+G48D
697	N.A.	5A2+F31V	983	N.A.	5P+ATG	1269	N.A.	5P-B9 (-147-157)
698	A.A	5A2+F31V	984	A.A	5P+ATG	1270	A.A	5P-B9 (-147-157)
699	N.A.	5A2+F31W	985	N.A.	5P+VTG	1271	N.A.	5P+L46R+P157S

700	A.A	5A2+F31W	986	A.A	5P+VTG	1272	A.A	5P+L46R+P157S
701	N.A.	5A2+F31Y	987	N.A.	5P+VTA	1273	N.A.	5P+L46H+P157H
702	A.A	5A2+F31Y	988	A.A	5P+VTA	1274	A.A	5P+L46H+P157H
703	N.A.	5A2+V58A	989	N.A.	5P+GTA	1275	N.A.	5P+L46R+H93P
704	A.A	5A2+V58A	990	A.A	5P+GTA	1276	A.A	5P+L46R+H93P
705	N.A.	5A2+V58C	991	N.A.	5P+VTGW	1277	N.A.	5P+L46R+H93P+F31L
706	A.A	5A2+V58C	992	A.A	5P+VTGW	1278	A.A	5P+L46R+H93P+F31L
707	N.A.	5A2+V58D	993	N.A.	5P+VTGWR	1279	N.A.	5P+L46R+H93P+K75E
708	A.A	5A2+V58D	994	A.A	5P+VTGWR	1280	A.A	5P+L46R+H93P+K75E
709	N.A.	5A2+V58E	995	N.A.	5P+VTGWE	1281	N.A.	5P+L46R+H93P+I76V
710	A.A	5A2+V58E	996	A.A	5P+VTGWE	1282	A.A	5P+L46R+H93P+I76V
711	N.A.	5A2+V58F	997	N.A.	5P+VTGWK	1283	N.A.	8S (5P+L46R+H93P+P157S+F31L)
712	A.A	5A2+V58F	998	A.A	5P+VTGWK	1284	A.A	8S (5P+L46R+H93P+P157S+F31L)
713	N.A.	5A2+V58G	999	N.A.	5P+VTGWQ	1285	N.A.	5P+L46R+H93P+P157S+K75E
714	A.A	5A2+V58G	1000	A.A	5P+VTGWQ	1286	A.A	5P+L46R+H93P+P157S+K75E
715	N.A.	5A2+V58H	1001	N.A.	5P+VTGWH	1287	N.A.	5P+L46R+H93P+P157S+I76V

716	A.A	5A2+V58H	1002	A.A	5P+VTGWH	1288	A.A	5P+L46R+H93P+P157S+I76V
717	N.A.	5A2+V58I	1003	N.A.	5P D1 (-157)	1289	N.A.	12S (8S+A51T+K75E+I76V+I107L)
718	A.A	5A2+V58I	1004	A.A	5P D1 (-157)	1290	A.A	12S (8S+A51T+K75E+I76V+I107L)
719	N.A.	5A2+V58K	1005	N.A.	5P D2 (-156-157)	1291	N.A.	11S (12-A51T)
720	A.A	5A2+V58K	1006	A.A	5P D2 (-156-157)	1292	A.A	11S (12-A51T)
721	N.A.	5A2+V58L	1007	N.A.	5P D3 (-155-157)	1293	N.A.	12S-K75E
722	A.A	5A2+V58L	1008	A.A	5P D3 (-155-157)	1294	A.A	12S-K75E
723	N.A.	5A2+V58M	1009	N.A.	5P D4 (-154-157)	1295	N.A.	12S-I76V
724	A.A	5A2+V58M	1010	A.A	5P D4 (-154-157)	1296	A.A	12S-I76V
725	N.A.	5A2+V58N	1011	N.A.	5P D5 (-153-157)	1297	N.A.	12S-I107L
726	A.A	5A2+V58N	1012	A.A	5P D5 (-153-157)	1298	A.A	12S-I107L

The polypeptides and coding nucleic acid sequences of Table 2 (SEQ ID NOS: 441-1298) all contain N-terminal Met residues (amino acids) or ATG start codons (nucleic acids). In some embodiments, the polypeptides and coding nucleic acid sequences of Table 2 are provided without N-terminal Met residues or ATG start codons (SEQ ID NOS: 1299-2156).

In certain embodiments, a polypeptide of one of the amino acid polymers of SEQ ID NOS: 441-2156 is provided. In some embodiments, polypeptides comprise a single amino acid difference from SEQ ID NO: 440. In some embodiments, polypeptides comprise two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30… 35… 40… 45… 50, or more) amino acid differences from SEQ ID NO: 440 and/or any of the amino acid polymers of SEQ ID NOS:441-2156. In some embodiments, polypeptides are provided comprising the sequence of one of the amino acid polymers of SEQ ID NOS: 441-2156 with one or more additions, substitutions, and/or deletions. In some embodiments, a polypeptide or a portion thereof comprises greater than 70% sequence identity (e.g., >71%, >75%, >80%,

>85%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, or >99%) with one or more of the amino acid polymers of SEQ ID NOS: 441-2156.

In certain embodiments, a nucleic acid from Table 2 is provided. In some embodiments, a nucleic acid encoding a polypeptide from Table 2 is provided. In some embodiments, a nucleic acid of the present invention codes for a polypeptide that comprises a single amino acid difference from SEQ ID NO: 440 and/or any of the amino acid polymers of SEQ ID NOS: 441-2156. In some embodiments, nucleic acids code for a polypeptide comprising two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30… 35… 40… 45… 50, or more) amino acid differences from SEQ ID NO: 440 and/or any of the polypeptides listed in Table 2. In some embodiments, nucleic acids are provided comprising the sequence of one of the nucleic acid polymers of SEQ ID NOS: 441-2156. In some embodiments, nucleic acids are provided comprising the sequence of one of the nucleic acid polymers of SEQ ID NOS: 441-2156 with one or more additions, substitutions, and/or deletions. In some embodiments, a nucleic acid or a portion thereof comprises greater than 70% sequence identity (e.g., >71%, >75%, >80%, >85%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, or >99%) with one or more of the nucleic acid polymers of SEQ ID NOS: 441-2156. In some embodiments, a nucleic acid or a portion thereof codes for an polypeptide comprising greater than 70% sequence identity (e.g., >71%, >75%, >80%, >85%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, or >99%) with one or more of the amino acid polymers of SEQ ID NOS: 441-2156. In some embodiments, nucleic acids are provided that code for one of the polypeptides of SEQ ID NOS: 441-2156. In some embodiments, nucleic acids are provided that code for one of the polypeptides of SEQ ID NOS: 441-2156 with one or more additions, substitutions, and/or deletions.

In some embodiments, a non-luminescent peptide or polypeptide and/or an interaction element, comprises a synthetic peptide, peptide containing one or more non-natural amino acids, peptide mimetic, conjugated synthetic peptide (e.g., conjugated to a functional group (e.g., fluorophore, luminescent substrate, etc.)).

The present invention provides compositions and methods that are useful in a variety of fields including basic research, medical research, molecular diagnostics, etc. Although the reagents and assays described herein are not limited to any particular applications, and any useful application should be viewed as being within the scope of the present invention, the following are exemplary assays, kits, fields, experimental set-ups, etc. that make use of the presently claimed invention.

Typical applications that make use of embodiments of the present invention involve the monitoring/detection of protein dimerization (e.g., heterodimers, homodimers), protein-protein

interactions, protein-RNA interactions, protein-DNA interactions, nucleic acid hybridization, protein-small molecule interactions, or any other combinations of molecular entities. A first entity of interest is attached to a first member of a non-luminescent pair and the second entity of interest is attached to the second member of a non-luminescent pair. If a detectable signal is produced under the particular assay conditions, then interaction of the first and second entities are inferred. Such assays are useful for monitoring molecular interactions under any suitable conditions (e.g., in vitro, in vivo, in situ, whole animal, etc.), and find use in, for example, drug discovery, elucidating molecular pathways, studying equilibrium or kinetic aspects of complex assembly, high throughput screening, proximity sensor, etc.

In some embodiments, a non-luminescent pair of known characteristics (e.g., spectral characteristics, mutual affinity of pair) is used to elucidate the affinity of, or understand the interaction of, an interaction pair of interest. In other embodiments, a well-characterized interaction pair is used to determine the characteristics (e.g., spectral characteristics, mutual affinity of pair) of a non-luminescent pair.

Embodiments described herein may find use in drug screening and/or drug development. For example, the interaction of a small molecule drug or an entire library of small molecules with a target protein of interest (e.g., therapeutic target) is monitored under one or more relevant conditions (e.g., physiological conditions, disease conditions, etc.). In other embodiments, the ability of a small molecule drug or an entire library of small molecules to enhance or inhibit the interactions between two entities (e.g., receptor and ligand, protein-protein, etc.) is assayed. In some embodiments, drug screening applications are carried out in a high through-put format to allow for the detection of the binding of tens of thousands of different molecules to a target, or to test the effect of those molecules on the binding of other entities.

In some embodiments, the present invention provides the detection of molecular interactions in living organisms (e.g., bacteria, yeast, eukaryotes, mammals, primates, human, etc.) and/or cells. In some embodiments, fusion proteins comprising signal and interaction (target) polypeptides are co-expressed in the cell or whole organism, and signal is detected and correlated to the formation of the interaction complex. In some embodiments, cells are transiently and/or stably transformed or transfected with vector(s) coding for non-luminescent element(s), interaction element(s), fusion proteins (e.g., comprising a signal and interaction element), etc. In some embodiments, transgenic organisms are generated that code for the necessary fusion proteins for carrying out the assays described herein. In other embodiments, vectors are injected into whole organisms. In some embodiments, a transgenic animal or cell (e.g., expressing a fusion protein) is used to monitor the biodistribution of a small molecule or a

biologic tethered (e.g., conjugated or genetically fused) to NLpeptide sequence that would form a complex in the subcellular compartments and/or tissues where it concentrates.

In some embodiments, a peptide (e.g., non-luminescent peptide) portion of a luminescent complex is employed as a protein tag (e.g., within cells). In such embodiments, a polypeptide (e.g., non-luminescent polypeptide) portion of a luminescent complex (e.g., capable of forming a luminescent complex with the non-luminescent peptide) is applied to cells (e.g., as part of a reagent) to detect/quantify the presence of proteins tagged with the non-luminescent peptide. For example, a protein of interest is fused to a high affinity NLpep (e.g., NLpep86). The NLpep is then transfected into cells of interest, a reagent containing NanoGlo+NLpoly11S is then added to cells+media, and luminescence is detected. This assay scheme is demonstrated in Figure 175. In some embodiments, the small size of the peptide is useful for protein tagging. In some embodiments, non-luminescent polypeptides used in such a system are stable enough to exist in a suitable buffer for extended periods of time (e.g., in the presence of the furimazine substrate). In certain embodiments, the non-luminescent polypeptide has minimal detectable luminescence in the absence of the complementing peptide (e.g., even in the presence of furimazine substrate). In some embodiments, optimized buffer conditions are utilized to meet criteria necessary for protein tagging. High affinity spontaneously polypeptides and peptides are useful in such systems, and have utility in, for example, immunoassays, detection of virus particles, the study of protein dynamics in living cells, etc. In some embodiments, such a system provides an extremely small protein tag (e.g., 11 amino acids) providing high sensitivity detection, stability (e.g., particularly under denaturing conditions), and/or a broad dynamic range.

The compositions and methods provided herein, as well as any techniques or technologies based thereon find use in a variety of applications and fields, a non-limiting list of example applications follows:

- Antibody-free Western Blot: For example, a protein of interest is fused to a non-luminescent peptide (e.g., by genetic engineering) and expressed by any suitable means. The proteins separated (e.g., by PAGE) and transferred to a membrane. The membrane is then washed with complimentary non-luminescent polypeptide (e.g. allowing a luminescent complex to form), and placed on imager (e.g., utilizing a CCD camera) with Furimazine (PBI-3939) atop the membrane, and the protein of interest is detected (e.g., via the luminescence of the luminescent complex).
- "LucCytochemistry": For example, a protein of interest is expressed fused to a non-luminescent peptide or polypeptide and then detected with a complimentary non-luminescent polypeptide or peptide in a fashion analogous to immunocytochemistry.

- Protein localization assay: For example, a localization signal is added to a non-luminescent polypeptide or polypeptide (e.g., via genetic engineering) and expressed in cells (e.g., a nuclear localization signal added would result in expression of the non-luminescent polypeptide in the nucleus). A complimentary non-luminescent peptide or polypeptide is fused to a protein of interest (e.g., via genetic engineering) and expressed in cells with the non-luminescent polypeptide or peptide. Luminescence is produced if the protein of interest localizes in the same subcellular compartment (e.g., the nucleus) as the signal-localized non-luminescent polypeptide.
- Protein Stability Assay: For example, a protein of interest is fused to a non-luminescent peptide or polypeptide (e.g., via genetic engineering) and incubated under one or more conditions of interest. A complimentary non-luminescent polypeptide or peptide is added (e.g., at various time points), and luminescence is used to quantify the amount of protein of interest (e.g., a proxy for stability).
- Protein Detection/Quantification: For example, a protein of interest fused to a non-luminescent peptide or polypeptide (e.g., via genetic engineering) and expressed and/or manipulated by any method. The complimentary non-luminescent polypeptide or peptide is then added to detect and/or quantify the protein of interest.
- Protein Purification: For example, a protein of interest is fused to a non-luminescent peptide or polypeptide (e.g., via genetic engineering) and expressed by any method. The mixture of proteins is passed through an immobilized complimentary non-luminescent polypeptide or peptide (e.g., on beads, on a column, on a chip, etc.), washed with suitable buffer and eluted (e.g., with a buffer of high ionic strength or low pH). A mutant form of the non-luminescent peptide or polypeptide that does not activate the luminescence of the complimentary non-luminescent peptide or polypeptide may be used to elute the protein of interest.
- Pull-down: For example, an immobilized, complimentary, non-luminescent polypeptide is used to isolate a protein of interest (and interacting proteins) that is fused to a non-luminescent peptide (e.g., via genetic engineering).
- G-Coupled Protein Receptor (GPCR) Internalization Assay: For example, a non-luminescent peptide or polypeptide is fused to a GPCR of interest (e.g., via genetic engineering) and expressed on the surface of cells. A complimentary non-luminescent polypeptide or peptide is added to the media of the cells and used to detect the GPCR on cell surface. A ligand is added to stimulate the internalization of the GPCR, and a decrease in luminescence is observed.

- Membrane Integrity Assay for Cell Viability: For example, when the cell membrane of a cell expressing a non-luminescent polypeptide become compromised, a non-luminescent peptide enters the cell (e.g., a peptide that otherwise can't cross the cell membrane), thereby forming a luminescent complex, and generating luminescence.
- 5-Hydroxymethyl Cytosine Detection: For example, a cysteine is added to a non-luminescent peptide and incubated with DNA and a methyltransferase. The methyltransferase catalyzes the addition of the thiol (cysteine) only onto cytosine residues that are 5-hydroxymethylated. Unincorporated peptide is then separated from the DNA (using any method possible), and a non-luminescent polypeptide is added to detect the peptide conjugated to the DNA.
- Formyl Cytosine Detection: For example, similar to the 5-hydroxymethyl cytosine detection above, this detection method uses chemistry with specific reactivity for formyl cytosine.
- Viral Incorporation: Nucleic acid coding for a non-luminescent peptide or polypeptide is incorporated into a viral genome, and the complementary non-luminescent polypeptide or peptide is constitutively expressed in the target cells. Upon infection of the target cells and expression of the non-luminescent peptide, the bioluminescent complex forms and a signal is detected (e.g., in the presence of substrate).
- Chemical Labeling of Proteins: A non-luminescent peptide is fused or tethered to a reactive group (e.g., biotin, succinimidyl ester, maleimide, etc.). A protein of interest (e.g., antibody) is tagged with the non-luminescent peptide through binding of the reactive group to the protein of interest. Because the peptide is small, it does not affect the functionality of the protein of interest. Complimentary non-luminescent polypeptide is added to the system, and a luminescent complex is produced upon binding to the polypeptide to the peptide.
- Protease Assay: For example, a peptide sequence that is recognized by a protease of interest can be joined to NLPep in such a way that prevents bioluminescence upon exposure to NLPoly. Ways to do this include attaching a luminescence quencher to the protease recognition sequence or binding the protease recognition sequence to NLPep in such a way that complementation is hindered. Upon activity of the protease to cleave the recognition sequence, the ability of NLPoly to complement to NLPep and emit luminescence is restored, and thus the system is a sensitive protease assay.
- RNA detection.
- Biomolecule Linker characterization: For example, a linker attached to a biomolecule such as an antibody can be evaluated for its stability under a set of conditions through

attaching NLPep to the molecule via the linker of interest. Over time, the production of free NLPep through linker degradation can be monitored by addition of NLPoly and furimazine and quantification of bioluminescence produced.

- Mutation assay: For example, a point mutation, a frameshift mutation, etc. introduced in vitro or in vivo results in either a gain of signal or loss of signal from a complementation pair. Such an assay could be used, for example, to test compounds for mutagenicity.
- Target engagement for peptide inhibitors: Use of low affinity NLpep-conjugated peptides (expressed in cells) to monitor target engagement of peptide-based inhibitors. NLpoly is tethered to the target of interest. Engagement results in loss of signal from luminescent complex.
- Gain of signal Protease biosensors: A protease cleavage site is expressed between NLpoly and a dark peptide NLpep (low affinity). Cleavage releases dark peptide allowing for high affinity NLpep to complement NLpoly.
- Gain of function protease assay: The sequence of an NLpep is engineered proximal to a cleavage site of a full length substrate for a protease (e.g., caspase, ADAM, etc). The peptide remains sterically inaccessible as long as the substrate remains intact and the peptide is "buried". Both the genetically engineered protease substrate and a NLpoly (e.g., NLpoly11S) are co-transfected into a target cell line. Luciferase activity is induced upon induction of protease activity which leads to the cleavage of the substrate and exposure of the activator peptide on the N- or C-terminus of one of the fragments. This principle is expandable to detect conformational changes and /or protein modifications as well.
- Intracellular analyte quantification using recombinant intrabodies: Antibody fragments expressed within cells as NLpoly or NLpep fusion. Complementary subunit is genetically fused to an analyte of interest. When analyte is present, antibody binds and luminescent complex is formed. The application is expandable to intracellular PTM (e.g. phosphorylation) biosensors, in which the intrabody only binds to the analyte when it has been phosphorylated (or otherwise bound by the modification-specific Ab).

The above applications of the compositions and methods of the present invention are not limiting and may be modified in any suitable manner while still being within the scope of the present invention.

The present invention also provides methods for the design and/or optimization of non-luminescent pairs/groups and the bioluminescent complexes that form therefrom. Any suitable

method for the design of non-luminescent pairs/groups that are consistent with embodiments described herein, and/or panels thereof, is within the scope of the present invention.

In certain embodiments, non-luminescent pairs/groups are designed de novo to lack luminescence individually and exhibit luminescence upon association. In such embodiments, the strength of the interaction between the non-luminescent elements is insufficient to produce a bioluminescent signal in the absence of interaction elements to facilitate formation of the bioluminescent complex.

In other embodiments, non-luminescent elements and/or non-luminescent pairs are rationally designed, for example, using a bioluminescent protein (e.g., SEQ ID NO: 2157) as a starting point. For example, such methods may comprise: (a) aligning the sequences of three or more related proteins; (b) determining a consensus sequence for the related proteins; (c) providing first and second fragments of a bioluminescent protein that is related to the ones from which the consensus sequence was determined, wherein the fragments are individually substantially non-luminescent but exhibit luminescence upon interaction of the fragments; (d) mutating the first and second fragments at one or more positions each (e.g., in vitro, in silico, etc.), wherein said mutations alter the sequences of the fragments to be more similar to a corresponding portion of the consensus sequence, wherein the mutating results in a non-luminescent pair that are not fragments of a preexisting protein, and (e) testing the non-luminescent pair for the absence of luminescence when unassociated and luminescence upon association of the non-luminescent pair. In other embodiments, first and second fragments of one of the proteins used in determining the consensus sequence are provided, mutated, and tested.

In some embodiments, a peptide of a luminescent pair is a ‘dark peptide,’ or one that binds to its complement (e.g., NLpoly) (e.g., with low or high affinity) but produces minimal or no luminescence (See figures 180-182). In some embodiments, a high affinity dark peptide finds use in inverse complementation, or gain of signal assays for measuring inhibitors. In some embodiments, a low affinity dark peptide is used to bring down background of NLpoly11S in a reagent for the detection of a high affinity peptide tag (e.g. NLpep86). Exemplary dark peptides are provided in Figure 180.

In some embodiments, a peptide of a luminescent pair is a ‘quencher peptide,’ or one that contains a quencher moiety (e.g., DAB), and the quencher absorbs the light/energy produced by both a NLpoly in isolation (e.g., the signal produced independent of a complementing NLpep) and a NLpoly-NLpep complex (e.g., the signal produced as a result of complex formation). Exemplary dark quencher peptides would have a suitable absorption spectrum and include DAB-161 (DAB-GWRLFKK), DAB-162 (DAB-GWALFKK),DAB-163 (DAB-

VTGWALFEEIL),DAB-164 (DAB-VTGYALFQEIL),DAB-165 (DAB-VTGYALFEQIL), and DAB-166 (DAB-VTGYALFEEIL); wherein DAB = Dabcyl (475nm quencher)+dPEG4 spacer.

In some embodiments, the above methods are not limited to the design and/or optimization of non-luminescent pairs. The same steps are performed to produce pairs of elements that lack a given functionality (e.g., enzymatic activity) individually, but display such functionality when associated. In any of these cases, the strength of the interaction between the non-luminescent pair elements may be altered via mutations to ensure that it is insufficient to produce functionality in the absence of interaction elements that facilitate formation of the bioluminescent complex.

EXPERIMENTAL

Example 1

Generation of Peptides

Peptide constructs were generated by one of three methods: annealing 5'-phosphorylated oligonucleotides followed by ligation to pF4Ag-Barnase-HALOTAG vector (Promega Corporation; cut with SgfI and XhoI) or pFN18A (Promega Corporation; cut with SgfI and XbaI), site directed mutagenesis using Quik Change Lightning Multi kit from Agilent or outsourcing the cloning to Gene Dynamics.

Example 2

Peptide Preparation

The peptides generated in Example 1 were prepared for analysis by inoculating a single colony of KRX E.coli cells (Promega Corporation) transformed with a plasmid encoding a peptide into 2-5 ml of LB culture and grown at 37ºC overnight. The overnight cultures (10 ml) were then diluted into 1L of LB and grown at 37ºC for 3 hours. The cultures were then induced by adding 10 ml 20% rhamnose to the 1L culture and induced at 25ºC for 18 hours.

After induction, 800 ml of each culture was spun at 5000xg at 4ºC for 30 minutes. The pellet generated was then resuspended in 80 ml Peptide Lysis Buffer (25mM HEPES pH 7.4, 0.1x Passive Lysis Buffer (Promega Corporation), 1ml/ml lysozyme and 0.03U/µl RQ1 DNase (Promega Corporation)) and incubated at room temperature for 15 minutes. The lysed cells were then frozen on dry ice for 15 minutes and then thawed in a room temperature bath for 15minutes. The cells were then spun at 3500xg at 4ºC for 30 minutes. The supernatants were aliquoted into 10ml samples with one aliquot of 50µl placed into a 1.5ml tube.

To the 50µl samples, 450µl H_2O and 167µl 4x SDS Loading Dye were added, and the samples incubated at 95ºC for 5 minutes. After heating, 5µl of each sample was loaded (in triplicate) onto an SDS-PAGE gel, and the gel run and stained according to the manufacturer's

protocol. The gel was then scanned on a Typhoon Scanner (excitation 532nm, emission 580nm, PMT sensitivity 400V). The resulting bands were quantified using the ImageQuant (5.2) software. Each of the three replicate intensities was averaged, and the average intensity of NLpep53-HT was defined at 12x concentration. The concentrations of all other peptides were relative to Pep53-HT.

Example 3

Peptide Analysis

All of the peptides generated in Examples 1-2 contained single mutations to the peptide sequence: GVTGWRLCKRISA (SEQ ID NO: 236). All of the peptides were fused to a HALOTAG protein (Promega Corporation). Peptides identified as "HT-NLpep" indicate that the peptide is located at the C-terminus of the HALOTAG protein. In this case, the gene encoding the peptide includes a stop codon, but does not include a methionine to initiate translation. Peptides identified as "NLpep-HT" indicate that the peptide is at the N-terminus of the HALOTAG protein. In this case, the peptide does include a methionine to initiate translation, but does not include a stop codon.

To determine the ability of the peptides to activate luminescence, individual colonies of KRX E.coli cells (Promega Corporation) was transformed with a plasmid encoding a peptide from Example 1, inoculated in 200µl of minimal medium (1x M9 salts, 0.1mM $CaCl_2$, 2mM $MgSO_4$, 1mM Thiamine HCl, 1% gelatin, 0.2% glycerol, and 100ul/ml Ampicillin) and grown at 37ºC overnight. In addition to the peptides, a culture of KRX E.coli cells expressing a wild-type (WT) fragment of residues 1-156 of the NanoLuc was grown. All peptides and the WT fragment were inoculated into at least 3 separate cultures.

After the first overnight growth, 10µl of culture was diluted into 190µl fresh minimal medium and again grown at 37ºC overnight.

After the second overnight growth, 10µl of the culture was diluted into 190µl of auto-induction medium (minimal medium + 5% glucose and 2% rhamnose). The cultures were then inducted at 25ºC for approximately 18 hours.

After induction, the small peptide mutant cultures were assayed for activity. The cultures containing the WT 1-156 fragment were pooled, mixed with 10 ml of 2x Lysis Buffer (50mM HEPES pH 7.4, 0.3x Passive Lysis Buffer, and 1mg/ml lysozyme) and incubated at room temperature for 10 minutes. 30µl of the lysed WT 1-156 culture was then aliquoted into wells of a white, round bottom 96-well assay plate (Costar 3355). To wells of the assay plate, 20µl of a peptide culture was added, and the plate incubated at room temperature for 10 minutes. After incubation, 50µl NANOGLO Luciferase Assay Reagent (Promega Corporation) was added, and

the samples incubated at room temperature for 10 minutes. Luminescence was measured on a GLOMAX luminometer with 0.5s integrations.

The results (See Table 3 and Figure 1) demonstrate various mutations in the peptide (relative to SEQ ID NO: 1) that altered (e.g., increased, decreased) the luminescence following complementation with the wild-type non-luminescent polypeptide. The increased luminescence is thought to stem from one (or a combination) of five main factors, any of which are beneficial: affinity between the non-luminescent peptide and non-luminescent polypeptide, expression of the peptide, intracellular solubility, intracellular stability, and bioluminescent activity. The present invention though is not limited to any particular mechanism of action and an understanding of the mechanism of action is not necessary to practice the present invention.

Table 3

Mutation	HT-NLPep	NLpep-HT	HT-Pep st. dev.	Pep-HT st. dev.
G157D	0.1137	0.5493	N.D.	N.D.
G157N	0.6415	3.3074	0.2512	1.4828
G157S	1.9937	1.7156	0.8554	1.0563
G157E	0.1959	1.4461	0.0811	0.3221
G157H	0.9380	0.5733	0.4366	0.2277
G157C	N.D.	0.0468	N.D.	0.0081
G157P	N.D.	0.0543	N.D.	0.0106
V158I	0.6075	1.6010	0.3283	0.6264
V158A	0.1348	0.1438	0.0561	0.0447
V158K	0.0770	0.1923	0.0323	0.0521
V158Q	0.0445	0.0397	0.0188	0.0160
V158S	0.0487	0.0838	0.0189	0.0251
T159V	0.5658	0.0455	0.2293	0.0005
T159K	0.0490	0.0307	0.0120	0.0103
T159Q	0.3979	0.0310	0.1063	0.0091
W161T	0.0028	0.0100	0.0007	0.0049

W161K	0.0002	0.0008	9.7E-06	0.0001
W161V	0.0086	0.0050	0.0062	0.0016
W161F	N.D.	0.0717	N.D.	0.0049
W161Y	N.D.	0.2154	N.D.	0.0103
W161E	N.D.	0.0012	N.D.	0.0002
L163I	N.D.	0.2923	N.D.	0.1198
L163V	0.1727	0.1190	0.0257	0.0288
L163T	0.0259	0.0262	0.0077	0.0122
L163Y	0.0512	0.1959	0.0126	0.1043
L163K	0.0885	0.0786	0.0130	0.0244
C164N	0.0874	0.1081	0.0097	0.0160
C164T	0.0116	0.0084	0.0029	0.0013
C164F	N.D.	13.3131	N.D.	3.6429
C164Y	N.D.	1.0092	N.D.	0.2592
C164S	N.D.	0.0202	N.D.	0.0029
C164H	N.D.	0.7597	N.D.	0.2149
C164M	N.D.	3.2618	N.D.	1.1763
C164A	N.D.	0.0858	N.D.	0.0196
C164Q	N.D.	0.0211	N.D.	0.0044
C164L	N.D.	1.0170	N.D.	0.2464
C164K	N.D.	0.0005	N.D.	0.0001
R166K	1.0910	1.2069	0.2266	0.5913
R166N	0.1033	0.1182	0.0289	0.0542
I167V	0.8770	1.0824	0.1113	0.2642
I167Q	0.0178	0.1172	0.0252	0.0150
I167E	0.2771	0.2445	0.0358	0.0456
I167R	0.0464	0.0469	0.0027	0.0084

I167F	0.2832	0.1793	0.0159	0.0683
A169N	0.9115	1.7775	0.1114	0.5901
A169T	0.9448	1.3720	0.0930	0.6021
A169R	0.9851	0.5014	0.2205	0.1895
A169L	1.1127	0.9047	0.1906	0.2481
A169E	0.8457	0.7889	0.1445	0.0819

Example 4

Generation of non-luminescent polypeptides

Using pF4Ag-NanoLuc1-156 (WT 1-156) as a template, error-prone PCR (epPCR) was performed using the Diversify PCR Random Mutagenesis Kit from Clontech. The resulting PCR product was digested with SgfI and XbaI and ligated to pF4Ag-Barnase (Promega Corporation), a version of the commercially-available pF4A vector (Promega) which contains T7 and CMV promoters and was modified to contain an E. coli ribosome-binding site. Following transformation into KRX E. coli cells (Promega Corporation) by heat shock at 42ºC, individual colonies were used to inoculate 200µl cultures in clear, flat bottom 96-well plates (Costar 3370).

Example 5

Non-luminescent polypeptide analysis

To determine the luminescence of the non-luminescent polypeptide mutants generated in Example 4, individual colonies of the KRX E.coli cells (Promega Corporation) transformed with a plasmid containing one of the non-luminescent polypeptide mutants from Example 4 was grown according to the procedure used in Example 3. The bacterial cultures were also induced according to the procedure used in Example 3.

To assay each non-luminescent polypeptide mutant induced culture, 30µl of assay lysis buffer (25mM HEPES pH 7.4, 0.3x Passive Lysis Buffer (Promega Corporation)), 0.006 U/µl RQ1 DNase (Promega Corporation) and 1x Peptide Solution (the relative concentration of the peptides were determined as explained in Example 2; from the relative concentration determined, the peptides were diluted to 1x in the lysis buffer) containing either the peptide fragment GVTGWRLCKRISA (SEQ ID NO: 18) or GVTGWRLFKRISA (SEQ ID NO: 106) were aliquoted into wells of a 96-well assay plate (Costar 3355). To the wells of the assay plate, 20µl of an induced non-luminescent polypeptide mutant culture was added, and the plate incubated at room temperature for 10 minutes. After incubation, 50µl of NANOGLO Luciferase Assay Reagent (Promega Corporation) was added, and the samples incubated at room temperature for 10 minutes. Luminescence was measured on a GLOMAX luminometer with 0.5s integrations.

The results (Table 4 and Figure 2) demonstrate numerous point mutations that improve the luminescence of the non-luminescent polypeptide upon complementation with two different peptides. Similar to the mutations in the peptide, these mutations in the non-luminescent polypeptide may stem from various factors, all of which are beneficial to the system as a whole.

Table 4

Mutation	V157D	F31I	L18Q	R11N
GVTGWRLCKRISA	4.98	4.1	3.81	3.37
st dev	0.48	0.37	0.29	0.67
GVTGWRLFKRISA	3.02	2.83	2.99	2.09
st dev	0.77	0.61	0.82	0.03
Mutation	Q32R	M106V	M106I	G67S
GVTGWRLCKRISA	1.52	1.3	1.27	1.22
st dev	0.2	0.22	0.04	0.26
GVTGWRLFKRISA	1.04	1.4	1.31	1.29
st dev	0.19	0.25	0.35	0.22
Mutation	F31L	L149M	N33K	I59T
GVTGWRLCKRISA	3.13	2.89	2.15	1.07
st dev	0.26	0.39	0.2	0.07
GVTGWRLFKRISA	2.86	2.16	1.76	1.35
st dev	0.7	0.26	0.08	0.37
Mutation	I56N	T13I	F31V	N33R
GVTGWRLCKRISA	0.44	2.18	2.12	2.1
st dev	0.05	0.75	0.09	0.18
GVTGWRLFKRISA	1.81	1.44	2.12	1.56
st dev	0.35	0.34	0.46	0.16
Mutation	V27M	Q20K	V58A	K75E
GVTGWRLCKRISA	1.99	4.43	1.88	2.08
st dev	0.09	0.84	0.6	0.47
GVTGWRLFKRISA	1.7	2.33	1.07	2.05
st dev	0.11	0.38	0.26	0.37
Mutation	G15S	G67D	R112N	N156D
GVTGWRLCKRISA	1.98	1.78	1.61	1.57
st dev	0.99	0.11	0.2	0.21
GVTGWRLFKRISA	2.34	1.57	1.45	1.21
st dev	0.82	0.17	0.47	0.26
Mutation	D108N	N144T	N156S	
GVTGWRLCKRISA	2.08	3.69	1.04	
st dev	0.6	1.12	0.29	
GVTGWRLFKRISA	1.88	2.26	1.4	
st dev	0.38	0.51	0.28	

***Units in Table 4 are RLU(mutant)/RLU(WT)**

Example 6

Glycine to Alanine Substitutions in non-luminescent polypeptide

The following example identified glycine residues within the non-luminescent polypeptide that can be substituted to alanine to provide an improved (e.g., greater luminescent signal) non-luminescent polypeptide. The substitutions were made singly (See Figure 3), or in composites (Figure 2). Non-luminescent polypeptides containing glycine to alanine substitutions were generated as described in Example 1.

Each single mutant colony was inoculated in 200µl Minimal Media (1x M9 salts, 0.1mM $CaCl_2$, 2mM $MgSO_4$, 1mM Thiamine HCl, 1% gelatin, 0.2% glycerol and 1x ampicillin) and incubated with shaking at 37ºC for 20 hours. 10µl of the culture was then added to 190µl of fresh Minimal Media and incubated again with shaking at 37ºC for 20 hours. 10µl of the second culture was then added to 190µl Auto-Induction Media (Minimal Media + 5% glucose + 2% rhamnose) and incubated with shaking at 25ºC for 18 hours to allow expression of the non-luminescent polypeptide.

To assay each mutant culture, 30µl of assay lysis buffer (50mM HEPES pH 7.5, 0.3x Passive Lysis Buffer (Promega Corporation)) and 0.006 U/µl RQ1 DNase (Promega Corporation)) containing non-luminescent peptide (1:10 dilution of NLpep9-HT (NLpep9 is SEQ ID NO: 17 and 18; HT is HaloTag E.coli clarified lysate) was added. The samples were shaken at room temperature for 10 minutes, and then 50µl NANOGLO Luciferase Assay Reagent (Promega Corporation) was added. The samples were incubated at room temperature for 10 minutes, and luminescence was measured on a GLOMAX luminometer with 0.5s integrations.

To generate the NLpep9-HT E.coli clarified lysate, 5ml LB was inoculated with a single E.coli colony of NLpep9-HT and incubated at 37ºC overnight. 500µl of the overnight culture was then diluted in 50mls LB and incubated at 37ºC for 3 hours. 500µl of 20% rhamnose was added and incubated at 25ºC for 18 hours. The expression culture was centrifuged at 3000xg for 30 minutes, and the cell pellet resuspended in 5ml peptide lysis buffer (25mM HEPES, pH 7.5, 0.1x Passive Lysis Buffer, 1mg/ml lysozyme, and 0.3U/µl RQ1 DNase) and incubated at room temperature for 10 minutes. The lysed sample was placed on dry ice for 15 minutes, thawed in a room temperature water bath and centrifuged at 3500xg for 30 minutes. The supernatant was the clarified lysate.

Figures 3 and 4 demonstrate the effects of the mutations on luminescence.

Example 7

Mutations in non-luminescent peptide

In the following example, mutations were made in the non-luminescent peptide based on alignment to other fatty acid binding proteins (FABPs) and were chosen based on high probability (frequency in FABPs) to identify a mutation that retains/improves activity (such as

NLpep2, 4, and 5) or establish that a mutation is not likely to be tolerated at that position (such as NLpep3). NLpep1-5 contain single mutations (See Table 1), and NLpep6-9 are composite sets of the mutations in NLpep2, 4, and 5 (See Table 1). Mutants were generated as described in Example 1.

Each mutant colony was inoculated in 200µl Minimal Media and incubated with shaking at 37ºC for 20 hours. 10µl of the culture was then added to 190µl of fresh Minimal Media and incubated again with shaking at 37ºC for 20 hours. 10µl of the second culture was then added to 190µl Auto-Induction Media and incubated with shaking at 25ºC for 18 hours to allow expression of the non-luminescent peptide mutant.

To assay each mutant culture, 30µl of assay lysis buffer (50mM HEPES pH 7.5, 0.3x Passive Lysis Buffer (Promega Corporation)) and 0.006 U/µl RQ1 DNase (Promega Corporation)) containing non-luminescent polypeptide (1:10 dilution of wild-type non-luminescent polypeptide E.coli clarified lysate) was added. The samples were shaken at room temperature for 10 minutes, and then 50µl NANOGLO Luciferase Assay Reagent (Promega Corporation) added. The samples were incubated at room temperature for 10 minutes, and luminescence was measured on a GLOMAX luminometer with 0.5s integrations.

Figure 1 shows the luminescence (RLUs) detected in each non-luminescent peptide mutant. The results demonstrate various positions that are able to tolerate a mutation without substantial loss in luminescence, as well as a few specific mutations that improve luminescence.

Example 8

Effect of Orientation of Fusion Tag on Luminescence

In the following example, luminescence generated by non-luminescent peptides with N- or C-terminus HaloTag protein was compared.

Single colony of each peptide-HT fusion was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7. Figure 6 and 7 demonstrate the luminescence (RLUs) detected in each peptide-HT fusion. The results demonstrate combinations of mutations that produce similar luminescence as NLpep1.

Example 9

Effect of Multiple Freeze-Thaw Cycles on non-luminescent peptides

1ml of NLpep9-HT was frozen on dry ice for 5 minutes and then thawed in a room temperature water bath for 5 minutes. 60µl was then removed for assaying. The freeze-thaw procedure was then repeated another 10 times. After each freeze-thaw cycle, 60µl of sample was removed for assaying.

To assay, 20µl of each freeze-thaw sample was mixed with 30µl of SEQ ID NO:2 and incubated at room temperature for 10 minutes. 50µl of NANOGLO Luciferase Assay Reagent was added, and the samples incubated at room temperature for 10 minutes. Luminescence was measured on a GLOMAX luminometer with 0.5s integrations. The results are depicted in Figure8 and demonstrate that NLpep can be subjected to multiple freeze-thaw cycles without a loss in activity (luminescence).

Example 10

Distinction of mutations in non-luminescent peptides

In the following example, TMR gel analysis was used to normalize the concentration of the non-luminescent peptide mutants to distinguish mutations that alter the expression from those that alter luminescence (e.g., altered luminescence may stem from altered binding affinity).

5ml of LB was inoculated with a single mutant peptide colony and incubated with shaking at 37ºC for 20 hours. 50µl of the overnight culture was diluted into 5ml of fresh LB and incubated with shaking at 37ºC for 3 hours. 50µl of 20% rhamnose was then added and incubated with shaking at 25ºC for 18 hours.

For TMR gel analysis, 79µl of each induced culture was mixed with 10µl 10x Fast Break Lysis Buffer (Promega Corporation), 10µl of a 1:100 dilution of HALOTAG TMR ligand (Promega Corporation) non-luminescent polypeptide and 10µl of RQ1 DNase and incubated at room temperature for 10 minutes. 33.3µl of 4x SDS-loading buffer was added, and the samples incubated at 95ºC for 5 minutes. 15µl of each sample was loaded onto an SDS gel and run according to the manufacturer's directions. The gel was then scanned on a Typhoon.
Each culture was diluted based on the TMR-gel intensity to normalize concentrations. 20µl of each diluted culture was then mixed with 30µl assay lysis buffer containing non-luminescent polypeptide (1:10 dilution of SEQ ID NO: 2 E.coli clarified lysate) and incubated with shaking at room temperature for 10 minutes. 50µl of NANOGLO Luciferase Assay Reagent was added, and the samples incubated at room temperature for 10 minutes. Luminescence was measured on a GLOMAX luminometer with 0.5s integrations (SEE FIG. 9).

Example 11

Site saturation in non-luminescent polypeptide

In the following example, positions 11, 15, 18, 31, 58, 67, 106, 149, and 157 were identified as sites of interest from screening the library of random mutations in wild-type non-luminescent polypeptide. All 20 amino acids at these positions (built on 5A2 non-luminescent mutant generated in Example 6 (SEQ ID NOS: 539 and 540) to validate with other mutations in the 5A2 mutant) were compared to determine the optimal amino acid at that position. Mutant non-luminescent polypeptides were generated as previously described in Example 1. Single

colony of each non-luminescent polypeptide mutant was grown according to the procedure used in Example 6. The bacterial cultures were also induced according to the procedure used in Example 6. Luminescence was assayed and detected according to the procedure used in Example 6 expect NLpep53 E.coli clarified lysate was used at 1:11.85 dilution.

Figures 10-18 demonstrate the effect of the mutations on the ability to produce luminescence with and without NLpep.

Example 12

Comparison of cysteine vs. proline as first amino acid in non-luminescent peptide

In the following example, a comparison of using cysteine or proline as first amino acid (after necessary methionine) in the non-luminescent peptide was performed. The mutant non-luminescent peptides were generated as previously described in Example 1. Single colony of each non-luminescent polypeptide mutant was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7.

Figure 19 demonstrates that both cysteine and proline can be used as the first amino acid of NLpep and produce luminescence.

Example 13

Identification of the optimal composite set of mutations for the non-luminescent peptide

In the following examples, an optimal composite set(s) of mutations for the non-luminescent peptide were identified. The mutant non-luminescent peptides were generated as previously described in Example 1.

1) For non-luminescent peptide composite mutants NLpep53, NLpep66, NLpep67, and NLpep68, a single colony of each was grown according to the procedure used in Example 10. The bacterial cultures were also induced according to the procedure used in Example 10. TMR gel analysis and luminescence was assayed and detected according to the procedure used in Example 10. The results in Figure 20 demonstrate the luminescence as well as the E. coli expression of NLpeps containing multiple mutations.

2) For non-luminescent peptide composite mutants NLpep53 and NLpeps 66-74, a single colony of each was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7. The results in Figure 21 demonstrate the luminescence of NLpeps containing multiple mutations.

3) For non-luminescent peptide

composite mutants NLpep53 and NLpeps 66-76, a single colony of each was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7 except the non-luminescent polypeptide was 5A2 or 5A2+R11E (1:10 dilution of E.coli clarified lysate). The results in Figure 22 demonstrate the luminescence of NLpeps containing multiple mutations with 5A2 or 5A2+R11E. These results also demonstrate the lower luminescence when the NLpoly mutation R11E is complemented with an NLpep containing E as the 9th residue (NLpep72, 75, and 76).

4) For non-luminescent peptide composite mutants NLpep1, NLpep69, NLpep78 and NLpep79, a single colony of each was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7 except the non-luminescent polypeptide was WT (1:10 dilution of E.coli clarified lysate). The results in Figure 23 demonstrate the luminescence of NLpeps containing multiple mutations.

Example 14

Composite non-luminescent polypeptide mutants

In the following example, 9 mutations from the library screens were combined into a composite clone (NLpoly1, SEQ ID NOS: 941,942), and then one of the mutations reverted back to the original amino acid (NLpoly2-10, SEQ ID NOS: 943-960) in order to identify the optimal composite set. Based on previous results of NLpoly1-10, NLpoly11-13 (SEQ ID NOS: 961-966) were designed and tested for the same purpose. Mutant NLpolys were generated as previously described in Example 1. Single colony of each non-luminescent polypeptide mutant was grown according to the procedure used in Example 6. The bacterial cultures were also induced according to the procedure used in Example 6. Luminescence was assayed and detected according to the procedure used in Example 6 expect NLpep53 E.coli clarified lysate was used at 1:11.85 dilution.

Figure 24 demonstrates the luminescence of NLpolys containing multiple mutations.

Example 15

Substrate specificity of non-luminescent polypeptide mutants

The following example investigates the substrate specificity of the non-luminescent polypeptide mutants. Luminescence generated from luminescent complexes formed from various non-luminescent polypeptide mutants, either Furimazine or coelenterazine as a substrate, and various non-luminescent peptides.

HEK 293 cells were plated at 100,000 cells/ml into wells of a 24 well plates containing 0.5ml DMEM+10% FBS (50,000/well). The cells were incubated in a 37ºC, 5% CO_2 incubator overnight. DNA for expression of each non-luminescent polypeptide mutant was transfected in duplicate. 1ug plasmid DNA containing a non-luminescent polypeptide mutant was mixed with OptiMEM (Life Technologies) to a final volume of 52ul. 3.3µl of Fugene HD (Promega Corporation) was added, and samples incubated for 15 minutes at room temperature. 25µl of each sample mixture was added to two wells and incubated overnight in a 37ºC, 5% CO_2 incubator overnight. After overnight incubation, the growth media was removed and 0.5ml DMEM (without phenol red) + 0.1% Prionex added. The cells were then frozen on dry ice (for how long) and thawed prior to detecting luminescence.

In Figures 25-26, luminescence was assayed and detected according to the procedure used in Example 6, except NLpep53 E.coli clarified lysate was used at 1:10 dilution and either Furimazine or coelenterazine in either NanoGlo Luciferase Assay buffer or DMEM were used. This data demonstrates the luminescence of NLpolys in NANOGLO and DMEM with either Furimazine or Coelenterazine as the substrate. This indicates the substrate specificity (Furimazine versus Coelenterazine) of the NLpoly in both NANOGLO and DMEM.

In Figure 27, luminescence was assayed and detected according to the procedure used in Example 6, except E.coli clarified lysate from various non-luminescent peptides (NLpep1, NLpep9, NLpep48, NLpep53, NLpep69 or NLpep76) were used at 1:10 dilution. In addition, either Furimazine or coelenterazine in either NanoGlo Luciferase Assay buffer were used. This data demonstrates the substrate specificity of NLpoly/NLpep pairs.

In Figure 28, luminescence was assayed and detected by separately diluting NLpep53-HT fusion 1:10 and the non-luminescent polypeptide lysates 1:10 in DMEM+0.1% Prionex. 20µl of non-luminescent peptide and 20µlnon-luminescent polypeptide were then combined and incubated for 10 minutes at room temperature. 40µl of NanoGlo Buffer with 100uM Furimazine or DMEM with 0.1% Prionex and 20uM Furimazine was then added to the samples, and luminescence detected on GloMax Multi. This data demonstrates the substrate specificity of NLpolys expressed in HEK293 cells.

In Figure 29, luminescence was assayed and detected by separately diluting NLpep1-HT, NLpep53-HT, NLpep69-HT or NLpep76-HT fusion 1:10 and the non-luminescent polypeptide lysates 1:10 in DMEM+0.1% Prionex. 20µl of non-luminescent peptide and 20µlnon-luminescent polypeptide were then combined and incubated for 10 minutes at room temperature. 40µl of NanoGlo Buffer with 100uM Furimazine or DMEM with 0.1% Prionex and 20uM Furimazine was then added to the samples, and luminescence detected on GloMax Multi. This

data demonstrates the luminescence of NLpolys expressed in mammalian cells and assayed with various NLpeps.

Example 16

Signal-to-background of non-luminescent polypeptide mutants with Furimazine or coelenterazine

The following example investigates signal-to-background of the non-luminescent polypeptide mutants. Luminescence generated from various non-luminescent polypeptide mutants was measured using either Furimazine or coelenterazine as a substrate as well as with various non-luminescent peptides.

HEK 293 cells were plated at 15,000 cells/well in 100µl DMEM+10% FBS into wells of 96-well plates. The cells were incubated in a 37ºC, 5% CO_2 incubator overnight. Transfection complexes were prepared by adding 0.66ug each of plasmid DNA for expression of a non-luminescent polypeptide mutant and a non-luminescent peptide mutant plasmid to a final volume of 31µl in OptiMem. 2µl Fugene HD was added to each transfection complex and incubated for 15 minutes at room temperature. For each peptide/polypeptide combination, 5µl of a transfection complex was added to 6 wells of the 96-well plate and grown overnight at 37C in CO_2 incubator. After overnight incubation, the growth media was removed and replaced with CO_2- independent media containing either 20uM coelenterazine or 20uM Furimazine . The samples were incubated for 10 minutes at 37ºC, and kinetics measured over the course of 1 hour at 37ºC on a GloMax Multi +. Figure 30 demonstrates the substrate specificity of various NLpoly/NLpep pairs when the NLpoly is expressed in mammalian cells.

Example 17

Luminescence and Substrate Specificity

The following example investigates the luminescence and substrate specificity of various non-luminescent polypeptide mutants with NLpep69 and using either Furimazine or coelenterazine as a substrate.

CHO cells were plated at 20,000 cells/ well in 100µl of DMEM+10% FBS into wells of 96-well plates. The cells were incubated in a 37ºC, 5% CO2 incubator overnight. Transfection complexes were prepared by adding 0.66ug each of plasmid DNA for expression of a non-luminescent polypeptide mutant and a non-luminescent peptide mutant plasmid to a final volume of 31µl in OptiMem. 2µl Fugene HD was added to each transfection complex and incubated for 15 minutes at room temperature. For each peptide/polypeptide combination, 5µl of transfection complex was added to 6 wells of the 96-well plate and grown overnight at 37C in CO_2 incubator. After overnight incubation, the growth media was removed and replaced with CO_2- independent media containing either 20uM coelenterazine or 20uM Furimazine . The samples were incubated

for 10 minutes at 37ºC, and kinetics measured over the course of 1 hour at 37ºC on a GloMax Multi +. Figure 31 demonstrates the substrate specificity when NLpolys are coexpressed in mammalian cells with NLpep69.

Example 18

Luminescence and Substrate Specificity Between Live-Cell and Lytic Conditions

The following example investigates the luminescence and substrate specificity of various non-luminescent polypeptide mutants with NLpep69, NLpep78 or NLpep79, using either Furimazine or coelenterazine as a substrate and under either lytic or live cell conditions.

HEK 293 cells were plated at 15,000 cells/well in 100µl DMEM+10% FBS into wells of 96-well plates. The cells were incubated in a 37ºC, 5% CO2 incubator overnight. Transfection complexes were prepared by adding 0.66ug each of plasmid DNA for expression of a non-luminescent polypeptide mutant and a non-luminescent peptide mutant plasmid to a final volume of 31µl in OptiMem. 2µl Fugene HD was added to each transfection complex and incubated for 15 minutes at room temperature. For each NLpoly-NLpep combination, 5µl of transfection complex was added to 6 wells of the 96-well plate and grown overnight at 37C in CO2 incubator. After overnight incubation, the growth media was removed and replaced with CO2- independent media containing either 20uM coelenterazine or 20uM Furimazine . The samples were incubated for 10 minutes at 37ºC, and kinetics measured over the course of 1 hour at 37ºC on a GloMax Multi +. Figures 32-34 demonstrate the substrate specificity of NLPolys coexpressed in mammalian cells with NLpep69, 78, or 79 in live-cell and lytic formats.

Example 19

Comparison of non-luminescent polypeptide mutants expressed in E.coli

A single colony of each non-luminescent polypeptide was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7 except NLpep78-HT or NLpep79-HT at 1:1,000 dilution was used. Figure 35 demonstrates the luminescence of NLpolys expressed in E. coli and assayed with NLpep78 or 79.

Example 20

Ability of non-luminescent polypeptide clones to produce luminescence without complementing non-luminescent peptide

A single colony of each non-luminescent polypeptide was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example 7 except no non-luminescent peptide was added to the assay buffer.

Figure 36 demonstrates the luminescence of NLpolys expressed in E. coli and assayed in the absence of NLpep.

Example 21

Substrate specificity of non-luminescent polypeptide mutants expressed in E.coli

A single colony of each non-luminescent polypeptide was grown according to the procedure used in Example 7. The bacterial cultures were also induced according to the procedure used in Example 7. Luminescence was assayed and detected according to the procedure used in Example except either Furimazine or coelenterazine was mixed with NANOGLO Assay Buffer. Figure 37 demonstrates the substrate specificity of NLpolys expressed in E. coli and assayed with NLpep78 or 79.

Example 22

Improved luminescence of non-luminescent polypeptide mutants with NLpep78

Complementation of the non-luminescent polypeptide mutants with NLpep78-HT was demonstrated in CHO and Hela cells.

CHO and Hela cells (CHO: 100,000 seeded the day prior to transfection; Hela: 50,000 seeded the day prior to transfection) were transfected with 5ng of a non-luminescent polypeptide mutant 5A2 or 5P or with wild-type non-luminescent polypeptide using Fugene HD into wells of a 24-well plate and incubated at 37ºC overnight. After the overnight incubation, the media was replaced with DMEM without phenol red, and the cells frozen at -80ºC for 30 minutes. The cells were then thawed and transferred to a 1.5ml tube. The cell lysates were then diluted 1:10 DMEM without phenol red, 20µl mixed with NLpep78 (NLpep78-HT7 E.coli lysate diluted 1:1,000 in DMEM without phenol red) and shaken at room temperature for 10 minutes. 40µl DMEM without phenol red and 20uM Furimazine were added and luminescence measured on a GloMax with a 0.5 second integration. Figure 38 demonstrates the luminescence of NLpolys expressed in mammalian cells and assayed with NLpep78.

Example 23

Non-luminescent polypeptide fusions and normalizing non-luminescent polypeptide concentrations

A comparison of raw and normalized luminescence from non-luminescent polypeptide fused to either firefly luciferase (Figure 39) or click beetle red luciferase (Figure 40) were performed to provide insight into how much benefit, e.g., in expression, solubility and/or stability, stems from the concentration of the non-luminescent polypeptide as well as complementation as a fusion non-luminescent polypeptide..

HEK293, Hela or CHO cells were transfected with 5ng 5P NLpoly-firefly luciferase fusion, 5P NLpoly-click beetle luciferase fusion, wild-type 5P-firefly luciferase fusion or wild-

type 5P-click beetle luciferase fusion according to the procedure in Example 22. Lysates were also prepared according to Example 22. The cell lysates were then diluted 1:10 DMEM without phenol red, 20µl mixed with NLpep78 (diluted 1:100 in DMEM without phenol red; E.coli lysate) and shaken at room temperature for 10 minutes. 40µl NanoGlo with 20uM Furimazine or Bright-Glo (Promega Corporation) was added and luminescence measured on a GloMax with 0.5 second integration. Figures 39 and 40 demonstrate the specific activity of 5P versus WT NLpoly expressed in mammalian cells and assayed with NLpep78.

Example 24

Complementation in live cells

This example demonstrates complementation in live-cells using either wild-type or 5P NLpoly. Hela cells plated into wells of 96-well plated, transfected with 0.5ng of wild-type or 5P non-luminescent polypeptide plasmid DNA using Fugene HD and incubated at 37ºC overnight. After the overnight incubation, the cells were then transfected with 0.5ng NLpep78-HT plasmid DNA using Fugene HD and incubated at 37ºC for 3 hours. The media was then replaced with CO_2-independent media+0.1% FBS and 20uM PBI-4377 and luminescence measured at 37ºC on a GloMax with 0.5 second integration. Figure 41 demonstrates the live-cell complementation between 5P or WT NLpoly and NLpep78.

Example 25

Complementation in cell-free extract

To demonstrate complementation in cell-free extract, 0.5ug NLpep78-HT and 0.5ug non-luminescent polypeptide mutant plasmid DNA were mixed with TNT rabbit reticulocyte lysate master mix (Promega Corporation) and incubated at 30ºC for 1 hour. 25µl of the cell-free expression extract was mixed with 25µl NanoGlo Luciferase Assay reagent and incubated at room temperature for 10 minutes. Luminescence was measured on a GloMax with 0.5 second integration. Figure 42 demonstrates luminescence from complementing NLpoly/NLpep pairs expressed in a cell-free format.

Example 26

Binding affinity of non-luminescent polypeptide expressed in mammalian cells with synthetic non-luminescent peptide

To demonstrate the binding affinity between non-luminescent polypeptide and non-luminescent peptide pairs, non-luminescent polypeptide lysates from Hela, HEK293 and CHO cells were prepared as previously described and diluted 1:10 PBS+0.1% Prionex. 4x concentrations of non-luminescent peptide (synthetic) were made in PBS+0.1% Prionex. 20µl of the non-luminescent polypeptide lysate was mixed with 20µl non-luminescent peptide and shaken at room temperature for 10 minutes. 40µl of NanoGlo Luciferase Assay Reagent or

PBS+0.1% Prionex with Furimazine was added and shaken at room temperature for 10 minutes. Luminescence was detected on a GloMax with 0.5s integration. Kd values were determined using Graphpad Prism, One Site-Specific Binding. Figure 43 and 44 demonstrate the dissociation constants measured under various buffer conditions (PBS for complementation then NanoGlo for detection, PBS for complementation and detection, NanoGlo for complementation and detection).

Example 27

Improved binding affinity when cysteine mutated to phenylalanine in non-luminescent peptide mutants

To demonstrate improved binding affinity in non-luminescent peptide mutants with a mutated cysteine at the 8th residue of the peptide, non-luminescent polypeptide mutant lysates from Hela, HEK293 and CHO cells were prepared as previously described and diluted 1:10 PBS+0.1% Prionex. 4x concentrations of non-luminescent peptide (NLpep) were made in PBS+0.1% Prionex+10mM DTT. 20µl of the non-luminescent polypeptide lysate was mixed with 20µl non-luminescent peptide and shaken at room temperature for 10 minutes. 40µl of NanoGlo Luciferase Assay Reagent was added and shaken at room temperature for 10 minutes. Luminescence was detected on a GloMax with 0.5s integration. Figure 45 demonstrates NLpep C8F mutation significantly improves the binding affinity for 5P.

Example 28Detectable luminescence of polypeptide variants without non-luminescent peptide in Hela cells

To demonstrate luminescence in non-luminescent polypeptide without non-luminescent peptide, Hela cells (10,000 seeded the day prior to transfection) in wells of a 96-well plate were transfected with varying amounts of non-luminescent polypeptide + pGEM-3zf Carrier DNA to a total of 50ng using Fugene HD and incubated 37ºC overnight. After incubation, the media was replaced with CO_2-independent media+0.1% FBS+20uM Furimazine and incubated at 37ºC for 10 minutes, and luminescence detected on a GloMax with 0.5s integration. Figure 46 demonstrates the luminescence of NLpoly WT or 5P in live Hela cells without NLpep after transfection of various amounts of plasmid DNA.

Example 29

Generation of additional non-luminescent polypeptide variants

Additional non-luminescent polypeptide variants: Ile-11 (Ile at residue 11), Val-11, Tyr-11, Glu-11, Glu-157, Pro-157, Asp-157, Ser-157, Met-149, Leu-106, NLpoly11, and NLpoly12 were generated as described below, and their expression analyzed. The additional non-luminescent polypeptide variants were made in the 5A2 non-luminescent polypeptide background.

Fresh individual colonies (KRX) of each additional non-luminescent polypeptide variants were picked and grown overnight in LB+ampicillin (100ug/ml) at 30ºC and then diluted 1:100 in LB+ampicillin and grown at 37ºC for 2.5 hours (OD600 ~0.5). Rhamnose was added to a final concentration of 0.2%, and the cells were split in triplicate and grown overnight at 25ºC for ~18 h. Cells were lysed using 0.5X Fast Break for 30 minutes at ambient temperature, snap-frozen on dry ice, and stored at -20°C. Upon fast thawing, soluble fractions were prepared by centrifugation at 10K for 15 min at 4ºC. Samples were assayed for luminescence on a Tecan Infinite F-500 luminometer.

Figure 49 demonstrates that total lysate and soluble fraction of each non-luminescent polypeptide variant as analyzed by SDS-PAGE. The data provides information about expression, solubility and stability of the additional non-luminescent polypeptide variants. A majority of the additional non-luminescent polypeptide variants produced more protein (total and soluble) than wild-type, but in many cases, the difference is subtle. Improved expression for NLpoly11 and NLpoly12 was more noticeable.

Example 30

Background luminescence of additional non-luminescent polypeptide variants

The background luminescence of the additional non-luminescent polypeptide variants generated in Example 29 was measured by incubating 25µl of non-luminescent polypeptide variant lysate with 25µl DMEM at room temperature for 10 minutes. 50µl NanoGlo Luciferase Assay Reagent was then added, and luminescence measured at 5 and 30 minutes on a Tecan Infinite F500. NLpep53 (Pep 53) alone and DMEM (DMEM) alone were used as controls. Figure 47 demonstrates that a majority of the additional non-luminescent polypeptide variants showed elevated background luminescence.

Example 31

Luminescence of additional non-luminescent polypeptide variants after complementation

Luminescence of the additional non-luminescent polypeptide variants generated in Example 28 was measured by incubating 25µl of non-luminescent polypeptide variant lysate with 25µl NLpep-53 at room temperature for 10 minutes50µl NanoGlo Luciferase Assay Reagent was then added, and luminescence measured at 5 and 30 minutes on a Tecan Infinite F500. NLpep53 (Pep 53) alone and DMEM (DMEM) alone were used as controls. Figure 48 demonstrates that the non-luminescent polypeptide variants Val-11, Glu-11, Glu-157, Pro-157, Asp-157, Ser-157 and Met-149 generated significantly more luminescence than parental 5A2.

Example 32

Correlation between increased background luminescence of non-luminescent polypeptide in the absence of non-luminescent peptide and amount of protein in soluble fraction

Individual colonies of the non-luminescent polypeptide variants 3P, 3E, 5P, 5E, 6P and 6E were picked and grown overnight in LB+ampicillin at 30ºC and then diluted 1:100 in LB+ampicillin and grown at 37ºC for 2.5 hours (OD600 ~0.5). Rhamnose was added to a final concentration of 0.2%, and the cells were split in triplicate and grown overnight at 25ºC for ~18 h. Cells were lysed using 0.5X Fast Break for 30 minutes at ambient temperature, snap-frozen on dry ice, and stored at -20ºC. Upon fast thawing, soluble fractions were prepared by centrifugation at 10K for 15 min at 4ºC. Samples were assayed for luminescence on a Tecan Infinite F-500. Figure 50A shows the total lysate and soluble fraction of each non-luminescent polypeptide variant. Figure 50B shows the background luminescence of each non-luminescent polypeptide variant. Figure 51 shows the luminescence generated with each non-luminescent polypeptide variant when complemented with 10 or 100nM NLpep78 (NVSGWRLFKKISN) in LB medium.

Example 33

Elongations and deletions of non-luminescent polypeptide

The non-luminescent polypeptide variant 5P was either elongated at the C-terminus by the addition of the residues VAT, AA, VTG, VT, VTGWR, VTGW, V, A, VA, GG, AT, GTA, ATG or GT or deletion of 1 to 7 residues at the C-terminus of 5P, e.g., D1=deletion of 1 residue, D2=deletion of 2 residues, etc. Background luminescence in E.coli lysates (Figure 52) and luminescence generated after complementation with NLpep78 (Figure 53; NVSGWRLFKKISN) or NLpep79 (Figure 54; NVTGYRLFKKISN) were measured. Figure 55 shows the signal-to-background of the non-luminescent polypeptide 5P variants. Figure 56 provides a summary of the luminescent results. Figure 57 shows the amount of total lysate and soluble fraction in each non-luminescent polypeptide 5P variant.

Example 34

Comparison of 5P and I107L non-luminescent polypeptide Variant

Figure 58 shows the amount of total lysate and soluble fraction of 5P and I107L (A), luminescence generated by 5P or I107L without non-luminescent peptide or with NLpep78 or NLpep79 (B) and the improved signal-to-background of I107L over 5P (C).

Example 35

Generation of 5P non-luminescent polypeptide mutants

Mutations identified in a screening of random mutations in the 5P non-luminescent polypeptide variant were generated as previously described. Each single 5P non-luminescent polypeptide mutant colony was inoculated in 200µl Minimal Media and incubated with shaking

at 37ºC for 20 hours. 10µl of the culture was then added to 190µl of fresh Minimal Media and incubated again with shaking at 37ºC for 20 hours. 10µl of the second culture was then added to 190µl Auto-Induction Media (Minimal Media + 5% glucose + 2% rhamnose) and incubated with shaking at 25ºC for 18 hours to allow expression of the non-luminescent polypeptide mutant. 10µl of the 5P non-luminescent polypeptide mutant expression culture was added to 40µl of assay lysis buffer containing NLpep78-HT (1:386 dilution) or NLpep79-HT (1:1,000 dilution) and shaken at room temperature for 10 minutes. 50µl of NanoGlo Assay Buffer containing 100uM coelenterazine was added and shaken at room temperature for 10 minutes. Luminescence was measured on GloMax with 0.5sec integration. Figures 59-62A shows background luminescence while Figures 59-62B and C show luminescence generated after complementation with NLpep78 or NLpep79.

Example 36

Binding affinity between elongated non-luminescent polypeptide variant and deleted non-luminescent peptide

The binding affinity between an elongated non-luminescent polypeptide variant, i.e., containing additional amino acids at the C-terminus, and a deleted non-luminescent peptide, i.e., deleted amino acids at the N-terminus.

Lysates of E.coli expressing non-luminescent polypeptide 5P/+V/+VT/+VTG prepared as previously described were diluted 1:2000 in PBS + 0.1% Prionex. 25µl of the diluted lysate was incubated with 25µl of NLpep78, NLpep80, NLpep81 or NLpep82 (diluted 0-500nM in dilution buffer) for 5 min at room temp. 50µl of Furimazine diluted to 1X with NanoGlo Assay Buffer was added to each sample and incubated for 10 minutes at room temperature. Luminescence was measured on a GloMax Multi with 0.5s integration time. Figure 63 demonstrates the binding affinity between NLpolys with additional amino acids at the C-terminus with NLpeps with amino acids deleted from the N-terminus.

Example 37

Binding affinity between non-luminescent polypeptide expressed in E.coli and synthetic non-luminescent peptide

Non-luminescent polypeptide LB lysates were prepared and diluted 1:100 into PBS+0.1% Prionex. 2X dilutions of synthetic NLpep78 were made in PBS+0.1% Prionex. 25µl of the diluted non-luminescent polypeptide lysate was mixed with 25µl of each dilution of non-luminescent peptide and incubated 3 minutes at ambient temperature. 50µl of NanoGlo Luciferase Assay Reagent was added, incubated for 5 minutes at room temperature, and luminescence measured on a GloMax Multi+. Figure 64 shows the calculated Kd values using one-site specific binding.

Example 38

Binding affinity between 5P non-luminescent polypeptide expressed in mammalian cells and NLpep80 or NLpep87

Lysates of CHO, HEK293T, or HeLa cells expressing NLpoly 5P were diluted 1:1000 in dilution buffer (PBS + 0.1% Prionex.) 25 µl of diluted lysate was incubated with 25 µl of NLpep80/87 (diluted 0-5 µM in dilution buffer) for 5 min at room temp. 50 µl of furimazine (diluted to 1X with NanoGlo buffer) was added to each well, and the plate was incubated for 10 min at room temp. Luminescence was then read on a GloMax Multi with 0.5s integration time (Figure 65).

Example 39

Binding affinity between 5P non-luminescent polypeptide expressed in E.coli and NLpep80 or NLpep87

Lysates of E.coli expressing NLpoly 5P were diluted 1:2000 in dilution buffer (PBS + 0.1% Prionex.) 25 µl of diluted lysate was incubated with 25 µl of NLpep80/87 (diluted 0-5 µM in dilution buffer) for 5 min at room temp. 50 µl of furimazine (diluted to 1X with NanoGlo buffer) was added to each well, and the plate was incubated for 10 min at room temp. Luminescence was then read on a GloMax Multi with 0.5s integration time (Figure 66).

Example 40

Complementation between a deleted non-luminescent polypeptide and elongated non-luminescent peptide

Complementation between a deleted non-luminescent polypeptide, i.e., amino acids deleted from the C-terminus, and an elongated non-luminescent peptide, i.e., amino acids added to the N-terminus, was performed. NLpep-HT E. coli clarified lysates as prepared as previously described in Example 6. The amount of NLpep-HT was quantitated via the HaloTag fusion. Briefly, 10µl of clarified lysate was mixed with 10µl HaloTag-TMR ligand (diluted 1:100) and 80µl water and incubated at room temperature for 10 minutes. 33.3µl 4x SDS Loading Buffer was added and incubated at 95ºC for 5 minutes. 15µl was loaded onto an SDS-PAGE gel and imaged on a Typhoon. Based on the intensities from the SDS-PAGE gel, non-luminescent peptides were diluted in PBS+0.1% Prionex non-luminescent peptides to make equivalent concentrations. The non-luminescent polypeptide lysates were then diluted 1:100 in PBS+0.1% Prionex. 20µl of diluted non-luminescent polypeptide and 20µl diluted non-luminescent peptide were mixed and shaken at room temperature for 10 minutes. 40µl NanoGlo Luciferase Assay Reagent was added and shaken at room temperature for 10 minutes. Luminescence was measured on a GloMax using 0.5sec integration. Figure 67 demonstrates the luminescence of

NLpolys with amino acids removed from the C-terminus with NLpeps with additional amino acids on the N-terminus.

Example 41

Binding affinity between 5P non-luminescent polypeptide expressed in Hela Cells and NLpep78 or truncated NLpep78 (NLpep80-87)

5P non-luminescent polypeptide lysate was prepared from Hela cells as previously described and diluted prepared 1:10 in PBS +0.1% Prionex. 4x concentrations (range determined in preliminary titration experiment) of non-luminescent peptide (synthetic peptide; by Peptide 2.0 (Virginia); made at either 5, 10, or 20 mg scale; blocked at the ends by acetylation and amidation, and verified by net peptide content analysis) was prepared in PBS +0.1% Prionex. 20µl 5P non-luminescent polypeptide and 20µl non-luminescent peptide were mixed and shaken at room temperature for 10 minutes. 40µl of NanoGlo Luciferase Assay reagent was added and shaken at room temperature for 10minutes. Luminescence was measured on GloMax with 0.5s integration. Figure 68 demonstrates the binding affinity and corresponding luminescence between 5P and truncated versions of NLpep78. The binding affinity is increased when 1 amino acid is removed from the N-terminus, the C-terminus, or 1 amino acid from each terminus. Removing more than 1 amino acid from either terminus lowers the affinity but does not always lower the Vmax to the same extent.

Example 42

Binding affinity between elongated non-luminescent polypeptide and truncated non-luminescent peptide

The binding affinity between an elongated non-luminescent polypeptide, i.e., one with 2 extra amino acids on C-terminus, and a truncated non-luminescent peptide, i.e., one with 2 amino acids removed from N-terminus (NLpep81), was determined.

Non-luminescent polypeptide lysate was prepared as previously described and diluted prepared 1:100 in PBS +0.1% Prionex. 2x dilutions of NLpep81 (synthetic peptide; by Peptide 2.0 (Virginia); made at either 5, 10, or 20 mg scale; blocked at the ends by acetylation and amidation, and verified by net peptide content analysis) was prepared in PBS +0.1% Prionex. 25µl non-luminescent polypeptide and 25µl of each non-luminescent peptide dilution were mixed and shaken at room temperature for 3 minutes. 50µl of NanoGlo Luciferase Assay reagent was added and shaken at room temperature for 5 minutes. Luminescence was measured on GloMax with 0.5s integration. Figure 69 shows the calculate Kd values using one-site specific binding.

Example 43

Binding affinity between elongated non-luminescent polypeptide and truncated non-luminescent peptide

The binding affinity between an elongated non-luminescent polypeptide, i.e., one with 3 extra amino acids on C-terminus, and a truncated non-luminescent peptide, i.e., one with 3 amino acids removed from N-terminus (NLpep82), was determined.

Non-luminescent polypeptide lysate was prepared and diluted prepared 1:100 in PBS +0.1% Prionex. 2x dilutions of NLpep82 (synthetic peptide; by Peptide 2.0 (Virginia); made at either 5, 10, or 20 mg scale; blocked at the ends by acetylation and amidation, and verified by net peptide content analysis) was prepared in PBS +0.1% Prionex. 25µl non-luminescent polypeptide and 25µl of each non-luminescent peptide dilution were mixed and shaken at room temperature for 3 minutes. 50µl of NanoGlo Luciferase Assay reagent was added and shaken at room temperature for 5 minutes. Luminescence was measured on GloMax with 0.5s integration. Figure 70 shows the calculate Kd values derived using one-site specific binding.

Example 44

Binding affinity between non-luminescent polypeptide clones expressed in E.coli and Synthetic NLpep78

Non-luminescent polypeptide variants were grown in M9 minimal media. Individual colonies were inoculated and grown overnight at 37ºC. Samples were diluted 1:20 in M9 minimal media and grown overnight at 37ºC. Samples were again diluted 1:20 in M9 induction media and grown overnight at 25ºC. Samples were pooled, and 100µl of the pooled cells were lysed with 400µl of PLB lysis buffer and incubate at room temperature for 10 minutes. The lysates were diluted 1:100 in PBS+0.1% Prionex. 2X dilutions of synthetic NLpep78 were made in PBS+0.1% Prionex. 25µl of non-luminescent polypeptide dilution was mixed with 25µl of each non-luminescent peptide dilution and incubated for 3 minutes at room temperature. 50µl of NanoGlo Luciferase Assay Reagent was added, incubated at room temperature for 5 minutes, and luminescence read on GloMax Multi+. Figure 71 shows the calculate Kd values derived using one-site specific binding.

Example 45

Determination of the effect of mutations on Km

Using diluted pooled lysates from Example 11, 25µl of non-luminescent polypeptide diluted lysate (1:100 in PBS+0.1% Prionex) was mixed with 25µl of 500nM NLpep78 for each sample and incubated at room temperature for 5 minutes. 2X dilutions of Furimazine in NanoGlo Luciferase Assay Buffer were prepared, and 50µl of non-luminescent peptide and non-luminescent polypeptide sample mixed with 50µl of NanoGlo/Furimazine dilutions.

Luminescence was measured after 5 minute incubation at room temperature. Figure 72 show the calculated Km derived using Michaelis-Menten.

Example 46

Demonstration of a three-component complementation

A tertiary complementation using 2 NLpeps and NLpoly 5P non-luminescent polypeptide is demonstrated. NLpoly 5P-B9 (5P with residues 147-157 deleted) and NLpep B9-HT (Met+ residues 147-157 fused to N-terminus of HT7) lysates were prepared.

A) NLpoly 5P-B9+ NLpoly B9 titration with NLpep78

NLpoly 5P-B9+ NLpoly B9 was titrated with NLpep78. 20µl 5P-B9 (undiluted) was mixed with 20µl peptideB9-HT (undiluted). Dilutions of NLpep78 (synthetic peptide, highest concentration = 100uM) were made in PBS +0.1% Prionex. 20µl NLpep78 was added to 40µl of the 5P-B9+peptideB9-HT mixture and shaken at room temperature for 10 minutes. 60µl NanoGlo Luciferase Assay Reagent was added and shaken at room temperature for 10 minutes. Luminescence was measured on GloMax with 0.5s integration.

B) NLpoly 5P-B9+ NLpep78 titration with NLpepB9-HT

20µl NLpoly 5P-B9 (undiluted) was mixed with 20µl NLpep78 (100uM). Dilutions of peptideB9-HT (highest concentration = undiluted) were made in PBS +0.1% Prionex. 20µl of peptideB9-HT was added to 40µl of the 5P-B9+NLpep78 mixture and shaken at room temperature for 10 minutes. 60µl NanoGlo Luciferase Assay Reagent was added and shaken at room temperature for 10 minutes. Luminescence was measured on GloMax with 0.5s integration.

Figure 73 demonstrates the feasibility of a ternary system consisting of 2 different NLpeps and a truncated NLpoly. Since all 3 components are non-luminescent without the other 2, this system could be configured such that each NLpep is fused (synthetically or genetic engineering) to a binding moiety and the truncated NLpoly used at high concentrations to produce light only in the presence of an interaction between the binding moieties, or such that each of the 3 components are fused to binding moieties to produce light only in the event of ternary complex formation.

Example 47

Complementation with NLpep88 (NLpep78 with Gly as 6th residue instead of Arg)

NLpep88-HT and 5P E. coli clarified lysates were prepared as previously described. Serial dilutions of NLpep88-HT lysate were made in PBS+0.1% Prionex. 20µl of 5P lysate and 20µl NLpep88-HT lysate were mixed and shaken at room temperature for 10 minutes. 40µl of

NanoGlo Luciferase Assay Reagent was added and shaken at room temperature for 10 minutes. Luminescence was measured on GloMax with 0.5s integration. Figure 74 demonstrates the importance of the arginine residue at the 6th position of the NLpep. While there is no increase in luminescence above 5P alone at lower concentrations of NLpep88, high concentrations of NLpep increased the luminescence suggesting a catalytically compromised complex and not a lack of interaction between 5P and NLpep88.

Example 48

Subcellular localization of NLpep78 and 79 as N-terminal fusions to HaloTag.

U2OS cells were plated and left to recover overnight at 37ºC. Cells were then transfected with HaloTag alone DNA construct or the HaloTag-NanoLuc peptide DNA constructs (all under the control of CMV promoter): P1-HT, P78-HT or P79-HT diluted 1:10 with carrier DNA (pSI) using FuGENE HD and incubated for 24 hours at 37ºC. Cells were then labeled with HaloTag-TMR ligand by the manufacturer's standard rapid labeling protocol and imaged. Figure 75 demonstrates that NLpep78 and 79 do not alter the intracellular localization of the HaloTag protein.

Example 49

Subcellular localization of non-luminescent polypeptide (WT and 5P)

U2OS cells were plated and left to recover overnight at 37ºC. Cells were either kept as non-transfection controls or transfected with the NanoLuc DNA constructs: FL, NLpoly (wt) or NLpoly(5P) diluted 1:10 with carrier DNA (pSI) using FuGENE HD and incubated for 24 hours at room temperature. Cells were fixed and subsequently processed for ICC. ICC was done using 1:5000 GS (PRO) primary antibody overnight at 4°C followed by an Alexa488 goat anti-rabbit secondary antibody. Figure 76 demonstrates that both NLpoly WT and NLpoly 5P localize uniformly in cells.

Example 50

Demonstration that non-luminescent polypeptide can easily and quickly detect non-luminescent peptide conjugated to a protein of interest.

99µl of NLpep53-HT E. coli clarified lysate was mixed with24.75µl 4x SDS loading buffer. 1:10 serial dilutions of the lysate-loading buffer mixture were made and incubated at 95ºC for 5 minutes. 15µl was loaded onto a SDS-PAGE gel. After gel completions, it was transferred to PVDF using iBlot and washed with 10mL NLpoly L149M *E. coli* clarified lysate at room temperature for 30 minutes. The membrane was then placed on a LAS4000 imager and 2mL NanoGlo® Luciferase Assay Reagent added. A 60 second exposure was taken (Figure 77).

Example 51

Site saturation at non-luminescent polypeptide positions 31, 46, 108, 144, and 157 in the context of 5P

Single amino acid change variants were constructed onto NLpoly 5P (pF4Ag vector background) at the sites according to table 5 below. In effect, the native residue was varied to each of the 19 alternative amino acids for a total of 95 variants.

Table 5

Position 31		Position 46		Position 108		Position 144		Position 157	
B1	Ala	E3	Ala	H5	Ala	C8	Ala	F10	Ala
C1	Cys	F3	Cys	A6	Cys	D8	Cys	G10	Cys
D1	Asp	G3	Asp	B6	Asp	E8	Asp	H10	Asp
E1	Glu	H3	Glu	C6	Glu	F8	Glu	A11	Glu
F1	Gly	A4	Phe	D6	Phe	G8	Phe	B11	Phe
G1	His	B4	Gly	E6	Gly	H8	Gly	C11	Gly
H1	Ile	C4	His	F6	His	A9	His	D11	His
A2	Lys	D4	Ile	G6	Ile	B9	Ile	E11	Ile
B2	Leu	E4	Lys	H6	Lys	C9	Lys	F11	Lys
C2	Met	F4	Met	A7	Leu	D9	Leu	G11	Leu
D2	Asn	G4	Asn	B7	Met	E9	Met	H11	Met
E2	Pro	H4	Pro	C7	Pro	F9	Asn	A12	Asn
F2	Gln	A5	Gln	D7	Gln	G9	Pro	B12	Gln
G2	Arg	B5	Arg	E7	Arg	H9	Gln	C12	Arg
H2	Ser	C5	Ser	F7	Ser	A10	Arg	D12	Ser
A3	Thr	D5	Thr	G7	Thr	B10	Ser	E12	Thr
B3	Val	E5	Val	H7	Val	C10	Val	F12	Val
C3	Trp	F5	Trp	A8	Trp	D10	Trp	G12	Trp
D3	Tyr	G5	Tyr	B8	Tyr	E10	Tyr	H12	Tyr

Individual colonies were grown in LB+amp and incubated overnight at 30ºC. A 5P control was also included. The overnight cultures were used to inoculate fresh LB+amp (1:100), and these cultures grew for 2 hours 45 minutes at 37ºC. Rhamnose was added to 0.2%, and the cultures left to grow/induce overnight at 25ºC. After 18 hours of induction, cells were lysed using 0.5X FastBreak (30 min ambient temperature), snap frozen on dry ice, and stored at -20°C. Following a fast thaw, samples were assayed in the absence and presence of Pep87 (aka NLpep 87).

For the (-) peptide reactions, 30 uL lysate was incubated with 30uL PBS pH 7.5 for 10 min and then 60 uL NanoGlo® Luciferase Assay reagent (Promega Corporation) added. After 5 minutes, luminescence was measured. For the (+) peptide reactions, 30uL lysate was incubated with 30uL of 8nM Pep87. After 10 min, 60 uL NanoGlo® Luciferase Assay reagent was added, and luminescence measured at 5 minutes.

Luminescence (RLU) data for the (-) peptide samples were normalized to the readings for the 5P control, and these results are presented in Figure 78. Luminescence (RLU) data for the

(+) peptide samples were also normalized to 5P, but then also normalized to the values in Figure 76 in order to represent signal to background (S/B; Figure 79).

Example 52

Use of the High Affinity Between NLpoly and NLpep for Protein Purification/Pull Downs

MAGNEHALOTAG beads (Promega Corporation; G728A) were equilibrated as follows: a) 1mL of beads were placed on magnet for ~30sec, and the buffer removed; b) the beads were removed from magnet, resuspended in 1mL PBS+0.1% Prionex, and shaken for 5min at RT; and c) steps a) and b) were repeated two more times NLpep78-HaloTag (*E. coli* clarified lysate) was bound to MAGNEHALOTAG beads by resuspending the beads in 1mL NLpep78-HT clarified lysate, shaking for 1hr at RT and placing on magnet for ~30sec. The lysate (flow through) was removed and saved for analysis. NLpoly 8S (*E. coli* clarified lysate) was bound to the NLpep78 bound-MagneHaloTag beads from the step above by resuspending the beads in 1.5mL 8S lysate, shaking for 1hr at RT and placing on a magnet for ~30sec. The lysate (flow through) was removed and saved for analysis. The beads were resuspended in 1mL PBS +0.1% Prionex, shaken for 5 min at RT, placed on magnet for ~30sec, and PBS (wash) removed. The beads were washed three more times.

To elute the bound peptide/polypeptide, the beads were resuspended in 500uL 1xSDS buffer and shaken for 5min at RT. The beads were then placed on a magnet for ~30sec; the SDS buffer (elution) removed and saved for analysis. The elution was repeated one more time.

The samples were then analyzed by gel. 37.5uL of sample (except elutions) was mixed with 12.5uL 4X SDS buffer and incubated at 95^0C for 5min. 5uL was loaded onto a Novex 4-20% Tris-Glycine gel and run at ~180V for ~50min. The gel was stained with SimplyBlue Safe Stain and imaged on a LAS4000 imager.

Figure 94 illustrates that the affinity of NLpoly and NLpep is sufficient to allow for purification from an E. coli lysate. As NLpoly 8S was purified from an *E. coli* lysate, it is reasonable to expect a protein fused to NLpoly 8S (or other variant described herein) could also be purified in a similar fashion. While in this example the NLpep was immobilized and used to purify NLpoly, it is also reasonable to expect a similar result if NLpoly were immobilized.

Example 53

Kinetics of NLpoly/NLpep Binding

2x concentrations of synthetic NLpep were made and diluted 2.7-fold nine times (10 concentrations) in PBS+0.1% Prionex. Final concentrations used in the assay were 30uM-3.9nM. WT NLpoly (*E.coli* clarified lysate; 1:10,000) or 11S (1:10,000,000) was diluted in NanoGlo+100uM Furmazine (Fz). 50uL of NLpep was placed into wells of white 96-well assay plate. 50uL NLpoly/NanoGlo/Fz was injected into the wells using the injector on GloMax®

Multi+ instrument, and luminescence measured every 3sec over 5min. k_{obs} was found by fitting data to: $Y = Y_{max}(1 - e^{-k_{obs}t})$ using Graphpad Prism. k_{on} and k_{off} were then fitted to: $k_{obs} = [NLpep]k_{on} + k_{off}$. Figure 95 illustrates the association and dissociation rate constants for the binding between NLpolys and NLpeps.

Example 54

NLpoly/NLpep Substrate Affinity

NLpoly was diluted into PBS+0.1% Prionex as follows: WT at $1:10^5$, 5P at $1:10^7$, and 11S at $1:10^8$. NLpep was diluted into PBS+0.1% Prionex as follows: 30uM for WT NLpoly studies or 3uM for NLpoly 5P and 11S studies. 50uL NLpoly/NLpep was incubated at RT for 5min, 50uL NanoGlo + Fz (ranging from 100uM to 1.2uM, 2X) added, and incubated for 10min at RT. Luminescence was measured on GloMax® Multi+ with 0.5sec integration. Km was derived using Graphpad Prism, Michaelis-Menton best-fit values. Figure 96 illustrates the Km values for various NLpoly/NLpep pairs.

Example 55

Substrate Effect on NLpoly/NLpep Affinity

11S (E. coli clarified lysate) was diluted into PBS+0.1% Prionex at $1:10^7$. Synthetic NLpep79 was diluted serially (1:2) from 800nM to 0.39nM (2X). 20uL 11S + 20uL NLpep79 were then mixed and incubated for 5min at RT. 40uL NanoGlo + 5uM or 50uM Fz was added and incubated another 5min at RT. Luminescence was measured on GloMax® Multi+ with 0.5sec integration. Kd was derived using Graphpad prism, One site-Specific binding value. Figure 97 illustrates that saturating concentrations of furimazine increase the affinity between 11S and NLpep79.

Example 56

Km for NLpoly 5A2:NLpep

NLpoly 5A2 was diluted into PBS+0.1% Prionex at $1:10^5$. NLpep (WT, NLpep 78 or NLpep79) was diluted into PBS+0.1% Prionex to 30uM. 50uL NLpoly/NLpep was incubated at RT for 5min. 50uL NanoGlo + Fz (ranging from 100uM to 1.2uM, 2X) was added and incubated for 10min at RT. Luminescence was measured on GloMax® Multi+ with 0.5sec integration. Km was derived using Graphpad Prism, Michaelis-Menton best-fit values. Figure 98 illustrates the Km values for NLpoly5A2 and NLpep WT, 78, and 79.

Example 57

Luminescence of NLpoly without NLpep

E. coli clarified lysate were prepared as described previously for NLpoly WT, 5A2, 5P, 8S and 11S. 50uL of each lysate and 50uL NanoGlo +Fz were mixed and incubated for 5min RT. Luminescence was measured on GloMax® Multi+ with 0.5sec integration. Figure 99 illustrates

that the ability of the NLpoly to produce luminescence in the absence of NLpep gradually increased throughout the evolution process resulting in ~500 fold higher luminescence for 11S than WT NLpoly.

Example 58

Improved Luminescence in E. coli Throughout Evolution Process

A single NLpoly colony of WT, 5A2, 5P, 8S or 11S was inoculated in 200uL minimal media and grown for 20hrs at 37^{0}C on shaker. 10uL of the overnight culture was diluted into 190uL fresh minimal media and grown for 20hrs at 37^{0}C on shaker. 10uL of this overnight culture was diluted into 190uL auto-induction media (previously described) and grown for 18hrs at 25^{0}C on shaker. The auto-induced cultures were diluted 50-fold (4uL into 196uL assay lysis buffer), 10uL expression culture added to 40uL of assay lysis buffer containing NLpep (synthetic; 1nM; WT, NLpep78, NL79 or NLpep80) and shaken for 10min at RT. 50uL NanoGlo + Fz was added, and samples shaken for 5 min at RT. Luminescence was measured on a GloMax luminometer with 0.5sec integration. Figure 100 illustrates the improvement in luminescence from E. coli-derived NLpoly over the course of the evolution process, an overall ~10^5 improvement (from NLpolyWT:NLpepWT to NLpoly11S:NLpep80).

Example 59

Improved Luminescence in HeLa Cells Throughout Evolution Process

50ng plasmid DNA expressing NLpoly WT, 5A2, 5P, 8S or 11S was transfected into HeLa cells into wells of a 12-well plate using FugeneHD. The cells were then incubated overnight at 37^{0}C/5%CO_2. The media was replaced with 500uL DMEM without phenol red, and the cells frozen at -80^{0}C for >30min. The cells were thawed and transferred to 1.5mL tubes. NLpep WT, NLpep78, NLpep79 or NLpep 80 (synthetic) were diluted to 10nM in PBS+0.1% Prionex, and 25ul mixed with 25uL of each of the NLpoly cell lysate. The samples were shaken for 10min at RT, and then 50uL NanoGlo+100uM Fz added and incubated for 5min at RT. Luminescence was measured on a GloMax luminometer with 0.5s integration. Figure 101 illustrates the improvement in luminescence from HeLa-expressed NLpoly over the course of the evolution process, an overall ~10^5 improvement (from NLpolyWT:NLpepWT to NLpoly11S:NLpep80).

Example 60

Improved Luminescence in HEK293 Cells Throughout Evolution Process

50ng plasmid DNA expressing NLpoly WT, 5A2, 5P, 8S or 11S was transfected into HEK293 cells into wells of a 12-well plate using FugeneHD. The cells were then incubated overnight at 37^{0}C/5%CO_2. The media was replaced with 500uL DMEM without phenol red, and the cells frozen at -80^{0}C for >30min. The cells were thawed and transferred to 1.5mL tubes.

NLpep WT, NLpep78, NLpep79 or NLpep 80 (synthetic) were diluted to 10nM in PBS+0.1% Prionex, and 25ul mixed with 25uL of each of the NLpoly cell lysate. The samples were shaken for 10min at RT, and then 50uL NanoGlo+100uM Fz added and incubated for 5min at RT. Luminescence was measured on a GloMax luminometer with 0.5s integration. Figure 102 illustrates the improvement in luminescence from HEK293-expressed NLpoly over the course of the evolution process, an overall ~10^4 improvement (from NLpolyWT:NLpepWT to NLpoly11S:NLpep80).

Example 61

Improved Binding Affinity Throughout Evolution

NLpoly WT, 5A2, 5P, 8S or 11S (E. coli clarified lysates) were diluted into PBS+0.1% Prionex as follows: WT 1:$10^{4;}$ 5A2 1:105; 5P 1:10^6; 8S 1:10^7; and 11S 1:10^7. NLpepWT, NLpep78, NLpep79 or NLpep80 (synthetic) were serially into PBS+0.1% Prionex to 4X concentration. 25uL NLpoly and 25uL NLpep were mixed and incubated for 10min at RT. 50uL NanoGlo+100uM Fz was added and incubated for 5min at RT. Luminescence was measured on a GloMax Multi+ with 0.5sec integration. Kd was determined using Graphpad Prism, One Site-Specific Binding, Best-fit values. Figure 103 illustrates a 10^4 fold improved affinity (starting affinity: NLpolyWT:NLpepWT, Kd~10uM) of K_d<1nM (NLpoly11S:NLpep86 or NLpoly11S:NLpep80) of the variants tested over wild-type.

Example 62

NLpoly luminescence

Single NLpoly variant colonies were inoculated with 200uL minimal media and grown for 20hrs at 37^{0}C on a shaker. 10uL of the overnight culture were diluted into 190uL fresh minimal media and grown for 20hrs at 37^{0}C on a shaker. 10uL of this overnight culture was then diluted into 190uL auto-induction media (previously described) and grown for 18hrs at 25^{0}C on a shaker. 10uL of this expression culture was mixed with 40uL of assay lysis buffer (previously described) without NLpep or NLpep78-HT (1:3,860 dilution) or NLpep79-HT (1:10,000 dilution) and shaken for10min at RT. 50uL of NanoGlo + Fz was added and again shake for 10min at RT. Luminescence was measured on GloMax® luminometer with 0.5sec integration. Figures 105-107 illustrate the luminescence of various NLpolys in the absence of NLpep.

Example 63

Solubility of NLpoly Variants

A single NLpoly variant colony (SEE FIG. 143) was inoculated into 5mL LB culture and incubated at 37^{0}C overnight with shaking. The overnight culture was diluted 1:100 into fresh LB and incubated at 37^{0}C for 3hrs with shaking. Rhamnose was added to the cultures to 0.2% and incubated 25^{0}C overnight with shaking. 900ul of these overnight cultures were mixed with

100uL 10X FastBreak Lysis Buffer (Promega Corporation) and incubated for 15min at RT. A 75uL aliquot (total) was removed from each culture and saved for analysis. The remaining culture from each sample were centrifuged at 14,000xrpm in a benchtop microcentrifuge at 4^0C for 15min. A 75uL aliquot of supernatant (soluble) was removed from each sample and saved for analysis. 25uL of 4x SDS buffer was added to the saved aliquots and incubated at 95^0C for 5min. 5ul of each sample was loaded onto a 4-20% Tris-Glycine SDS gel and run at ~190V for ~50min. The gel was stained with SimplyBlue Safe Stain and imaged on a LAS4000. Figure 143 shows a protein gel of total lysates and the soluble fraction of the same lysate for the NLpoly variants.

Example 64

Dissociation constants

NLpoly variant lysate (SEE FIG. 144; prepared as described previously) was diluted 1:10 into PBS +0.1% Prionex. 4x concentrations of NLpep78 (synthetic NLpep78) were made in PBS +0.1% Prionex. 20uL NLpoly variant lysate and 20uL NLpep were mixed and shaken for 10min at RT. 40uL NanoGlo/Fz was added and shaken for 10min at RT. Luminescence was measured on a GloMax® luminometer with 0.5s integration. Kd determined using Graphpad Prism, One site-specific binding, best-fit values. Figure 144 illustrates dissociation constants of NLpep78 with various NLpolys.

Example 65

Comparison of luminescence generated by cells expressing different combinations of FRB and FKBP fused to NLpoly5P and NLpep80/87

HEK293T cells (400,000) were reverse-transfected with 1 µg pF4A Ag FKBP or 1 µg pF4A Ag FRB vectors expressing N- or C-terminal fusions of NLpoly5P and/or NLpep80/87 using FuGENE HD at a DNA-to-FuGENE HD ratio of 1:4. 24-hours post transfection, cells were trypsinized and re-plated in opaque 96-well assay plates at a density of 10,000 cells per well. 24-hours after plating, cells were washed with PBS and then incubated with or without 20nM rapamycin for 15, 60 or 120 min in phenol red-free OptiMEMI. 10µM furimazine substrate with or without 20nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figures 108 (15 min induction), 109 (60 min induction) and 110 (120 min induction) illustrate a general increase in induction over time, with NLpoly5P and NLpep80 combinations generating the most luminescence. Individual components contribute minimally to signal.

Example 66

Comparison of luminescence generated by cells expressing different combinations of FRB

and FKBP fused to NLpoly5P and NLpep80/87

Although similar to Example 65, this example tested all 8 possible combinations of FRB and FKBP fused to NLpoly/NLpep as well as used less total DNA. HEK293T cells (400,000) were reverse-transfected with a total of 0.001 µg pF4A Ag FRB-NLpoly5P and 0.001 µg pF4A Ag FKBP-NLpep80/NLpep87 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with 0 or 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with 0 or 50nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figures 111 illustrates that NLpep80 combinations generated the highest luminescence and that all configurations respond to rapamycin treatment.

Example 67

Comparison of luminescence generated by FRB or FKBP fusions expressed in the absence of binding partner

HEK293T cells (400,000) were reverse-transfected with a total of 0.001 µg pF4A Ag FRB-NLpoly5P or pF4A Ag FKBP-NLpep80/NLpep87 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with 0 or 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with 0 or 50nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 112 illustrates that the individual components generate a low basal level of luminescence that is not responsive to rapamycin treatment.

Example 68

Comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep80/87 DNA

HEK293T (400,000) cells were reverse-transfected with a total of 2, 0.2, 0.02, or 0.002 µg pF4A Ag FRB-NLpoly5P and pF4A Ag FKBP-NLpep80 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 2 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 20nM rapamycin for 2 h. 10µM furimazine substrate (final

concentration on cells) with or without 20nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 113 illustrates that transfection with less DNA decreases overall luminescence but increases fold induction.

Example 69

Comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P or FKBP-NLpep80/87 DNA in the absence of binding partner

HEK293T cells (400,000) were reverse-transfected with a total of 2, 0.2, 0.02, or 0.002 µg pF4A Ag FRB-NLpoly5P or pF4A Ag FKBP-NLpep80 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 2 µg. 24-hours post-transfection, 10,000 cells were replated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 20nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 20nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 114 illustrates that lower DNA levels do not change overall luminescence of cells transfected with individual components.

Example 70

Comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep80/87 DNA

HEK293T cells (400,000) were reverse-transfected with a total of 0.2, 0.02, 0.002, or 0.0002 µg pF4A Ag FRB-NLpoly5P and pF4A Ag FKBP-NLpep80/NLpep87 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 2 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 115 illustrates that luminescence above background, as determined in Examples 69 and 71, and rapamycin induction can be achieved with DNA levels down to 2.5 pg.

Example 71

Comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P or FKBP-NLpep80/87 DNA in the absence of binding partner

HEK293T cells (400,000) were reverse-transfected with a total of 0.2, 0.02, 0.002, or 0.0002 µg pF4A Ag FRB-NLpoly5P or pF4A Ag FKBP-NLpep80/NLpep87 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 2 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 116 illustrates no significant change in luminescence generated by individual components when less DNA was used.

Example 72

Comparison of luminescence generated by cells transfected with varying amounts of FRB-NLpoly5P and FKBP-NLpep80 or FKBP-NLpep87 DNA after treatment with rapamycin for different lengths of time

HEK293T cells (400,000) were reverse-transfected with a total of 2, 0.2, 0.02, or 0.002 µg pF4A Ag FRB-NLpoly5P and pF4A Ag FKBP-NLpep80 or FKBP-NLpep87 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 2 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 20nM rapamycin for 5/15/30/60/120 min. 10 µM furimazine substrate (final concentration on cells) with or without 20nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 117 and 118 illustrates a decline in luminescence with less DNA and an increase in rapamycin induction over time.

Example 73

Comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly5P or FRB-NLpoly5A2 with FKBP-NLpep80/87/95/96/97

In this example, the assay was performed in both a two-day and three-day format. For the 2 day assay, 20,000 HEK293T cells were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly5P or FRB-NLpoly5A2 and pF4A Ag FKBP-NLpep80/87/95/96/97 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM

rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time.

For 3 day assay, 400,000 HEK293T cells were reverse-transfected with a total of 0.002 µg pF4A Ag FRB-NLpoly5P and pF4A Ag FKBP-NLpep80/87/95/96/97 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figures 119 and 120 illustrate similar levels of luminescence in both the 2 day and 3 day assays. Assays performed with NLpoly5A2 showed greater rapamycin induction relative to NLpoly5P, and assays performed with NLpoly5A2 and NLpep96 showed greatest rapamycin induction of all tested combinations.

Example 73

Comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly5A2 or FRB-NLpoly11S with FKBP-NLpep101/104/105/106/107/108/109/110

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly5A2/11S and pF4A Ag FKBP-NLpep101/104/105/106/107/108/109/110 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 121 illustrates that, of tested combinations, NLpoly11S with NLpep101 showed the greatest rapamycin induction and one of the strongest rapamycin-specific luminescent signals.

Example 74

Comparison of luminescence generated by cells transfected with different combinations of FRB-NLpoly5A2 or FRB-NLpoly11S with FKBP-NLpep87/96/98/99/100/101/102/103

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly5A2/11S and pF4A Ag FKBP-NLpep87/96/98/99/100/101/102/103 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 122 illustrates that the NLpoly11S and NLpep101 combination produces the highest induction while maintaining high levels of specific luminescence.

Example 75

Comparison of luminescence generated by cells transfected with different levels of FRB-NLpoly11S and FKBP-NLpep87/101/102/107 DNA

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.01, 0.1, 1, or 10ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep87/101/102/107 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 1.5 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 123 illustrates NLpoly11S with NLpep101 produces the overall lowest luminescence in untreated samples at all tested DNA levels, and the combination maintains relatively high levels of luminescence in rapamycin-treated samples.

Example 76

Comparison of luminescence generated by cells transfected with different levels of FRB-NLpoly5A2 and FKBP-NLpep87/101/102/107 DNA

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.01, 0.1, 1, or 10ng pF4A Ag FRB-NLpoly5A2 and pF4A Ag FKBP-NLpep87/101/102/107 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 1.5 h. 10 µM furimazine substrate (final concentration on cells) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s

integration time. Figure 124 illustrates that NLpoly5A2 generates higher luminescence in untreated samples than NLpoly11S shown in example 75.

Example 77

Rapamycin dose response curve showing luminescence of cells expressing FRB-NLpoly5P and FKBP-NLpep80/87 DNA

HEK293T cells (400,000) were reverse-transfected with a total of 0.001 µg pF4A Ag FRB-NLpoly5P and 0.001 µg pF4A Ag FKBP-NLpep80/NLpep87 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1 µg. 24-hours post-transfection, 10,000 cells were re-plated in opaque 96-well assay plates and incubated an additional 24 hours. Cells were washed with PBS and then incubated in phenol red-free OptiMEMI with 0 to 500nM rapamycin for 2 h. 10 µM furimazine substrate (final concentration on cells) with 0 to 500nM rapamycin in OptiMEM was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Kd was calculated with GraphPad Prism version 5.00 for Windows. Figure 125 illustrates a rapamycin-specific increase in luminescence.

Example 78

Rapamycin dose response curve showing luminescence of cells expressing FRB-NLpoly5A2 and FKBP-NLpep87/101 DNA

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly5A2/11S and pF4A Ag FKBP-NLpep87/101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with 0 to 1 µM rapamycin for 1.5 h. 10 µM furimazine substrate (final concentration on cells) with 0 to 1 µM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 126 illustrates a sigmoidal dose response to rapamycin with NLpoly5A2/NLpep101 and NLpoly11S/NLpep101 combinations. While combinations with NLpep87 show an increase in luminescence with rapamycin, the collected data points deviate more from the sigmoidal curve.

Example 79

Comparison of luminescence generated by cells expressing FRB-11S and FKBP-101 and treated with substrate PBI-4377 or furimazine

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1/1/10ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in

each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with 0 or 50nM rapamycin for 1.5 h. 10 µM furimazine or PBI-4377 substrate (final concentration on cells) with 0 to 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time. Figure 127 illustrates a decrease in luminescence and fold induction with the PBI-4377 substrate compared to the furimazine substrate.

Example 80

Time course of cells expressing FRB-NLpoly11S/5A2 and FKBP-NLpep87/101 conducted in the presence or absence of rapamycin

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S/5A2 and pF4A Ag FKBP-NLpep87/101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 50nM rapamycin and 10 µM furimazine was added either manually or via instrument injection. Luminescence was immediately measured on a GloMax Multi with 0.5s integration time. Figure 128 and 129 illustrate that, of all combinations tested, NLpoly11S with NLpep101 has the lowest luminescence at time 0, hits a luminescent plateau faster and has the largest dynamic range.

Example 81

Luminescence generated by FRB-NLpoly11S and FKBP-NLpep101 as measured on two different instruments

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 50nM rapamycin was added for 20 min. 10 µM furimazine (final concentration on cells) in OptiMEMI with 0 or 50nM rapamycin was added and incubated for an additional 5 min. Luminescence was immediately measured on a GloMax Multi with 0.5s integration time and on the Varioskan Flash with 450nM band pass filter. Figure 130 illustrates that the rapamycin-specific induction of FRB-NLpoly11S and FKBP-NLpep101 can be measured on different instruments.

Example 82

Images showing luminescence of cells expressing FRB-NLpoly11S and FKBP-NLpep101 at various times after treatment with rapamycin

HeLa cells (500,000) were reverse transfected with 1 µg pF4 Ag FRB-NLpoly11S and 1 µg pF4 Ag FKBP-NLpep101 using FuGENE HD at a DNA to FuGENE ratio of 1 to 4. Cells were transfected in 35 mm glass bottom culture dishes (MatTek #p35gc-1.5-14-C). 24 hours post-transfection, cells were washed with PBS and then incubate with 10 µM furimazine in OptiMEM for 5 min. 50nM rapamycin in OptiMEMI was added to cells and luminescent images were acquired with LV200 at 10s intervals for a total of 20 min. Instrument was at 37oC, objective was 60X, gain was 200 and exposure was 600ms. Figure 131 illustrates that imaging can detect an increase in cellular luminescence in cells expressing FRB-NLpoly11S and FKBP-NLpep101 following rapamycin treatment.

Example 83

Quantitation of the signal generated by individual cells expressing FRB-NLpoly11S and FKBP-NLpep101 at various times after treatment with rapamycin

HeLa cells (500,000) were reverse transfected with 1 µg pF4 Ag FRB-NLpoly11S and 1 µg pF4 Ag FKBP-NLpep101 using FuGENE HD at a DNA to FuGENE ratio of 1 to 4. Cells were transfected in 35 mm glass bottom culture dishes (MatTek #p35gc-1.5-14-C). 24 hours post-transfection, cells were washed with PBS and then incubate with 10 µM furimazine in OptiMEM for 5 min. 50nM rapamycin in OptiMEMI was added to cells, and luminescent images were acquired with LV200 at 10s intervals for a total of 20 min. Instrument was at 37^0C, objective was 60X, gain was 200, and exposure was 600ms. The signal intensity of every cell in the field of view was analyzed with Image J software over the entire time period. Figure 132 illustrates that signal generated by individual cells can be measured and that the increase in signal by each cell parallels the increase observed in the 96-well plate assay shown in Figures 128 and 129.

Example 84

Comparison of luminescence in different cell lines expressing FRB-NLpoly11S and FKBP-NLpep101

HEK293T, HeLa, or U2-OS cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 50nM rapamycin was added for 20 min. 10 µM furimazine (final concentration on cells) in OptiMEMI with 0 or 50nM rapamycin was added and incubated for an additional 5 min. Luminescence was immediately measured on a GloMax Multi with 0.5s integration time. Figure 133 illustrates similar levels of luminescence generated

in the absence and presence of rapamycin in three different cells lines transfected with FRB-NLpoly11S and FKBP-NLpep101.

Example 85

Comparison of luminescence generated by cells expressing FRB-NLpoly11S and FKBP-NLpep101 after treatment with the rapamycin competitive inhibitor FK506

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 20nM rapamycin was added for 20 min. FK506 inhibitor in OptiMEM was added to cell at final concentration of 5 µM and incubated for 3 or 5 hours. Furimazine in OptiMEM was added to cells for a final concentration of 10 µM on cells. Luminescence was immediately measured on a GloMax Multi with 0.5s integration time. Figure 134 illustrates a decrease in rapamycin-induced luminescence after treatment with the competitive inhibitor FK506.

Example 86

Luminescence generated by cells expressing FRB-NLpoly11S and FKBP-NLpep101 after treatment with the rapamycin competitive inhibitor FK506

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 20nM rapamycin was added for 2.5 hours. FK506 inhibitor in OptiMEM was added to cell via injector at final concentration of 0, 1 or 10 µM in OptiMEM with 10 µM. Luminescence was measured every 10 min for 4 hours on a GloMax Multi set to 37oC with 0.5s integration time. Figure 135 illustrates that by 200 s, FK506 inhibitor can reduce luminescence close to levels of untreated cells.

Example 87

Luminescence generated by cells transfected with different combinations of V2R-NLpoly5A2 or V2R-NLpoly11S with NLpep87/101-ARRB2 in the presence or absence of the V2R agonist AVP

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1, 1, or 10ng pF4A Ag V2R-NLpoly11S and pF4A Ag ARRB2-NLpep87/101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total

DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 1 µM AVP and 10 µM furimazine was added for 25 min. Luminescence was then measured on a GloMax Multi with 0.5s integration time.

Figure 136 illustrates that V2R-NLpoly11S with NLpep101 generates the greatest AVP-specific increase in luminescence. Combinations with NLpep87 show no significant response to AVP.

Example 88

Time course showing luminescence generated by cells transfected with V2R-NLpoly5A2 or V2R-NLpoly11S and NLpep87/101-ARRB2 after treatment with AVP

HEK293T cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 0.1 or 1ng pF4A Ag V2R-NLpoly11S or 1ng pF4A Ag V2R-NLpoly5A2 and pF4A Ag ARRB2-NLpep87/101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 1 µM AVP and 10 µM furimazine was added either manually (Figure 137) or via instrument injection (Figure 138). Luminescence was then measured on a GloMax Multi every 5 min for 25 min with 0.5s integration time at room temperature (Figures 137 and 138) or 37^{0}C (Figure 139). Figures 137 and 138 illustrate a time-dependent increase in AVP-induced luminescence for V2R-NLpoly11S with NLpep101-ARRB2 that begins to peak at 600 s. Combinations with V2R-NLpoly5A2 and NLpep87 do not show a significant increase in luminescence over time. Figure 139 illustrates that at 37^{o}C all NLpoly11S and NLpep101 combinations tested show a time-dependent increase in AVP-induced luminescence that levels out around 200s.

Example 89

Comparison of luminescence in different cell lines expressing V2R-NLpoly11S and NLpep101-ARRB2

HEK293T, HeLa, or U2-OS cells (20,000) were reverse-transfected in opaque 96-well assay plates with a total of 1ng pF4A Ag V2R-NLpoly11S and pF4A Ag ARRB2-NLpep87/101 using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. 24 hours-post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 0 or 1 µM AVP was added for 20 min. Furimazine in OptiMEM was then added to a final concentration of 10 µM on cells, and luminescence was measured on a GloMax Multi with 0.5s integration time.

Figure 140 illustrates similar luminescence levels in three different cell lines expressing V2R-NLpoly11S and NLpep101-ARRB2 in the presence and absence of AVP.

Example 90

Luminescence of cells expressing V2R-NLpoly11S and NLpep101-ARRB2 at various times

after treatment with AVP

HeLa cells (500,000) were reverse transfected with 1 µg pF4 Ag V2R-NLpoly11S and 1µg pF4 Ag ARRB2-NLpep101 using FuGENE HD at a DNA to FuGENE ratio of 1 to 4. Cells were transfected in 35 mm glass bottom culture dishes (MatTek #p35gc-1.5-14-C). 24 hours post-transfection, cells were washed with PBS and then incubate with 10 µM furimazine in OptiMEM for 5 min. 1µM AVP in OptiMEMI was added to cells, and luminescent images were acquired with LV200 at 15s intervals for a total of 30 min. Instrument was at 37^{0}C, objective was 60X or 150X, gain was 600, and exposure was 1s or 2s. Figures 141 and 142 illustrate that imaging can detect the increase in luminescence and formation of punctate in individual cells after treatment with AVP.

Example 91

Dissociation Constants for NLpeps

NLpoly 5P E. coli clarified lysate (prepared as described previously) was diluted 1:1,000 into PBS +0.1% Prionex. 4x concentrations of NLpep78-HT (E. coli clarified lysate prepared as described previously) were made in PBS +0.1% Prionex. 20uL NLpoly 5P and 20uL NLpep78 were mixed and shaken for 10min at RT. 40uL NanoGlo/Fz was added and shaken for 10min at RT. Luminescence was measured on GloMax luminometer with 0.5s integration. Kd was determined using Graphpad Prism, One site-specific binding, best-fit values. Figure 80 compares the dissociation constants for an NLpep consisting of either 1 or 2 repeat units of NLpep78.

Example 92

Affinity Between NLpoly 5A2 and NLpep86

NLpoly 5A2 lysate (prepared as described previously after transfecting CHO cells) was diluted 1:10 into PBS +0.1% Prionex. 4x concentrations of NLpep86 (synthetic NLpep) were made in PBS +0.1% Prionex. 20uL NLpoly and 20uL NLpep were mixed and shaken for 10min at RT. 40uL NanoGlo/Fz was added and shaken for 10min at RT. Luminescence was measured on GloMax luminometer with 0.5s integration. Kd was determined using Graphpad Prism, One site-specific binding, best-fit values. Figure 81 illustrates the affinity between NLpoly 5A2 and NLpep86.

Example 93

Luminescence of NLpoly variants

A single colony of various NLpolys were inoculated individually into 200uL minimal media and grown for 20hrs at 37^{0}C on shaker. 10uL of overnight culture was diluted into 190uL fresh minimal media and grown for 20hrs at 37^{0}C on shaker. 10uL of this overnight culture was diluted into 190uL auto-induction media (previously described) and grow for 18hrs at 25^{0}C on shaker. 10uL of the expression culture was mixed with 40uL of assay lysis buffer (previously

described) without NLpep or with NLpep78-HT (1:3,860 dilution) or NLpep79-HT (1:10,000 dilution). The mixtures were shaken for 10min at RT, 50uL NanoGlo + Fz added and shaken again for 10min at RT. Luminescence was measured on a GloMax luminometer with 0.5sec integration. Figure 82 demonstrates the luminescence from NLpoly variants without an NLpep or with NLpep78 or NLpep79. The results show that the NLpoly variant 11S (12S-51) has improved luminescence over the other variants.

Example 94

Dissociation Constants and Vmax Values for NLpolys with 96 variants of NLpeps

NLpeps were synthesized in array format by New England Peptide (peptides blocked at N-terminus by acetylation and at C-terminus by amidation; peptides in arrays were synthesized at ~1 mg scale) (Table 6). Each peptide was lyophilized in 3 separate plates. Each well from 1 of the 3 plates of peptides was dissolved in 100uL nanopure water, and the A260 measured and used to calculate the concentration using the extinction coefficient of each peptide. The concentration was then adjusted based on the purity of the peptide, and nanopure water was added to give a final concentration of 750uM.

Peptides were diluted to 12.66uM (4X) in PBS+0.1% Prionex and then diluted serially 7 times (8 concentrations total) in 0.5 log steps (3.162 fold dilution). NLpolys 5P, 8S, 5A2 or 11S were diluted into PBS+0.1% Prionex as follows: 5P 1:2,000; 8S 1:10,000; 11S 1:150,000, 5A2 1:1,000. 25uL each NLpep + 25uL each NLpoly were mixed and incubated for 30min at RT. 50uL NanoGlo+100uM Fz was added and incubated for 30min at RT. Luminescence was measure on a GloMax Multi+ with 0.5sec integration. Kd/Vmax were determined using Graphpad Prism, One site-specific binding, best-fit values. Figures 83-90 illustrate the dissociation constant and Vmax values from NLpolys with the 96 variant NLpeps. The results indicate specific mutations in the NLpeps that exhibit lower binding affinity without loss in Vmax.

Table 6. Peptide Array 1

	Sequence
array1.1	VTGWRLCERIL
array1.2	VSGWRLFKKIS
array1.3	VTGYRLFKKIS
array1.4	ISGWRLFKKIS
array1.5	ASGWRLFKKIS
array1.6	GSGWRLFKKIS
array1.7	KSGWRLFKKIS
array1.8	LSGWRLFKKIS
array1.9	QSGWRLFKKIS
array1.10	SSGWRLFKKIS
array1.11	TSGWRLFKKIS

array1.12	VVGWRLFKKIS
array1.13	VKGWRLFKKIS
array1.14	VIGWRLFKKIS
array1.15	VEGWRLFKKIS
array1.16	VAGWRLFKKIS
array1.17	VQGWRLFKKIS
array1.18	VHGWRLFKKIS
array1.19	VSAWRLFKKIS
array1.20	VSSWRLFKKIS
array1.21	VSGFRLFKKIS
array1.22	VSGWKLFKKIS
array1.23	VSGWQLFKKIS
array1.24	VSGWELFKKIS
array1.25	VSGWALFKKIS
array1.26	VSGWRIFKKIS
array1.27	VSGWRVFKKIS
array1.28	VSGWRTFKKIS
array1.29	VSGWRYFKKIS
array1.30	VSGWRKFKKIS
array1.31	VSGWRFFKKIS
array1.32	VSGWRLAKKIS
array1.33	VSGWRLDKKIS
array1.34	VSGWRLEKKIS
array1.35	VSGWRLGKKIS
array1.36	VSGWRLHKKIS
array1.37	VSGWRLIKKIS
array1.38	VSGWRLKKKIS
array1.39	VSGWRLLKKIS
array1.40	VSGWRLMKKIS
array1.41	VSGWRLNKKIS
array1.42	VSGWRLQKKIS
array1.43	VSGWRLRKKIS
array1.44	VSGWRLSKKIS
array1.45	VSGWRLTKKIS
array1.46	VSGWRLVKKIS
array1.47	VSGWRLWKKIS
array1.48	VSGWRLYKKIS
array1.49	VSGWRLFEKIS
array1.50	VSGWRLFVKIS
array1.51	VSGWRLFSKIS
array1.52	VSGWRLFRKIS
array1.53	VSGWRLFTKIS
array1.54	VSGWRLFNKIS
array1.55	VSGWRLFQKIS
array1.56	VSGWRLFKRIS
array1.57	VSGWRLFKQIS
array1.58	VSGWRLFKEIS

array1.59	VSGWRLFKAIS
array1.60	VSGWRLFKKVS
array1.61	VSGWRLFKKLS
array1.62	VSGWRLFKKAS
array1.63	VSGWRLFKKFS
array1.64	VSGWRLFKKES
array1.65	VSGWRLFKKTS
array1.66	VSGWRLFKKIL
array1.67	VSGWRLFKKIA
array1.68	VSGWRLFKKIE
array1.69	VSGWRLFKKIV
array1.70	VSGWRLFKKIG
array1.71	VSGWRLFKKIH
array1.72	VSGWRLFKKIT
array1.73	VVGYRLFKKIS
array1.74	VKGYRLFKKIS
array1.75	VIGYRLFKKIS
array1.76	VEGYRLFKKIS
array1.77	VAGYRLFKKIS
array1.78	VQGYRLFKKIS
array1.79	VHGYRLFKKIS
array1.80	VTAYRLFKKIS
array1.81	VTSYRLFKKIS
array1.82	VTGYRIFKKIS
array1.83	VTGYRVFKKIS
array1.84	VTGYRTFKKIS
array1.85	VTGYRYFKKIS
array1.86	VTGYRKFKKIS
array1.87	VTGYRFFKKIS
array1.88	ISGWRLMKNIS
array1.89	ASGWRLMKKES
array1.90	VSGWRLMKKVS
array1.91	ISGWRLLKNIS
array1.92	ASGWRLLKKES
array1.93	VSGWRLLKKVS
array1.94	ISGWRLAKNIS
array1.95	ASGWRLAKKES
array1.96	VSGWRLAKKVS

Example 95

Solubility of NLpoly Variants

A single NLpoly 5A2, 12S, 11S, 12S-75, 12S-107 or 5P-B9 colony was inoculated into 5mL LB culture and incubated at 37^0C overnight with shaking. The overnight culture was diluted 1:100 into fresh LB and incubated at 37^0C for 3hrs with shaking. Rhamnose was added to the cultures to 0.2% and incubated 25^0C overnight with shaking. 900ul of these overnight cultures were

mixed with 100uL 10X FastBreak Lysis Buffer (Promega Corporation) and incubated for 15min at RT. A 75uL aliquot (total) was removed from each culture and saved for analysis. The remaining culture from each sample was centrifuged at 14,000xrpm in a benchtop microcentrifuge at 4^0C for 15min. A 75uL aliquot of supernatant (soluble) was removed from each sample and saved for analysis. 25uL of 4x SDS buffer was added to the saved aliquots and incubated at 95^0C for 5min. 5ul of each sample was loaded onto a 4-20% Tris-Glycine SDS gel and run at ~190V for ~50min. The gel was stained with SimplyBlue Safe Stain and imaged on a LAS4000. Figure 91 shows a protein gel of total lysates and the soluble fraction of the same lysate for the NLpoly variants. With the exception of 5A2, all variants exhibit a percentage of NLpoly in the soluble fraction.

Example 96

Solubility and Dissociation Constant of NLpoly Variants

A single NLpoly colony (listed in Figure 92) was inoculated into 5mL LB culture and incubated at 37^0C overnight with shaking. The overnight culture was diluted 1:100 into fresh LB and incubated at 37^0C for 3hrs with shaking. Rhamnose was added to the cultures to 0.2% and incubated 25^0C overnight with shaking. 900ul of these overnight cultures were mixed with 100uL 10X FastBreak Lysis Buffer (Promega Corporation) and incubated for 15min at RT. A 75uL aliquot (total) was removed from each culture and saved for analysis. The remaining culture from each sample was centrifuged at 14,000xrpm in a benchtop microcentrifuge at 4^0C for 15min. A 75uL aliquot of supernatant (soluble) was removed from each sample and saved for analysis. 25uL of 4x SDS buffer was added to the saved aliquots and incubated at 95^0C for 5min. 5ul of each sample was loaded onto a 4-20% Tris-Glycine SDS gel and run at ~190V for ~50min. The gel was stained with SimplyBlue Safe Stain and imaged on a LAS4000. Figure 92 shows a protein gel of total lysates and the soluble fraction of the same lysate for NLpoly variants as well a table containing the dissociation constants for the same variants.

Example 97

Substrate Specificity for NLpoly 5P and 11S with NLpep79

E. coli clarified lysates were prepared for NLpoly 5P or 11S as described previously. The NLpoly lysates were then serially diluted in steps of 10-fold into PBS+0.1% Prionex. 25uL NLpoly and 25uL synthetic NLpep79 (400nM, 4X) were mixed and incubated for 10 min at RT. 50uL NanoGlo + 100uM Fz was added, incubated for10min at RT, luminescence measured on a GloMax Multi+ with 0.5sec integration. Figure 93 shows the substrate specificity for 5P and 11S with NLpep79 and demonstrates that 11S has superior specificity for furimazine than 5P.

Example 98

Solubility of NLpoly variants from Various Steps of Evolution

A single NLpoly WT, 5A2, 5P, 8S or 11S colony was inoculated into 5mL LB culture and incubated at 37^{0}C overnight with shaking. The overnight culture was diluted 1:100 into fresh LB and incubated at 37^{0}C for 3hrs with shaking. Rhamnose was added to the cultures to 0.2% and incubated 25^{0}C overnight with shaking. 900ul of these overnight cultures were mixed with 100uL 10X FastBreak Lysis Buffer (Promega Corporation) and incubated for 15min at RT. A 75uL aliquot (total) was removed from each culture and saved for analysis. The remaining culture from each sample was centrifuged at 14,000xrpm in a benchtop microcentrifuge at 4^{0}C for 15min. A 75uL aliquot of supernatant (soluble) was removed from each sample and saved for analysis. 25uL of 4x SDS buffer was added to the saved aliquots and incubated at 95^{0}C for 5min. 5ul of each sample was loaded onto a 4-20% Tris-Glycine SDS gel and run at ~190V for ~50min. The gel was stained with SimplyBlue Safe Stain and imaged on a LAS4000. Figure 104 shows a protein gel of total lysates and the soluble fraction of the same lysate for NLpoly variants from various steps of the evolution process. These results demonstrate that the solubility of NLpoly was dramatically increased in the evolution process.

Example 99

Chemical Labeling of Proteins

The non-luminescent peptides (NLpeps) of the present invention can be used to chemically label proteins. An NLpep of the present invention can be synthesized to contain a reactive group, e.g., biotin, succinimidyl ester, maleimide, etc., and attached (e.g., conjugated, linked, labeled, etc.) to a protein, e.g., antibody. The NLpep-labeled protein, e.g., NLpep-antibody, can then be used in a variety of applications, e.g., ELISA. The interaction/binding of the NLpep-labeled protein, e.g., NLpep-antibody, to its target/binding partner would be detected by adding an NLpoly of the present invention and NanoGlo® assay reagent. The luminescence generated by the interaction of the NLpep-labeled protein and NLpoly would correlate to the interaction of the NL-labeled protein to its target/binding partner. This concept could allow for multiple NLpeps to be attached to a single protein molecule thereby resulting in multiple NLpep-labeled protein/NLpoly interactions leading to signal amplification.

Example 100

Detection of post-translational protein modification using HaloTag-NLpep by Western Blotting

Several proteins can be posttranslationally modified by AMPylation or ADP-ribosylation. In AMPylation, AMP is added to the target protein by a phosphodiester bond using ATP as the donor molecule. Similarly, in ADP-ribosylation, an ADP-ribose moiety is added to target proteins through a phosphodiester bond using NAD+ as the donor molecule. It has been shown that the N6-position of both ATP and NAD+ can be used to tag linkers without affecting the

posttranslational event. If a N6-modified chloroalkane-ATP or -NAD+ is used to perform the AMPylation or ADP-ribosylation reaction, the target proteins would be modified to contain the chloroalkane-ATP or -NAD+.

The N6-modified ATP/NAD has been used in combination with click-chemistry to develop in-gel fluorescent-based detection systems. Detection of these post-translational modifications by western blotting techniques requires antibodies, which are often not specific or not available. An alternative approach could be to combine the properties of HaloTag® technology and the high luminescence of NanoLuc® luciferase (NL).

Upon post-translational modification of target proteins with chloroalkane-ATP (for AMPylation) or chloroalkane-NAD+ (for ADP-ribosylation) using either cell lysate or purified proteins, samples can be resolved by SDS-PAGE and transferred to PVDF membrane. Following blocking, the blot can be incubated with HaloTag-NLpep. HaloTag will bind to the post-translationally-modified proteins. In the next step, the NLpoly and furimazine could be added to the blot to detect the bioluminescence. This detection method is an alternative to a chemiluminescent-based approach for detection of western blots. A chemiluminescent-based approach could involve incubation HaloTag-protein G fusions (as a primary) in the next step any secondary antibody-linked to HRP could be used followed by ECL reaction.

Example 101

Post translational Modification Assays

Post translational modifications (PTMs) of proteins are central to all aspects of biological regulation. PTMs amplify the diverse functions of the proteome by covalently adding functional groups to proteins. These modifications include phosphorylation, methylation, acetylation, glycosylation, ubiquitination, nitrosylation, lipidation and influence many aspects of normal cell biology and pathogenesis. More specifically, histone related PTMs are of great importance. Epigenetic covalent modifications of histone proteins have a strong effect on gene transcriptional regulation and cellular activity. Examples of post translational modification enzymes include but not limited to, Kinases/Phosphatases, Methyltransferases (HMT)/Demethylases (HDMT), Acetyltransferases/Histone Deacetylases, Glycosyltransferases/Glucanases and ADP-Ribosyl Transferases. Under normal physiological conditions, the regulation of PTM enzymes is tightly regulated. However, under pathological conditions, these enzymes activity can be dysregulated, and the disruption of the intracellular networks governed by these enzymes leads to many diseases including cancer and inflammation.

The non-luminescent peptides (NLpep) and non-luminescent polypeptides (NLpoly) of the present invention can be used to determine the activity of PTM enzymes by monitoring changes in covalent group transfer (e.g. phosphoryl, acetyl) to a specific peptide substrate linked

to an NLpep of the present invention. The NLpep will be linked through peptide synthesis to small PTM enzyme specific peptide and used as a substrate for the PTM enzyme.

A) PTM Transferase assays (HAT)

Once the PTM enzyme reaction has occurred, an aminopeptidase can be used to degrade the non-modified peptide (NLpep; control). The modified (acetylated) peptide (NLpep-PTM enzyme substrate) would be degraded at a very slow rate or would not be degraded at all as the aminopeptidase activity is known to be affected by a PTM. Once the aminopeptidase reaction is complete, the NLpoly is added with the NanoGlo® assay reagent containing Furimazine. Luminescence would be generated from the sample where PTM occurred via the interaction of the NLpep and NLpoly. If no PTM occurred, the NLpep would be degraded, and no interaction between the NLpep and NLpoly would occur, thereby no luminescence would be generated. This concept is exemplified in Figure 197 for a general transferase enzyme concept and in Figure 145 for H3K4/9 acetyltransferases.

The reaction would be performed under optimal enzyme reaction condition using the histone peptide substrate linked to NLpep of the present invention and Acetyl-CoA or SAM as the acetyl or methyl group donor. A buffer containing aminopeptidase or a mixture of aminopeptidases would be added to degrade specifically all the non-modified substrates. A buffer containing a NLpoly of the present invention and an aminopeptidase inhibitor would be added. NanoGlo® assay reagent would be added, and luminescence detected. Luminescence generated would be proportional to the amount of non-degraded NLpep present, and therefore would correlate with the amount of methylated or acetylated substrates, thereby indicating the amount of methyl or acetyl transferase activity. The assay can also be applied to PTM such as phosphorylation, glycosylation, ubiquitination, nitrosylation, and lipidation.

B) PTM Hydrolase Assays (HDMT)

In a similar concept to A) can be used for Histone Demethylases (HDMT). However, instead of an aminopeptidase, a PTM-specific antibody can be used to create activity interference. An NLpep of the present invention could be linked through peptide synthesis to small methylated peptide and used as a substrate for the hydrolase. Once a hydrolase reaction has been completed, an anti-methyl antibody can be added to the reaction. This antibody will bind specifically to the methylated peptide (control). The peptide product generated by the HDMT will not bind to the antibody. Then, an NLpoly of the present invention can be added. If the antibody interferes with the interaction of NLpep and NLpoly, no luminescence will be generated. If there was hydrolysis of the PTM by the demethylase, the NLpep and NLpoly will interact, and luminescence will be

generated. This concept is exemplified in Figure 198 for a general hydrolase enzyme concept and in Figure 146 H3K4/9 demethylases.

The concept of aminopeptidase degradation of the non-modified substrate can also be used for a hydrolase assay except it would be a loss of signal assay instead of a gain of signal. The reaction would be performed under optimal enzyme reaction condition using a modified (methylated or acetylated) histone peptide substrate linked to an NLpep of the present invention. A buffer containing an antibody capable of recognizing the methyl or acetyl group would be added. A buffer containing an NLpoly of the present invention would be added. The NLpoly would interact with NLpep not bound to the antibody. NanoGlo® assay reagent would be added, and luminescence detected. The luminescence generated would be proportional to the amount of NLpep not bound to the antibody, and therefore would correlate with the amount of demethylated or deacetylated substrate, thereby indicating the amount of demethylase or deacetylase activity. Both hydrolase assay concepts can also be applied to PTM hydrolases such as phosphatases, glucanases and deubiquitinases.

In another version of these concepts, the PTM transfer or hydrolysis on the peptide-NLpep would be alone sufficient to reduce or enhance the interaction of NLpep with NLpoly and therefore decrease or increase the luminescence signal without the need of aminopeptidase or antibody.

The method of the present invention was used to assay a representative transferase, the Tyrosine Kinase SRC using the following NLpep-SRC substrate peptide: YIYGAFKRRGGVTGWRLCERILA. SRC enzyme was titrated in 10µl Reaction Buffer A (40mM Tris 7.5, 20mM MgCl2 and 0.1mg/ml BSA) in the presence of 150µM ATP and 2.5µM NLpep-Src substrate and incubated for 1 hour at 23°C. After incubation, 10µl of Amino-peptidase M (APM) reagent (40mM Tris 7.5, 0.1mg/ml BSA and 50mU APM) was added, mixed for 2 minutes on an orbital shaker, and then incubated at 37°C for 2 hours. To the samples, 30µl of NLpoly Reagent was added, and the samples were incubated at room temperature. NLpoly Reagent contained the NLpoly fragment and an Aminopeptidase inhibitor. After 30 minutes, 50µl NanoGlo® assay reagent was added and the luminescence was recorded after 3 minutes on a luminometer. It was found that an increase in SRC kinase enzyme activity is correlated with an increase in luminescence over background (Figure 199). Only background activity was found when SRC was not present indicating that the non-phosphorylated NLpep-SRC substrate peptide was digested resulting in no light production by the NLpoly fragment, thus demonstrating use of the method of the present invention to monitor the activity of a transferase enzyme such as a kinase.

Example 102

Detection of specific RNAs (noncoding RNA or mRNA) of interest in mammalian cells, cell lysate or clinical sample

The non-luminescent peptide (NLpep) and non-luminescent polypeptide (NLpoly) of the present invention can be tethered to an RNA binding domain (RBD) with engineered sequence specificity. The specificity of the RBD can be changed with precision by changing unique amino acids that confers the base-specificity of the RBD. An example of one such RBD is the human pumilio domain (referred here as PUM). The RNA recognition code of PUM has been very well established. PUM is composed of eight tandem repeats (each repeat consists of 34 amino acids which folds into tightly packed domains composed of alpha helices). Conserved amino acids from the center of each repeat make specific contacts with individual bases within the RNA recognition sequence (composed of eight bases). The sequence specificity of the PUM can be altered precisely by changing the conserved amino acid (by site-directed mutagenesis) involved in base recognition within the RNA recognition sequence. For detection of specific RNAs in the cell, PUM domains (PUM1 and PUM2) with customized sequence specificities for the target RNA can be tethered to a NLpep and NLpoly of the present invention (e.g., as a genetic fusion protein via genetic engineering) and can be expressed in mammalian cells. PUM1 and PUM2 are designed to recognize 8-nucleotide sequences in the target RNA which are proximal to each other (separated by only few base pairs, determined experimentally). Optimal interaction of PUM1 and PUM2 to their target sequence is warranted by introducing a flexible linker (sequence and length of the linker to be determined experimentally) that separates the PUM and the non-luminescent peptide and non-luminescent polypeptide. Binding of the PUM1 and PUM2 to their target sequence will bring the NLpep and NLpoly into close proximity in an orientation that results in a functional complex formation capable of generating bioluminescent signal under our specific assay condition. A functional bioluminescent complex would not be generated in the absence of the RNA target due to the unstable interaction of the NLpep and NLpoly pairs that constitutes the complex.

A similar strategy can also be used for detecting RNA in clinical sample in vitro. The NLpep-PUM fusion proteins with customized RNA specificity can be expressed and purified from suitable protein expression system (such as E.coli or mammalian protein expression system). Purified components can be added to the biological sample along with suitable substrate and assay components to generate the bioluminescent signal.

Example103

DNA oligo-based detection of specific RNA (noncoding RNA or mRNA) in clinical sample or mammalian cell lysate

A non-luminescent peptide (NLpep) and non-luminescent polypeptide (NLpoly) of the present invention can be attached to oligonucleotides complementary to the target RNA with suitable linker (amino acids or nucleotides). Functional assembly of bioluminescent complex occurs only when sequence specific hybridization of DNA oligo to their target RNA brings the NLpep and NLpoly into close proximity in an ideal conformation optimal for the generation of a bioluminescent signal under the assay conditions. The detection can also be achieved through a three-component complementation system involving two NLpeps and a third NLpoly. For example, two NLpep-DNA conjugates will be mixed with the target RNA. Functional assembly of the bioluminescent complex is achieved by subsequent addition of the third NLpoly. Thus, if a detectable signal is produced under specific assay conditions using a clinical sample or cell lysate, the presence of target RNA in such a sample is inferred. Such assays are useful for detecting RNAs derived from infectious agents (viral RNAs) and specific RNA biomarkers (implicated in many disease conditions such as various forms of cancers, liver diseases, and heart diseases), and could provide a new avenue for diagnosis and prognosis of many disease conditions.

Example 104

In-vivo imaging

Biotechnology-derived products (Biologics), including antibodies, peptides and proteins, hold great promises as therapeutics agents. Unlike small molecule drugs, biologics are large molecules with secondary and tertiary structures and often contain posttranslational modifications. Internalization, intracellular trafficking, bio-distribution, pharmacokinetics and pharmacodynamics (PK/PD), immunogenicity, etc. of biologics differ significantly from small molecule drugs, and there is a need for new tools to 'track' these antibodies *in vivo*. Conventional chemical labeling with enzyme reporters (HRP, luciferase, etc.) or small fluorescent tags can significantly alter the therapeutic value of the biologics and are not ideal for *in vivo* imaging using biologics. Radioisotope-labeling for PET-based imaging is also not convenient.

The NLpolys and NLpeps described herein offer a novel solution for *in vivo* imaging of biologics. The NLpep can be genetically encoded into a biologic therapeutics without any synthetic steps. Genetic encoding allows precise control over amount of peptide per biologic molecule as well as its position, thereby minimizing any perturbation to its therapeutic value. For imaging, a NLpoly along with substrate, e.g., furimazine, can be injected into the animal. If the NLpep-biologic and NLpoly interact, luminescence would be generated. Alternatively, a transgenic animal expressing NLpoly can be used as a model system.

Example 105

BRET Applications

This concept fundamentally measures three moieties coming together. Two of the NLpolys and/or NLpeps form a complex, and the third moiety, which is either fluorescent or bioluminescent, provides an energy transfer component. If the complex formed is bioluminescent, both bioluminescence and energy transfer (i.e., BRET) can be measured. If the complex formed is fluorescent, the magnitude of energy transfer can be measured if the third component is a bioluminescent molecule.

A) This example demonstrates a fluorescent dye attached to a NLpep. Alternatively, a fluorescent protein could be fused, e.g., a fusion protein, with a NLpoly or NLpep (created from a genetic construct).

E. coli clarified lysate of NLpoly WT was prepared as described previously. 40uL NLpoly WT lysate was mixed with 10uL of **PBI-4730** (NLpep1) or **PBI-4877** (NLpep1-TMR) and incubated for 10min at RT. 50uL 100uM furimazine in 50mM HEPES pH 7.4 was added and incubated for 30min at RT. Luminescence was measured over 400-700nm on TECAN M1000.

Figure 147 illustrates very efficient energy transfer from the NLPoly/NLPep complex (donor) to TMR (acceptor), and the corresponding red shift in the wavelength of light being emitted.

B) This example demonstrates using the BRET in detection, such as detecting small molecule concentration or enzymatic activity. Because energy transfer is strongly dependent on distance, the magnitude of energy transfer can often be related to the conformation of the system. For instance, insertion of a polypeptide that chelates calcium can be used to measure calcium concentration through modulation of energy transfer.

An enzyme that also changes the distance, either through causing a conformational change of the sensor as above or through cleavage of the sensor from the fluorescent moiety, can be measured through a system as described herein. A NLpoly or NLpep bound to a fluorescent moiety gives energy transfer when the NLpoly and NLpep interact. One example of this is a peptide sensor that has been made wherein the NLpep is conjugated to a fluorescent TOM dye via a DEVD linker (Caspase-3 cleavage site). When exposed to the NLpoly, energy transfer is observed. When exposed to Caspase-3, energy transfer is eliminated, but luminescence at 460nm remains.

NLpoly 5A2 and NL-HT (NanoLuc fused to HaloTag) were purified. 20uL of 8pM NL-HT was mixed with 20uL of 100nM PBI-3781 (See, e.g., U.S. Pat. App. Ser. No. 13/682,589, herein incorporated by reference in its entirety) and incubated for 10min at RT. 40uL NanoGlo+100uM furimazine was added, and luminescence measured over 300-800nm on TECAN M1000.

20uL of 33ng/uL NLpoly 5A2 was mixed with 20uL of ~500uM PBI-5074 (TOM-NCT-NLpep). 40uL NanoGlo+100uM furimazine was added, and luminescence measured over 300-800nm on TECAN M1000.

Figure 148 illustrates energy transfer from the NLPoly/NLPep complex (donor) to TOM-dye (acceptor), and the corresponding red shift in the wavelength of light being emitted.

C) Ternary Interactions

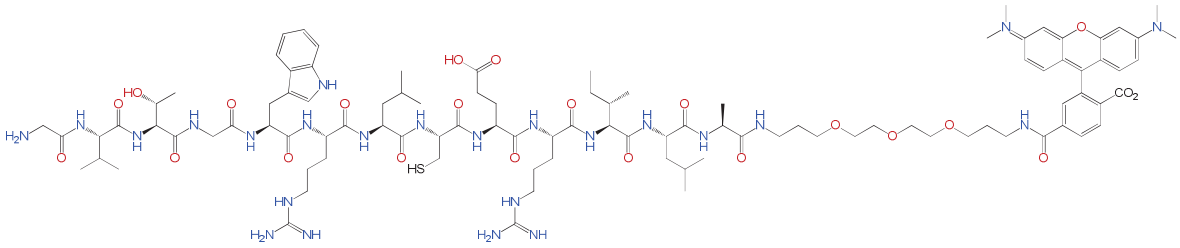
The energy transfer with an NLpoly and NLpep can also be used to measure three molecules interacting. One example would be a GPCR labeled with NLpoly and a GPCR interacting protein with NLpep that forms a bioluminescent complex when they interact. This allows measurement of the binary interaction. If a small molecule GPCR ligand bearing an appropriate fluorescent moiety for energy transfer interacts with this system, energy transfer will occur. Therefore, the binary protein-protein interaction and the ternary drug-protein-protein interaction can be measured in the same experiment. Also, the fluorescent molecule only causes a signal when interacting with a protein pair, which removes any signal from the ligand interacting with an inactive protein (Figure 149).

Example 106

6-Tetramethylrhodamine-PEG3-NH_2:

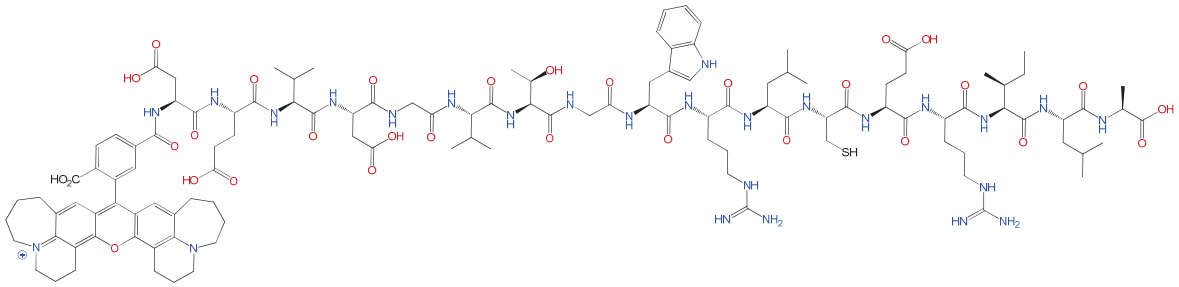
To a solution of 6-Tetramethylrhodamine succidimidyl ester (0.25g, 0.5 mmol) in DMF (5 mL), 1-Boc-4,7,10-trioxatridecan-1,13-diamine (0.15g, 0.5 mmol) was added followed by diisopropylethylamine (0.25mL, 1.4 mmol). After stirring for 16 h, the reaction was analyzed by HPLC to confirm complete consumption of the 6-tetramethylrhodamine succidimyl ester. The reaction was concentrated to a pink film, which was dissolved in a combination of triisopropylsilane (0.2mL) and trifluoroacetic acid (4mL). The pink solution was stirred for 2h, after which analytical HPLC confirmed complete consumption of starting material. The reaction was concentrated to dryness to provide crude 6-Tetramethylrhodamine-PEG3-NH2 as a pink film.

H-GVTGWRLCERILA-PEG-TMR (**PBI-4877**):



The fully protected peptide Boc-GVTGWRLCERILA-resin was synthesized by standard solid phase peptide synthesis using Fmoc techniques, then cleaved from the resin using dichloroacetic acid to liberate the fully protected peptide as a white solid. To a solution of 6-Tetramethylrhodamine-PEG3-NH2 (0.05g, 0.08mmol) in DMF (1.5mL), this Boc-GVTGWRLCERILA-OH (0.2g, 0.07mmol), 1-hydroxyazabenzotriazole (11mg, 0.08mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (15mg, 0.08 mmol) and diisopropylethylamine (0.28mL, 0.16mmol) was added . After stirring for 30min, the reaction was concentrated, and the resulting crude was partitioned between CH_2Cl_2 and water, the layers separated and the organic layer was washed with water and brine, dried over sodium sulfate and concentrated. The resulting pink solid was dissolved in a combination of triisopropylsilane (0.2mL) and trifluoroacetic acid (4mL). After stirring for 3h, the reaction was concentrated, and the resulting pink film was purified with reverse phase HPLC using a gradient of ACN in 0.1% aqueous TFA to provide PBI 4877 as a pink powder: MS (M+) calcd 2088.5, found 2089.1.

TOM-DEVDGVTGWRLCERILA-OH (PBI-5074):



The fully protected peptide H-DEVDGVTGWRLCERILA-resin was synthesized by standard solid phase peptide synthesis using Fmoc techniques. While still on the resin, a solution of 6-TOM (**PBI-3739**) succidimidyl ester was added and allowed to react with the free N-terminus. The peptide was then cleaved from the resin and fully deprotected using trifluoroacetic acid (TFA) to provide a blue solid. This solid was purified with reverse phase HPLC using a gradient of ACN in 0.1% aqueous TFA to provide PBI 5074 as a blue powder: MS (M+Z/2) calcd 1238.9, found 1238.8.

Example 107

Complementation Comparison Between a Synthetic, N-terminal Fusion and C-terminal Fusion of NLPep78

Fusions of NLpep78-HaloTag (78-HT) and HaloTag-NLPep78 (HT-78) were quantitated, with a GST-HaloTag® fusion (GST-HT) as a control, by labeling *E. coli* lysates with the HaloTag-TMR® ligand, separated by SDS-PAGE, and scanned on Typhoon. A standard curve

was then created using known concentrations of GST-HT standard, and band intensities of 78-HT and HT-78 were used to determine their concentrations.

E. coli lysates containing NLpoly11S were diluted 1:10^7 into PBS pH 7 + 0.1% Prionex. Serial dilutions of 78-HT, HT-78, and synthetic NLpep78 were made in PBS pH 7 + 0.1% Prionex. 20uL NLpoly11S and 20uL of one of the NLPep were mixed and incubated at ambient temperature for 5 minutes. 40uL NanoGlo® reagent (Promega Corporation) + 100uM Fz were added, and the samples incubates at ambient temperature for 5 min. Luminescence was measured on GlomaxMulti+ using 0.5s integration. Data was fit to one-site, specific binding using GraphPad Prism to determine Bmax and Kd.

The results (Figure 150) compare the binding of NLpoly11S to synthetic NLPep78 and NLPep78 at the N- or C-terminus of a fusion partner (HaloTag). The binding affinities were not found to change significantly, but Bmax was reduced when NLPep78 was at the C-terminus.

Example 108

Complementation Comparison Between a Synthetic, N-terminal Fusion and C-terminal Fusion of NLPep79

Fusions of NLpep79-HaloTag (79-HT) and HaloTag-NLPep79 (HT-79) were quantitated, with a GST-HaloTag® fusion (GST-HT) as a control, by labeling E. coli lysates with the HaloTag-TMR® ligand, separated by SDS-PAGE, and scanned on Typhoon. A standard curve was then created using known concentrations of GST-HT standard, and band intensities of 79-HT and HT-79 were used to determine their concentrations.

E. coli lysates containing NLpoly11S were diluted 1:10^7 into PBS pH 7 + 0.1% Prionex. Serial dilutions of 79-HT, HT-79, and synthetic NLpep79 were made in PBS pH 7 + 0.1% Prionex. 20uL NLpoly11S and 20uL of one of the NLPep were mixed and incubated at ambient temperature for 5 minutes. 40uL NanoGlo® reagent (Promega Corporation) + 100uM Fz were added, and the samples incubates at ambient temperature for 5 min. Luminescence was measured on GlomaxMulti+ using 0.5s integration. Data was fit to one-site, specific binding using GraphPad Prism to determine Bmax and Kd.

The results (Figure 151) compare the binding of NLpoly11S to synthetic NLPep79 and NLPep79 at the N- or C-terminus of a fusion partner (HaloTag). The binding affinities were not found to change significantly, but Bmax was reduced when NLPep79 was at the C-terminus.

Example 109

Spectral Scan of NLpoly11S with NLPep86 Compared to PBI-4877 (NLPep1-fluorophore)

Purified NLpoly11S was diluted to 1nM in PBS pH 7 + 0.01% Prionex + 1mM DTT. NLPep86 or PBI-4877 was diluted to 40uM in PBS pH 7 + 0.01% Prionex + 1mM DTT. 25uL NLpoly11S and 25uL NLPep86 or PBI-4877 were mixed and then incubated at ambient

temperature for 10min. 50uL buffer (PBS pH 7 + 0.01% Prionex + 1mM DTT) + 100uM Fz was then added. Luminescence was measured on Tecan Infinite M1000: 300-800nm, every 5nm, bandwidth 10nm, gain 127, integration 0.5s, z-position 22,000um.

The results demonstrate (Figure 152) that the NLPep can be conjugated to small molecules such as fluorescent dyes and retain interaction with NLpoly11S to produce luminescence. It also demonstrates efficient energy transfer and the ability to alter the emission spectra.

Example 110

Spectral Scan of NLpoly11S with NLPep86 Compared to PBI-5434 (fluorophore- NLPep1)

Purified NLpoly11S was diluted to 1nM in PBS pH 7 + 0.01% Prionex + 1mM DTT. NLPep86 or PBI-5434 was diluted to 40uM in PBS pH 7 + 0.01% Prionex + 1mM DTT. 25uL NLpoly11S and 25uL NLPep86 or PBI-5434 were mixed and then incubated at ambient temperature for 10min. 50uL buffer (PBS pH 7 + 0.01% Prionex + 1mM DTT) + 100uM Fz was then added. Luminescence was measured on Tecan Infinite M1000: 300-800nm, every 5nm, bandwidth 10nm, gain 127, integration 0.5s, z-position 22,000um.

The results demonstrate (Figure 153) that the NLPep can be conjugated to small molecules such as fluorescent dyes and retain interaction with 11S to produce luminescence. This, along with the results with PBI-4877 in Example 109, also suggests that the terminus and/or the linker length used for the conjugation can significantly affect the energy transfer.

Example 111

Spectral Scan of NLpoly11S with NLPep86 Compared to PBI-5436 (fluorophore- NLPep1)

Purified NLpoly11S was diluted to 1nM in PBS pH 7 + 0.01% Prionex + 1mM DTT. NLPep86 or PBI-5436 was diluted to 40uM in PBS pH 7 + 0.01% Prionex + 1mM DTT. 25uL NLpoly11S and 25uL NLPep86 or PBI-5436 were mixed and then incubated at ambient temperature for 10min. 50uL buffer (PBS pH 7 + 0.01% Prionex + 1mM DTT) + 100uM Fz was then added. Luminescence was measured on Tecan Infinite M1000: 300-800nm, every 5nm, bandwidth 10nm, gain 127, integration 0.5s, z-position 22,000um.

The results demonstrate (Figure 154) that the NLPep can be conjugated to small molecules such as fluorescent dyes and retain interaction with 11S to produce luminescence. It also demonstrates efficient energy transfer and the ability to alter the emission spectra.

Example 112

Comparison of Km Values for 11S with Various NLPeps in Affinity Buffer

Purified NLpoly11S was diluted to 40pM in PBS pH 7 + 0.01% Prionex + 1mM DTT +0.005% Tergitol (affinity buffer) or NanoGlo assay reagent (Promega Corporation). NLPeps (NLpep86, 78, 99, 101, 104, 128 and 114) were diluted to 400uM (NLPep to 1mM) in affinity

buffer or NanoGlo assay reagent. 300uL NLpoly11S and 300uL of an NLPep were mixed and incubated at ambient temperature for 30min. 50ul was then added to a well of white 96-well plates. 50ul affinity buffer + 2x Fz (12.5uM diluted 2-fold 7 times) or 50ul NanoGlo + 2x Fz (100uM diluted 2-fold 7 times) was added to each well, and luminescence measured on a Glomax Multi+ using 0.5s integration. Km was determined using GraphPad Prism, Michaelis-Menten.

The results demonstrate substrate binding in affinity buffer (Figure 155) or NanoGlo assay buffer (Figure 156) to the complex between NLpoly11S and various NLPeps. The determined Km values do not fluctuate significantly with the indicated NLPeps.

Example 113

NLPep1 Binding Affinity to NLpoly11S at Various Concentrations of Furimazine

Purified NLpoly156 and NLpoly11S to 40pM in affinity buffer (PBS pH 7 + 0.01%prionex + 1mM DTT + 0.005%tergitol). Synthetic NLPep1 (WT) was diluted to 560uM for NLpoly156 or 80uM for NLpoly11S in affinity buffer and then serially diluted 3-fold to make 8 concentrations. 350uL NLPep1 and 350uL NLPoly156 or 11S were mixed and then incubated at ambient temperature for 30min. 50uL was then aliquoted into a well of white 96-well assay plate. Fz was added to affinity buffer to 40, 20, 10, 5, 2.5 and 1.25uM, 50uL Fz/affinity buffer added to each well and incubated at ambient temperature for 2min. Luminescence was measured on a Glomax Multi+ with 0.5s integration. GraphPad Prism and 1-site specific binding was used to calculate Kd at each concentration of Fz.

The results (Figure 157) indicate the change in affinity (NLPoly/NLPep) with increasing concentrations of Fz.

Example 114

Furimazine Km Values for NLpoly156/NLPep1 and NLpoly11S/NLPep1 at Various Concentrations of NLPep1

Purified NLpoly156 and NLpoly11S were diluted to 40pM in affinity buffer (PBS pH 7 + 0.01%prionex + 1mM DTT + 0.005%tergitol). Synthetic NLPep1 (WT) was diluted to 560uM for NLpoly156 or 80uM for NLpoly11S in affinity buffer and then serially diluted 3-fold to make 8 concentrations. 50uL was then aliquoted into a well of white 96-well assay plate. Fz was added to affinity buffer to 40, 20, 10, 5, 2.5 and 1.25uM, 50uL Fz/affinity buffer added to each well and incubated at ambient temperature for 2min. Luminescence was measured on a Glomax Multi+ with 0.5s integration. GraphPad Prism and 1-site specific binding was used to calculate Kd at each concentration of NLPep1.

The results (Figure 158) indicate the change in affinity (NLPoly/NLPep) with increasing concentrations of NLPep1.

Example 115

Comparison of Maximal Activity for NLPoly156/NLPep1, NLPoly11S/NLPep1, and NanoLuc® luciferase

Purified NLPoly156, NLPoly11S, or NanoLuc® luciferase (Nluc) were diluted to 40pM in affinity buffer (PBS pH 7 + 0.01%prionex + 1mM DTT + 0.005%tergitol). Synthetic NLPep1 (WT) was diluted to 560uM for NLPoly156 or 80uM for NLPoly11S in affinity buffer and then serially diluted 3-fold to make 8 concentrations. 350uL NLPep1 (or affinity buffer) and 350uL NLPoly (or Nluc) were mixed and then incubated at ambient temperature for 30min. 50uL was then aliquoted into a well of white 96-well assay plate. Fz was added to affinity buffer to 40, 20, 10, 5, 2.5 and 1.25uM, 50uL Fz/affinity buffer added to each well and incubated at ambient temperature for 2min. Luminescence was measured on a Glomax Multi+ with 0.5s integration. GraphPad Prism and Michaelis-Menton equation was used to calculate Vmax at each concentration of NLPep (input calculated Vmax values at each concentration of NLPep1 into 1-site specific binding to calculate Bmax). GraphPad Prism and 1-site specific binding was used to calculate Bmax at each concentration of Fz (input calculated Bmax values at each concentration of Fz into Michaelis-Menton equation to calculate Vmax).

The results (Figure 159) demonstrate the maximal activity of NLPoly156 or NLPoly11S upon activation by NLPep1 to the maximal activity of NanoLuc luciferase.

Example 116

Luminescent Values Resulting from Titrating NLpoly11S with Various NLPeps

Purified NLPoly11S was diluted to 40pM in PBS pH 7 + 0.01% Prionex + 1mM DTT +0.005% Tergitol (affinity buffer). Synthetic NLPeps (NLPep86, 78, 79, 99, 101, 104, 114, 128 or wt) were diluted in affinity buffer as follows: NLPep86 = 60nM, NLPep78 = 280nM, NLPep79 = 800nM, NLPep99 = 4uM, NLPep101 = 34uM, NLPep104 = 20uM, NLPep128 = 4uM, NLPep114 = 4.48mM and NLPepWT = 20uM. 25uL NLPoly11S and 25uL an NLPep were mixed and then incubated at ambient temperature for 30min. 50ul affinity buffer + 20uM Fz was then added to each mixture, and luminescence measured on a GlomaxMulti+ using 0.5s integration. Bmax and Kd values were determined using GraphPad Prism and 1 site specific binding.

The results (Figure 160) demonstrate ~100,000-fold range of affinities using NLPoly11S and various NLPeps. Minimal loss in Bmax was observed between the high affinity and low affinity NLPeps.

Example 117

Western Blot of NLPoly156, NLPoly11S, and NanoLuc® luciferase After Transfection intoHEK293T Cells

On day 1, a transfection mixture of 2ng NLPoly156, NLPoly11S or NanoLuc® luciferase (Nluc) DNA, 1ug pGEM3Zf(+) carrier DNA, 4ul Fugene HD (Promega Corporation) and Phenol red-free OptiMEM to 100ul was made and incubated at RT for 10 minutes. The transfection mixture was then transferred to one well of 6 well plate, and 2 ml of HEK293T cells at 400,000 cells/ml (800,000 cells total) was added. The cells were incubated overnight at 37°C.

On day 2, the cells were washed with phenol red-free DMEM, 500uL phenol red-free DMEM added to each well, and the cells frozen at -70°C for at least 30 min. The cells were then thawed, 500uL transferred to microcentrifuge tube, and 20ul mixed with 80uL of 1.25x SDS loading buffer and incubated at 95°C for 5min. 10ul was loaded onto 10% Bis-Tris NuPAGE gel with MES running buffer. Protein was transferred to PVDF using iBlot, and the membrane washed in methanol. The membrane was then blocked in TBST + 5% BSA for 1hr at ambient temperature, washed 3 times in TBST and then incubated with 10mL TBST + 2uL rabbit anti-Nluc polyclonal antibody + 2uL rabbit anti-β-actin polyclonal antibody (Abcam #ab8227) at 4°C overnight.

On day 3, the membrane was washed 3 times in TBST, incubated with 10mL TBST + 2uL anti-rabbit HRP conjugated antibody for 1hr at ambient temperature, washed again 3 times with TBST and incubated with 12mL ECL Western Blotting Substrate for 1 min. Chemiluminescence was imaged with LAS 4000 Image Quant.

The results (Figure 161) show the expression level of NLPoly compared to full-length NanoLuc® luciferase. NLPoly156 does not express as well as NanoLuc® luciferase (Nluc), whereas NLPoly11S expresses similarly to Nluc.

Example 118

Determination of the Influence of NLPoly11S/NLPep114 Affinity on the Interaction Between a β-lactamase (SME) and β-lactamase Inhibitory Protein (BLIP) and Comparison Between Affinity Values Measured Through 11S/114 and β-lactamase Activity

Protein purification

pF1K-signal-6H-SME, pF1K-signal-6H-SME-11S, pF1K-signal-6H-BLIPY50A, and pF1K-signal-6H-BLIPy50A-114 (Promega Flexi vectors for T7 promoter-based expression of recombinant protein in E. coli; the signal refers to the native signal peptide for either SME or BLIP) were induced with rhamnose to express in the periplasm of KRX cells at 25°C for 18-20hrs. Cells were pelleted and resuspended in B-Per lysis reagent (Pierce; 1/50th culture volume) and incubated at ambient temperature for 15min. Lysate was then diluted by addition of 1.5x volume 20mM Tris pH 8 + 500mM NaCl and centrifuged at 12,000xg for 10min. The supernatant was transferred to a clean tube, 1mL RQ1 DNase (Promega Corporation) added and centrifuged again at 12,000xg for 10min. Supernatant was purified over HisTALON column

Clontech) with 25mM Tris pH 8 and 500mM NaCl loading buffer and eluted with 25mM Tris pH 8, 500mM NaCl and 50mM imidazole. Eluted protein was dialyzed into 25mM Tris pH 7.5 and 25mM NaCl and purified over HiTrap Q FF column (GE Healthcare) with 25mM Tris pH 7.5 and 25mM NaCl loading buffer and eluted with 25mM Tris pH 7.5 and 125mM NaCl. Ionic strength was adjusted to final concentration of 150mM NaCl, and concentrated using a VivaSpin concentrator.

Assay

BLIPY50A and BLIPY50A-114 were diluted to 312.5nM in affinity buffer (PBS pH 7 0.01%prionex 0.005%tergitol 1mM DTT), and then serially dilute 1.5-fold. SME and SME-11S were diluted to 0.2nM in affinity buffer. 11.11uL SME and 88.89uL BLIP were mixed and then incubated at ambient temperature for 2hrs. 90uL of the mixture was transferred to a clear 96-well plate with 10uL of 100uM Nitrocefin (Calbiochem in affinity buffer). 90uL of SME-11S/BLIPY50A-114 was transferred to a white 96-well plate with 10uL of 100uM Fz (in affinity buffer). Absorbance (nitrocefin) was measured at 486nm every 15sec over 30min, and luminescence (Fz) was measure every 2min over 30min.

For nitrocefin, initial velocities were fit using Excel. Initial velocities vs. BLIP concentration were plotted. Fit Ki using E_free=[E]−([E_0]+[I_0]+K_app−√(([E_0]+[I_0]+K_app)^2−(4[E_0][I_0])))/2 and K_app=K_i (1+([S])/K_M)

For Fz, Kd using RLU=($Bmax$ × [$BLIP$−114])/([$BLIP$−114]+K_D) was fit.

The results (Figure 162) compares the affinity of a protein interaction (the β-lactamase SME and its inhibitor BLIPY50A) as unfused proteins to the affinity when NLPoly and NLPep are fused to them and demonstrates the affinity between NLPoly11S and NLPep114 does not result in an increased apparent affinity for the SME/BLIPY50A interaction. This also demonstrates the use of NLPoly11S and NLPep114 to measure an equilibrium binding constant for a protein interaction, and the affinity measured through NLPoly11S and NLPep114 is consistent with the affinity measured by activity of the target protein (SME).

Example 119

Comparison of luminescence generated by cells expressing different combinations of FRB-NLPoly11S with FKBP-NLPep101 and 111-136

HEK293T cells (20,000) were reverse-transfected into wells of opaque 96-well assay plates with a total of 1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP- NLpep101 or 111-136 plasmid DNA using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. Twenty-four hours-post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without

50nM rapamycin for 1.5 h. 10 μM furimazine substrate (final concentration) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then read on a GloMax Multi with 0.5s integration time.

Figure 163 demonstrates that, of tested combinations, NLpoly11S with NLpep114 shows the greatest rapamycin induction and one of the strongest rapamycin-specific luminescent signals.

Example 120

Comparison of luminescence generated by cells expressing different combinations of FRB-NLpoly11S with FKBP-NLpep114 and 137-143

HEK293T cells (20,000) were reverse-transfected into wells of opaque 96-well assay plates with a total of 1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep114 or 137-143 plasmid DNA using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1μg. Twenty-four hours post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 1.5 h. 10 μM furimazine substrate (final concentration) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then read on a GloMax Multi with 0.5s integration time.

Figure 164 demonstrates that, of tested combinations, NLpoly11S with NLpep114 shows the greatest rapamycin induction and one of the strongest rapamycin-specific luminescent signals.

Example 121

Rapamycin dose response curves of cells expressing FRB-NLpoly11S and FKBP-NLpep78/79/99/101/104/114/128

HEK293T cells (20,000) were reverse-transfected into wells of opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep78/79/99/101/104/128 plasmid DNA using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1μg. Twenty-four hours post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with 0 to 300nM rapamycin for 2 h. 10 μM furimazine substrate (final concentration) with 0 to 300nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then read on a GloMax Multi with 0.5s integration time. Graphpad Prism was used to fit data to sigmoidal curve and calculate EC50 values.

Figure 165 shows a sigmoidal dose response to rapamycin for NLpoly11S with NLpep78/79/99/101/104/114/128. Of the combinations plotted, NLpoly11S with NLpep114 shows the greatest dynamic range.

Example 122

Response of cells expressing FRB-NLpoly11S and FKBP-78/79/99/101/104/114/128 to the rapamycin competitive inhibitor FK506

HEK293T cells (20,000) were reverse-transfected into wells of opaque 96-well assay plates with a total of 0.1ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep78/79/99/101/104/114/128 plasmid DNA using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. Twenty-four hours post transfection, cells were washed with PBS and then phenol red-free OptiMEMI with 10nM rapamycin was added for 2 h. FK506 inhibitor in OptiMEM was added to cells at final concentrations of 0 to 50 µM and incubated for 3h. Furimazine in OptiMEM was added to cells for a final concentration of 10 µM on cells. Luminescence was immediately read on a GloMax Multi with 0.5s integration time. Graphpad Prism was used to plot data, fit to a sigmoidal curve, and calculate IC50 values.

Figure 166 demonstrates dose-dependent decreases in rapamycin-induced signal of FRB-NLpoly11S and FKBP-78/79/99/101/104/114/128 with the rapamycin competitive inhibitor, FK506.

Example 123

Comparison of luminescence generated by cells transfected with different ratios of FRB-NLpoly11S and FKBP-NLpep114

HEK293T cells (20,000) were reverse-transfected into wells of opaque 96-well assay plates with 1ng pF4A Ag FRB-NLpoly11S and 0.01, 0.1, 1, 10, or 100ng pF4A Ag FKBP-NLpep114 plasmid DNA using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 8. HEK293T cells (20,000) were also reverse transfected with 1ng pF4A Ag FKBP-NLpep114 and 0.01, 0.1, 1, 10, or 100ng pF4A Ag FRB-NLpoly11S. In both situations, pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. Twenty-four hours post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin for 1.5 h. 10 µM furimazine substrate (final concentration) with or without 50nM rapamycin in OptiMEMI was added directly to each well and incubated at room temperature for 5 min. Luminescence was then read on a GloMax Multi with 0.5s integration time.

Figure 167 demonstrates that a DNA ratio of 1:1 generated the greatest rapamycin induction, although a significant induction was observed at all DNA ratios tested.

Example 124

Comparison of luminescence generated by cells expressing NLpoly11S/NLpep114 fusions of FRB/FKBP in different orientations and with different linker lengths

HEK293T cells (20,000) were transfected into wells of 96-well plates with vectors expressing combinations of N- and C-terminal fusions of pF4Ag NLpoly11S and pF4Ag NLpep114 with FRB or FKBP. In these constructs, NLpoly11S/NLpep114 were separated from their fusion partners with either a 4, 10, or 15 serine/glycine linker. 0.1ng NLpoly11S and NLpep114 DNA was transfected per well at a DNA-to-FugeneHD ratio of 1 to 8. Twenty-four hours post transfection, cells were washed with PBS and then incubated in phenol red-free OptiMEMI with or without 50nM rapamycin in OptiMEMI for 2 h. 10 μM Furimazine substrate was then added, and following a 5 min incubation at room temperature, the plate was read using a GloMax Multi with 0.5s integration time.

Figure 168 illustrates a rapamycin-specific increase in RLU regardless of fusion orientation or linker length.

Example 125

Comparison of rapamycin dose response curve and time course generated by FRB-NLpoly11S/FKBP-NLpep114 and split firefly complementation systems

HEK293T cells (800,000) were transfected into wells of 6-well plates with a total of 20ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep114 or 750ng pF4A Ag N-Fluc(1-398)-FRB and FKBP-C-Fluc(394-544) using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1μg. Twenty-four hours post transfection, 20,000 cells were re-plated into wells of opaque 96-well assay plates and incubated an additional 24 h.

For dose response experiments (Figure 169A), NLpoly11S/NLpep114-expressing cells were treated with 0-1μM rapamycin in phenol red-free OptiMEMI for 3 h and then incubated with 10μM furimazine for 5 min before recording luminescence on GloMax Multi. Cells expressing N-Fluc(1-398)/C-Fluc(394-544) were incubated with 0-1 μM rapamycin in phenol red-free for 2 h, followed by an additional 1 h incubation in the presence of 4 mM D-Luciferin, prior to recording luminescence on GloMax Multi.

For time course experiments (Figure 169B), NLpoly11S/NLpep114-expressing cells were treated with 0 or 50nM rapamycin in phenol red-free OptiMEMI was added via GloMax Multi injector, and luminescence was immediately measured. Cells expressing N-Flu(1-398)/C-Flu(394-544) were treated with 4 mM D-luciferin in phenol red-free OptiMEMI for 1 h followed by addition of 0 or 50nM rapamycin via injector and measurement of luminescence by GloMax Multi. Curves were fit using GraphPad Prism 6 software.

Figure 169A-B demonstrate that both NLpoly11S/NLpep114 and split firefly complementation systems respond in a rapamycin-dependent manner, generating sigmoidal dose response curves and similar EC50 values. The NLpoly11S/NLpep114 system displays faster association kinetics and a higher maximum signal.

Example 126

Comparison of FK506 dose response curve and time course generated by FRB-NLpoly11S/FKBP-NLpep114 and split firefly complementation systems

HEK293T cells (800,000) were transfected into wells of 6-well plates with a total of 20ng pF4A Ag FRB-NLpoly11S and pF4A Ag FKBP-NLpep114 or 750ng pF4A Ag N-Fluc(1-398)-FRB and FKBP-C-Fluc(394-544) using FuGENE HD at a DNA-to-FuGENE ratio of 1 to 4. pGEM-3Zf(+) DNA was added to bring total DNA in each transfection to 1µg. Twenty-four hours post transfection, 20,000 cells were re-plated into wells of opaque 96-well assay plates and incubated an additional 24 h. Cells were then treated with 0 or 20nM rapamycin in phenol red-free OptiMEMI for 3 h.

For FK506 dose response experiments (Figure 170A), cells were incubated with 0 to 100µM FK506 inhibitor in phenol red-free OptiMEMI for 5 h, treated with 10µM furimazine, and then read with GloMax Multi in luminescence mode with 0.5s integration time.
For time course experiments (Figure 170B), cells were treated with 10 µM FK506 in phenol red-free OptiMEMI containing 10 µM furimazine and luminescence was immediately read with GloMax Multi.

Figure 170A-B demonstrates that the NLpoly11S/NLpep114 and split firefly complementation systems show a dose-dependent decrease in light output following treatment with the FK506 inhibitor. The loss of signal in the NLpoly11S/NLpep114 system begins at an earlier time point, is more rapid, and is more complete than the split firefly system.

Example 127

Western blot showing expression levels of FKBP-NLpep114 and FKBP-Fluc(394-544)

HEK293T cells (200,000) were transfected with 0 to 30ng of pF4Ag NLpep114-FKBP or pF4Ag FKBP-Fluc(394-544) DNA using FugeneHD at a DNA to Fugene ratio of 1 to 8. Forty-eight hours post-transfection, cells were harvested with 1X SDS gel loading buffer. Samples were separated on a 4-10% Tris-HCl SDS-PAGE gel and transferred to PVDF membrane. The membrane was blocked in 5% BSA in TBST for 1h and then incubated with anti-FKBP (Abcam #ab2918) overnight. Secondary antibody incubation with horse radish peroxidase-conjugated donkey anti-rabbit IgG was performed for 1h and then the blot was developed using ECL Western Blotting Substrate (Promega Corporation) and the Image Quant LAS 4000 system.

Figure 171 demonstrates similar expression levels of FKBP-NLpep114 and FKBP-Fluc(394-544) at equal levels of transfected DNA.

Example 128

Dose- and time-specific inhibition of NLpoly11S-BRD4 and Histone H3.3-NLpep114 interaction by IBET-151

HEK293T cells (20,000) were transfected into wells of a 96-well white assay plate with 10ng of pF4Ag Histone H3.3-NLpep114 and NLpoly11S-NLpoly11S using Fugene HD at a DNA to Fugene ratio of 1 to 8.

For dose response experiment (Figure 172A), cells were treated with 0 to 10μM IBET-151 in phenol red-free OptiMEMI for 4 h at 37°C and then treated with 10μM furimazine for 5 min before reading luminescence with GloMax Multi.

For time course experiment (Figure 172B), cells were pre-incubated with 10μM furimazine for 5 min, treated with 0-500nM IBET-151 and immediately placed in a GloMax Multi for luminescent measurements every 5 min.

Figure 172A-B demonstrates a dose-dependent decline in luminescence upon treatment with the BRD4 inhibitor IBET-151that occurs within 3 hours of treatment, consistent with literature reports.

Example 129

RAS/CRAF, BRAF/BRAF, and CRAF/BRAF dimerization in response to GDC0879

HEK293T cells (20,000) were co-transfected into wells of 96-well assay plates with combinations of pF4Ag NLpoly11S-BRAF, NLpoly11S-CRAF, NLpep114-KRAS, or NLpep114-BRAF using a total of 0.1ng DNA per well and Fugene HD at a ratio of 1 to 4. Twenty-four hours post-transfection, cells were treated with 0 to 10μM of the BRAF inhibitor GDC0879 in phenol red-free OptiMEMI for 4h. Furimazine substrate in phenol red-free OptiMEMI was added to 10μM, and luminescence was read immediately with GloMax Multi set to 0.5s integration time.

Figure 173 demonstrates a dose dependent increase of RAS/CRAF, BRAF/BRAF and CRAF/BRAF dimerization in response to BRAF inhibitor GDC0879.

Example 130

Twelve synthetic peptides (Figure 180) were examined for their ability to structurally complement three different versions of NLpoly11S (i.e. 11S, 11S-amino acid 157, 11S-amino acids 156 and 157). Stocks of NLpoly were made to 35 nM in NanoGlo reagent and stocks of NLpep were made to 12.5 nM in PBS pH 7.2. Equal volumes were mixed and samples measured for luminescence on a Tecan Infinite F500 reader (100 msec integration time; 10 min time point) (Figure 200).

Example 131

Spontaneously interacting peptide NLpep86

Purified NLPoly11S was diluted to 40pM in PBS pH 7 + 0.01% Prionex + 1mM DTT +0.005% Tergitol (affinity buffer). Synthetic NLPeps (NLPep86, WT, 114) were diluted in affinity buffer as follows: NLPep86 = 60nM, NLPep114 = 4.48mM and NLPepWT = 20uM. 25uL NLPoly11S and 25uL an NLPep were mixed and then incubated at ambient temperature for 30min. 50ul affinity buffer + 20uM Fz was then added to each mixture, and luminescence measured on a GlomaxMulti+ using 0.5s integration. Bmax and Kd values were determined using GraphPad Prism and 1 site specific binding.

Figure 174 demonstrates ~100,000-fold range of affinities using NLPoly11S and various NLPeps. Pep 86 is an example of a spontaneously interacting peptide (with LSP 11S), and Pep 114 is shown for reference as a low affinity interacting peptide.

Example 132

Titration of high affinity peptide in vitro

Purified NLpoly11S (HaloTag purification/*E.coli* expression; pFN18K) and synthetic peptide NLpep86 (obtained from Peptide 2.0) were titrated at a linear dynamic range using 33nM NLpoly11S in Nano-Glo® assay buffer to 3.3 fM – 100 nM high affinity NLpep86. For a 30 kDa protein, this corresponds to LOD of 10 fg.

Figure 176 demonstrates the broad linear range and ability to detect femptamolar concentrations of the high affinity peptide tag (NLpep86). This rivals most sensitive Western Blot (WB)+ Enhanced Chemiluminescence (ECL) kits

Example 133

Western Blot-Like Utility of NLpoly and NLpep

A titration of HaloTag (HT7)-NLpep 80 (80) or NLpep80-HaloTag (HT7) were run on an SDS page gel. The HaloTag® protein was imaged with HaloTag-TMR ligand (Promega Corporation) on a Typhoon scanner. The samples were transferred to a membrane and PBS pH 7 + 0.1% Prionex + NLpoly11S (E. coli lysate diluted 1:1,000) was used to blot the membrane. NanoGlo/Fz was then added to the membrane and it was imaged on a ImageQuant.

Figure 177 demonstrates the sensitivity of detecting proteins tagged with a high affinity NLPep using NLpoly11S. Figure 177 also compares the detection using NLPep/NLPoly to the detection using fluorescently labeled HaloTag.

Example 134

Stability of an NLpoly11S Reagent

100nM NLpoly11S was incubated in NanoGlo assay buffer (Promega Corporation)+100uM furimazine and assayed with equal amounts of diluted NLpep86.

As a control, NanoGlo assay buffer+100uM furimazine was used to assay an equal volume of diluted NanoLuc® luciferase (Promega Corporation).

The results (Figure 178) demonstrate that an NLpoly11S reagent (containing Fz) has similar stability compared to the commercial NanoGlo® assay reagent (also containing Fz).

Example 135

Titration of DNA for High Affinity NLpep78-HT7 fusion

HEK293 cells (200,000/ml) were reverse transfected with 10-fold dilutions of DNA (starting with 100ng) from a high affinity peptide, NLpep78, fused to HaloTag® protein (HT7). 100ul of each transfection was plated in triplicate into wells of a 96-well plate. Twenty-four hours post-transfection, 100ul NanoGlo® assay buffer containing 100nM NLpoly11S and 100ul furimazine was added and mixed. Luminescence was measured10 minutes after reagent addition on a GloMax luminometer.

The results (Figure 179) demonstrate the broad linear range similar to Example 131/Fig 27. This is essentially a similar experiment to what was done in Example 131 except that this examples uses recombinantly expressed peptide (fused to HaloTag) in a mammalian cell.

Example 136

Preliminary results (array peptides)

In Figure 183A, 50nM NLpoly11S was mixed with 7.5μM NLpep114 and 37.5 μM dark peptide (DP) candidate (Q-162, A-162, K-162 or E-162). NanoGlo® assay reagent (Promega Corporation) was added and incubated for 5 minutes. Luminescence was detected.
In Figure 183B, 50nM NLpoly11S in assay buffer (PBS pH7 + 0.01% Prionex + 1 mM DTT + 0.005% Tergitol) was mixed with 7.5μM NLpep114 (also in assay buffer) and variable amounts of dark peptide (DP) candidates Q-162 or K-162 (also in assay buffer). NanoGlo® assay reagent (Promega Corporation) was added and incubated for 5 minutes. Luminescence was detected on a Tecan Infinite F500 reader; 100 ms integration time; 5 min time point used.

Panel A indicates that each of the peptide candidates (at 7.5 uM) can inhibit the binding between NLpoly11S and NLpep114, as indicated by less bioluminescence. Note these “dark” peptides do generate some luminescence, thus the increased signal compared to no peptides at all.

Panel B indicates that with the Lys-162 and Gln-162 peptides the inhibition is dose-dependent.

Example 137

High purity (>95%) dark peptides

In Figure 184A, 5nM NLpoly11S was mixed with 500nM NLpep114 and variable amounts of a dark peptide (DP) candidate Q-162 or A-162 (n=3). NanoGlo® assay reagent (Promega Corporation) was added and incubated for 5 minutes. Luminescence was detected.

In Figure 184B, 5nM NLpoly11S in assay buffer was mixed with variable amounts of dark peptide (DP) candidates Q-162 or A-162 in assay buffer (no NLpep114)(n=3). NanoGlo® assay reagent (Promega Corporation) was added and incubated for 5 minutes. Luminescence was detected.

The results (Figure 184A and B) substantiate the results from Example 135, but there is greater confidence here because the peptides are more pure. These results also suggest that of the dark peptides variants tested the Ala peptide is the most potent as an inhibitor.

Example 138

Inhibition of Circularly Permuted NanoLuc® Luciferase by Dark Peptides

To determine whether "high affinity/low activity" NLpeps (a.k.a. Dark Peptides) can compete with the intramolecular interaction (i.e., protein folding) between NanoLuc® luciferase (Nluc) residues 1–156 and 157–169 in the context of circularly permuted Nluc (CP Nluc).

CP NLuc: NLuc 157–169---33aa-linker---Nluc 1–156

Dark peptides: VTGWRLCERIL (wt)

1. Gln-162 VSGWQLFKKIS
2. Ala-162 VSGWALFKKIS

Recombinant CP Nluc was prepared as a soluble fraction of an *E. coli* 5x-concentrated lysate (T7-promoter; overnight expression). A 10,000-fold dilution of the CP Nluc in Assay Buffer (PBS pH 7/0.01% Prionex/1mM DTT/0.005% Tergitol) was used. Synthetically-derived dark peptides were prepared across a range of concentrations, also in the Assay Buffer. Reactions were set up using 30µL of CP NLuc and 60µL of Dark peptide and assayed by adding 90µL NanoGlo® assay reagent (Promega Corporation). Luminescence was measured (5 min) on a Tecan Infinite F500 reader (100 ms integration). Three replicates were used for Dark peptide samples. Two replicates were used for buffer controls (acetic acid from peptide stocks).

Figure 185 demonstrates a dose-response of the dark peptides with CP Nluc. Figure 186 demonstrates a time course of dark peptide (56 µM peptide) with CP Nluc.

The results indicate that both dark peptides, particularly the Ala162 version, are able to significantly inhibit generation of luminescence by CP Nluc (Ala162 >2 logs; Gln162 >1 log). This indicates that a CP Nluc approach has utility for inverse complementation.

Example 139

Dark Peptides in Cells

In this example, the following constructs were used:

-Four dark peptide vectors: pF4Ag + FKBP-dark peptide Ala-162, Leu-162, Gln-162 and Val-162

-Two non-dark peptide vectors: pFc5K2 FKBP-NLpep114 (low affinity peptide) and pFc5K2 FKBP-NLpep80 (high affinity peptide)

-One NLpoly vector: pFc5K2 FRB-NLpoly11S

All constructs harbored a CMV promoter for mammalian cell expression. All fusions constructs contained a 10aa Gly-Ser flexible linker.

Serial dilutions of the dark peptide constructs, Ala-162 (A), Leu-162 (L), Gln-162 (Q) and Val-162 (V), were made in OptiMem and additionally contained carrier DNA (pGEM-3Z).

For transfection containing NLpoly11S only, 20ul of diluted dark peptide was mixed with 20ul NLpoly11S, 60ul OptiMem and 8uL Fugene. For transfections containing NLpoly11S and NLpep114 or NLpep80, 20ul of diluted dark peptide was mixed with 20ul NLpoly11S (10ng/ul), 20ul NLpep114 or NLpep80 (10ng/ul), 40ul OptiMem and 8ul Fugene. All were incubated at RT for 15 minutes. 5ul of each transfection, in triplicate, was added to wells of two, 96-well plates (one +Rapamycin one without Rapamycin). 100ul of HEK293T at 200,000 cells/ml in DMEM+10% FBS were then added to the wells, and the transfected cells incubated overnight at 37^0C

The medium was then removed from the cells, and the cells washed with 200 µl DPBS. 50 µl of 50nM rapamycin was added, and the cells incubated for 1 h at 37^oC. 20µl of 5mM furimazine in 5 ml phenol red-free OptiMEMI + 50 nM rapamycin was diluted, 50µl added directly to the cells and incubated for 5 min in GloMax Multi+. Luminescence was measured on the GloMax.

Figure 187 demonstrates that the dark peptides, when fused to FKBP, can reduce the background signal of NLpoly11S (i.e., FRB-NLpoly11S). Taken together Figures 188–190 demonstrate that the dark peptides, when fused to FKBP, can 1) compete with the folding of full length NanoLuc (i.e., FRB-NanoLuc or NanoLuc-FRB) and 2) compete with both low and high affinity peptides (also FKBP fusions) for binding to NLpoly11S (i.e. FRB-NLpoly11S), and as a result reduce the total luminescence being produced and detected in live cells.

Example 140

Virology Applications

In addition to enabling measurement of viral titers, spontaneously interacting NLpeps also enable studying re-assortment of viruses (e.g., influenza). Re-assortment of viruses refers to the formation of new “hybrid” viruses from dual infections e.g. H1N1, H5N1, H3N2 (H is hemagglutinin; N is neuraminidase); bird, human, pig, chicken (most common in pigs)

Because of its segmented nature, the influenza genome can be readily shuffled in host cells infected with more than one virus. When a cell is infected with influenza viruses from different species, reassortment can result in progeny viruses that contain genes from strains that normally infect birds and genes from strains that normally infect humans, leading to the creation of new strains that have never been seen in most hosts. Moreover, because at least 16 different subtypes and nine different neuraminidase subtypes have been characterized, many different combinations of capsid proteins are possible. Of these subtypes, three subtypes of hemagglutinin (H1, H2, and H3) and two subtypes of neuraminidase (N1 and N2) have caused sustained epidemics in the human population. Birds are hosts for all influenza A subtypes and are the reservoir from which new HA subtypes are introduced into humans (Palese, 2004).

The application of the present system for detecting re-assortment is that the two components of spontaneously interacting NLpeps are be put into different viral particles, or the large component in cells and the small component in a virus, and the presence of both elements (e.g., being present in a cell) is detected by luminescence.

Example 141

Validating the Use of Spontaneously Interacting NLpep86 as an Epitope Tag for Proteins Degraded by the Proteasome

Experiments were conducted during development of embodiments of the present invention to validate the use of NLpep86 as a tag to monitor expression levels of proteins degraded by the proteasome. To do this, Nlpep86 was fused to firefly luciferase variants that were also fused to either one or more PEST, CL1 or ubiquitin sequences (pBC21, 22, 24-29). Each of these constructs is expected to undergo proteasome-mediated turnover to varying degrees following expression from a mutant CMV promoter (d1CMV).

The constructs pBC21,22, 24-29 and control constructs expressing untagged firefly luciferase or untagged firefly luciferase fused to a PEST sequence (ATG082 and ATG083) were transiently transfected into HELA cells plated at 10,000 cells per well in a 96-well plate using 100 µLof DMEM + 10% FBS. The following day, 10 µL of a transfection mixture (920ul OptiMEM I + 5 ug of the respective construct + 15ul Fugene HD) was added per well and cells were allowed to incubate for 48 hours in a 37 °C incubator containing 5% CO2. Protein expression levels were quantified in replicate wells for each construct by detecting firefly luciferase activity or by adding a detection reagent containing NLpoly11S (purified NLpoly11S added to NanoGlo®). A good correlation was observed between the NLpep86 and Fluc signals in each case, suggesting that NLpep86 detection can be used to monitor fusion protein expression levels for proteins degraded by the proteasome.

BC21 MVSGWRLFKKIS-GGSGGGGSGG-Fluc(high affinity)

BC22 MVSGWRLFKKIS-GGSGGGGSGG-FlucP(high affinity)

BC24 pFC15A/MVSGWRLFKKIS-GGSGGGGSGG-Fluc-CL1

BC25 MVSGWRLFKKIS-GGSGGGGSGG-Fluc-PEST12opt(high affinity)

BC26 MVSGWRLFKKIS-GGSGGGGSGG-Fluc-CP(high affinity)

BC27 UBQ G76V-VGKLGRQDP-Fluc(EDAKNIKK..)-GGSGGGGSGG-VSGWRLFKKIS(high affinity)

BC28 UBQ-RGKLGRQDP-Fluc(EDAKNIKK..)-GGSGGGGSGG-VSGWRLFKKIS(high affinity)

BC29 UBQ-LGKLGRQDP-Fluc (EDAKNIKK..)-GGSGGGGSGG-VSGWRLFKKIS(high affinity)

ATG083 D1 FlucP; pF4Ag CMV Luc2-PEST

ATG082 D1Fluc; pF4Ag CMV Luc2

After a 48 hour incubation, 100uL NanoGlo® NLpep11S reagent (90ul of NLpoly11S in 50ml of NanoGlo® assay reagent was added to each well and incubate for 3 minutes with shaking. Luminescence was then read on GloMax luminometer (0.5 sec/well).

The results in Figure 191 demonstrate that the signal from Fluc and NLpep86 appear to reflect each other with respect to relative brightness and have similar RLUs. BC21, BC25 and BC29 are the brightest constructs of the BC series with BC21 appearing the brightest in this experiment BC24, 26 and 27 are the least bright which is predicted from the engineered destabilization.

Example 142

This example demonstrates that a known complementing peptide can be used as a linker between the same or a different complementing NLpep and NLpoly11S (e.g., NLpep78(2X)).

HEK293T cells (20,000) were transfection with a mixture containing 20 μL of NLpep78-HaloTag (HT) or NLpep78(2x)-HT DNA, 80 μL of phenol-free Opimex and 8 μL of FugeneHD. Cells were grown overnight at 37°C and assayed at 24h using NanoGlo® assay reagent (Promega Corporation) containing 33nM purified NLpoly11S.

The results (Figure 192) demonstrate that a tandem binding peptide can be used and that it may suffice as a linker.

Example 143

Comparison of the Specific Activities of Wild-Type Oplophorus luciferase residues 1-156, NLpoly11S and NanoLuc in HEK293T lysates

Each clone was inserted into pFN21A HaloTag® CMV Flexi® Vector (Promega G2821), and lysates were prepared as follows: 3ml HEK293T cells that have been diluted to a concentration of 200,000 cells/ml (600,000 cells total) were plated into each well of a 6-well plate and grown overnight at 37°C in a CO_2 incubator. The following day transfection complexes of each DNA were prepared by combining 6.6µg of DNA, Opti-MEM® (Life Technologies 11058-021) to a final volume of 310µl and 20µl of FuGENE® HD (Promega E231a). The transfection complexes were incubated for 20 minutes and then 150µl of each complex added in duplicate to cells. The cells were grown overnight 37°C in a CO_2 incubator. The following day, the cells were washed cells with DPBS (Life Technologies 14190-144), and 1ml fresh DPBS added. Cells were frozen to lyse and then thawed for testing. Duplicate transfection reactions lysates were combined.

To quantitate the level of protein expression for each sample, each sample was labeled with HaloTag® TMR Ligand (Promega Corporation) as follows: HaloTag® TMR Ligand (Promega G8251) was diluted 1:100 into water to a concentration of 0.05mM;100µl of each lysate was mixed with 2µl of diluted TMR ligand and incubated for 30 minutes at RT; 20µl of SDS loading dye was added, and the samples heated to 95°C for 5 minutes. 10µl and 20µl of each sample was loaded onto a polyacrylamide gel (Bio-Rad, 4–15% Criterion™ Tris-HCl Gel #345-0030.), run at 200V for 1 hour and then quantitated using ImageQuant™ LAS 4000 (GE). Both NLpoly11S and NanoLuc® luciferase (Nluc) expressed approximately 4-fold higher than 1-156.

In order to compare the specific activities of Nluc (full length enzyme) to wt Oplophorus1-156 and NLpoly11S in combination with wt Oplophorus 157-169 peptide (binary proteins), substrate titrations were run for all of the samples, but for the binary samples substrate titrations were run at multiple peptide concentrations. Using this format, it was possible to calculate a Vmax value for Nluc and both Vmax and Bmax values for NLpoly11S and wt Oplophorus 1-156. Three separate experiments were run using this format and Vmax and Bmax values were normalized to the Vmax of NanoLuc. Relative specific activities (calculated as averages of Vmax and Bmax) are normalized to NanoLuc.

Sample	**Relative specific activity (*with wt 157-169 peptide)**
NanoLuc	1.00
wt 1-156	0.07*
11S	0.18*

Example 144

Effect of NLpoly and NLpep on Intracellular Half-life of FlucP

To determine if appending either NLpoly11S or NLpep114 to Luc2-PEST alters the intracellular half-live as measured by decay of signal after cycloheximide (CHX) treatment.

Day 1: Plate Hela cells in 6 well plates. Plate 3 ml of cells (200,000/ml) into two 6 well plates. Grow overnight. DMEM+10%FBS.

Constructs containing FlucP, wt 157-169 FlucP, NLpoly11S, NLpep114 FlucP and pBC22 (all pF4Ag D1-CMV) were transfected into HeLa cells. Briefly, 33ul of DNA (3.3ug) was added to 122ul of OptiMem, mixed and 9.9ul of FuGENE®HD added. The transfection mixtures were then incubated at RT for 20 minutes, and150ul added to cells. After an overnight incubation, cells were replated at 10,000 cells/well and incubated again overnight.

After incubation, the growth media was removed and replaced with either 0.4mM cycloheximide (CHX) or control (DMSO). At each time point, ONE-Glo™ assay reagent was added, incubated at RT for 3 minutes and luminescence measured on Tecan GENios Pro luminometer.

Figure 193 demonstrates that none of the NLpoly or NLpep components tested interfere with the normal intracellular degradation of a reporter enzyme (FlucP).

Example 145

Extracellular Protease Activity Assay

In some embodiments, the present invention provides an assay for extracellular protease (e.g. caspase) activity. A quenched peptide is provided (e.g., high affinity peptide such as NLpep86) that can only be accessed and refolded into an active luciferase with an NLpoly, e.g., NLpoly11S, upon removal of the quencher moiety by a protease (e.g. caspase)(Figure 194). NLpoly11S and furimazine are introduced to the assay as a reagent and then samples are measured for bioluminescence.

Example 146

Medially Attached Pro-Groups (isopeptides and glycosylated amino acids)

Assays are provided for measuring the activity of an enzyme through using a ProNLpep. This configuration of ProNLpep is a NLpep with one of the internal amino acids conjugated to a group that prevents the complementation of the peptide to an NLpoly. When this ProNLpep encounters an enzyme that removes the blocking group (e.g., caspase 1 in the case of WEHD or a glycosidase in the case of the serine glycoside), the ability of the NLpep to complement to an NLpoly is restored (Figure 195). In the presence of furimazine, this results in production of light in proportion to the activity of the enzyme of interest. Because each enzymatic cleavage results

in the formation of a luciferase, the sensitivity of this system for assaying small concentrations of enzyme is expected to be high.

Example 147

Linker Evaluation

Assays are provided measuring the release of cargo from an antibody. An NLpep is attached to an antibody, protein, peptide, or transporter recognition moiety in such a manner that prevents it from associating with a NLpoly to form a luciferase. Upon a stimulus, such as cellular internalization, the linker between the antibody, protein, peptide, or transporter recognition moiety and the NLpep is cleaved, due to intracellular reducing potential, and the NLpep is released (Figure 196). The NLpep can now complement with an NLpoly to form a luciferase, and the light generated will be proportional to the cleavage of the linker. This provides a system to measure the release of a compound from an antibody, which is a surrogate for cytotoxic drug delivery from Antibody Drug Conjugates. The linker can be cleaved through any manner known in the art, such as through intracellular proteases or pH sensitivity. Again, because a luciferase is generated through every cleavage, this is expected to be a sensitive method for assaying cleavage.

Example 148

The use of antibodies to target and destroy diseased cells has shown significant therapeutical promise and occurs through a process called Antibody-dependent cell-mediated cytotoxicity (ADCC). There are many ways to monitor ADCC activity, including crosslinking of different cells types or monitoring gene transcription using specific luciferase reporters expressed in the effector cells. A potential alternative readout to the ADCC mechanism of action could be in the monitoring of specific protein:protein interactions induced or disrupted after the binding of therapeutical antibodies to their target antigens or receptors presented on the cell surface. In some embodiments, the specific protein:protein interactions are monitored using the system of the present invention, which provides a readout in the time frame of minutes versus hours which is required by other methods.

Example 149

Immunoassays

Embodiments of the presnt invention find use in homogeneous immunoassays, for example, as depicted in Figure 201, where the NLpep and NLpoly are fused to binding moieties (e.g., A and B). The binding moieties A and B may comprise many different components, making up several different formats of immunoassays than can be utilized as target specific assays or more generalized reagents to be used in immunoassays. The binding moieties will only come into close proximity in the presence of the target, thus bringing the NLpep and NLpoly into

close proximity resulting in production of luminescence upon substrate addition. Table 7 lists exemplary of binding moieties (Mie et al. The Analyst. 2012 Mar 7;137(5):1085-9.;

Stains et al. ACS chemical biology. 2010 Oct 15;5(10):943-52.; Ueda et al. Journal of immunological methods. 2003 Aug;279(1-2):209-18. Ueda et al. Nature biotechnology. 1996 Dec;14(13):1714-8.; Lim et al. Analytical chemistry. 2007 Aug 15;79(16):6193-200.; Komiya et al. Analytical biochemistry. 2004 Apr 15;327(2):241-6.; Shirasu et al. Analytical sciences : the international journal of the Japan Society for Analytical Chemistry. 2009 Sep;25(9):1095-100.; herein incorporated by reference in their entireties).

Table 7.

(A) binding moiety	(B) Binding moiety	Example from literature	Reference
Domain of Protein A	Domain of Protein A	Rluc fragments fused to B-domain of protein A to detect E.coli with primary anti-coli Ab + fusion complexed rabbit anti-mouse IgG	Mie et al, Analyst, 2012
Protein A	Protein A		
Protein G	Protein G		
Domain of protein G	Domain of protein G		
Polyclonal Ab	Polyclonal Ab: either same or second pAB recognizing same target		
mAb	mAb to same target recognizing different epitope		
scFv	scFv from an antibody recognizing different epitope on same target	Omnitarg and Herceptin Fluc fusions for HER2 detection; b-gal fusions human serum albumin antibodies	Stains et al, ACS Chem Biol, 2010; Komiya et al, Analytical Biochemistry, 2004
Receptor domain 1	Receptor domain 2 to same target	Flt-1 domain 1 and 2 Fluc fusions for VEGF detection	Stains et al, ACS Chem Biol, 2010
Ab variable heavy chain	Ab variable light chain of same antibody	b-gal chain fusions for HEL ; alkaline phosphatase and thioredoxine fusions for benzaldehyde	Ueda et al, J of Immunological Methids, 2003; Shirasu et al, Analytical Sciences, 2009
Mix and match: pAb, mAb, scFv, receptor domain, Vh, Vl	Mix and match: pAb, mAb, scFv, receptor domain, Vh, Vl	CD4 receptor domain and scFv of anti-gp120 antibody Fluc fusions for HIV detection;	Stains et al, ACS Chem Biol, 2010

In some embodiments in which the binding moieties are comprised of protein A, protein G, or domains of protein A or G, the immunoassay system utilizes the NLpoly and NLpep fusions to complex with antibodies prior to addition with the sample containing the target. Antibodies bind non-covalently to protein A and G naturally. Introduction of a covalent coupling between the antibody and the fusions are introduced in the complex formation step. The NLpep/NLpoly-protein A/G/domain fusions binding moieties can be complexed to the antibodies in various formats, for example:

- individually with two different specific antibodies targeting two different proteins to determine if proteins exist in complex;
- together with a single target specific polyclonal antibody;
- together with secondary antibody (e.g., rabbit anti mouse IgG) to bind to sample preincubated with primary antibody (e.g., target specific mouse IgG); and
- individually with two antibodies targeting two different epitopes on the same target protein.

As described in Table 7, in some embodiments, the binding moieties are target specific antibodies, domains of target specific antibodies, receptor domains that bind target ligands, or a combination of antibodies, antibody domains, and target receptor domains.

In some embodiments, targets are monitored in samples which include but are not limited to blood, plasma, urine, serum, cell lysates, cells (primary or cell lines), cell culture supernatant, cerebral spinal fluid, bronchial alveolar lavage, tissue biopsy samples, chemical compounds, etc.

Methods describe analysis of targets which include but are not limited to: proteins, small molecules and compounds, haptens, peptides, hormones, heterodimeric protein-protein interactions, cell surface antigens, interactions between receptors and ligands, proteins in complex, viruses and viral components, bacteria, toxins, synthetic and natural drugs, steroids, catecholamines, eicosanoids, protein phosphorylation events, etc.

Applications include but are not limited to detection or quantitation of target for clinical disease monitoring, diagnostics, therapeutic drug monitoring, biological research, pharmaceuticals, compound detection and monitoring in the food/beverage/fragrance industry, viral clade identification, etc.

Additional applications include high throughput screening of molecules capable of disrupting the interactions of target with its receptor thus resulting in a loss of signal assay. There are several proposed formats for use of NLpep/NLpoly in immunoassays. In some embodiments, these are performed homogeneously and supplied as a kit, as separate diagnostic and research kit components, or as stand-alone reagents customizable to the individual's assay.

In other embodiments, homogeneous immunoassay utilizing NLpep/NLpolyutilize variations of the HitHunter or CEDIA technology (Yang et al. Analytical biochemistry. 2005 Jan 1;336(1):102-7.; Golla and Seethala. Journal of biomolecular screening. 2002 Dec;7(6):515-25.; herein incorporated by reference in their entireties). In such assays, components include: target specific antibody, NLpoly, NLpep-recombinant target fusion, and substrate. The NLpoly and NLpep-recombinant target fusion form a luminescent complex when the NLpep is not bound to the target specific antibody. Upon addition of the test sample to the assay components, the amount of luminescence is directly proportional to the target concentration in the test sample as the target present in the test sample will compete with the NLpep-recombinant target fusion on the antibody (e.g., gain of signal indicates the presence of the target).

Example 150

Exemplary configurations for NLpoly11S in spontaneous complementation

Various configurations of NLpoly11S may find use in spontaneous complementation assays or systems. Such configurations may include: deletions at the C-term (e.g., to reduce background luminescence), N- and/or C-terminal appendages (e.g., based on whether they are to be purified by His or HaloTag), etc. For example, the appendage left by HaloTag when it's a N-terminal tag is SDNIAI. Exemplary configurations include: SDNAIA-11S (HaloTag purification); SDN-11S; SDNAIA-11S, with single del at C-term; SDN-11S, with single del at C-term; SDNAIA-11S, with double del at C-term; SDN-11S, with double del at C-term; SDNAIA-11S, with triple del at C-term; SDN-11S, with triple del at C-term; 6His-AIA-11S; 6His-11S; 6His-AIA-11S with single del at C-term; 6His-11S with single del at C-term; 6His-AIA-11S with double del at C-term; 6His-11S with double del at C-term; 6His-AIA-11S with triple del at C-term; 6His-11S with triple del at C-term; 11S-6His; 11S-6His, minus C-term 11S residue; 11S-6His, minus last two C-term 11S residues; 11S-6His, minus last three C-term 11S residues; 11S-HT7, minus C-term 11S residue; 11S- HT7, minus last two C-term 11S residues; 11S- HT7, minus last three C-term 11S residues; 6His-HT7-AIA-11S; 6His-HT7 -11S; 6His-AIA-HT7 -11S (with single, double, triple 11S C-term dels); 6His -HT7 -11S (with single, double, triple 11S C-term dels); 11S-HT7-6His; 11S-HT7-6His (with single, double, triple 11S C-term dels); and Ternary 11S.

Example 151

Protein interactions for binary complementation studies

The binary complementation system described herein has been used to analyze a wide variety of protein interactions (See Table 8).

Table 8. Protein Interactions for Binary Complementation Studies

Interaction	Status
FRB/FKBP	Tested
V2R/ARRB2	Tested
V2R Homodimerization	Tested
BRD4/H3.3	Tested
L3MBTL3/BCLAF1	Tested
GR Homodimerization	Tested
RAS/RAF	Tested
p53/MDM2	Tested
EGFR/GRB2	Tested
BCL2/BIM/BAX	Tested
MYC/MAX	Tested
CUL1/NEDD8	In Progress
EZH2/SUZ12/EED	In Progress
GABAA Multimerization	In Progress

Example 152

Dissociation Constants and Bmax Values for NLpolys with 108 variants of NLpeps (array#2)

NLpeps were synthesized in array format by New England Peptide (peptides blocked at N-terminus by acetylation and at C-terminus by amidation; peptides in arrays were synthesized at ~2 mg scale) (Table 9). Each peptide was lyophilized in 2 separate plates. Each well from 1 of the plates of peptides was dissolved in 100uL nanopure water, and the A260 measured and used to calculate the concentration using the extinction coefficient of each peptide. The concentration was then adjusted based on the purity of the peptide, and nanopure water was added to give a final concentration of 800uM.

Table 9. Peptide array 2 sequences

	Sequence
array2.1	VTGYRLFKKIS
array2.2	VTGYRLFKKAS
array2.3	VTGYRLFKKES
array2.4	VTGYRLFKQIS
array2.5	VTGYRLFKQAS
array2.6	VTGYRLFKQES
array2.7	VTGYRLFKEIS
array2.8	VTGYRLFKEAS
array2.9	VTGYRLFKEES
array2.10	VTGYRLFQKIS

array2.11	VTGYRLFQKAS
array2.12	VTGYRLFQKES
array2.13	VTGYRLFQQIS
array2.14	VTGYRLFQQAS
array2.15	VTGYRLFQQES
array2.16	VTGYRLFQEIS
array2.17	VTGYRLFQEAS
array2.18	VTGYRLFQEES
array2.19	VTGYRLFEKIS
array2.20	VTGYRLFEKAS
array2.21	VTGYRLFEKES
array2.22	VTGYRLFEQIS
array2.23	VTGYRLFEQAS
array2.24	VTGYRLFEQES
array2.25	VTGYRLFEEIS
array2.26	VTGYRLFEEAS
array2.27	VTGYRLFEEES
array2.28	VTGYRLFKKIL
array2.29	VTGYRLFKKAL
array2.30	VTGYRLFKKEL
array2.31	VTGYRLFKQIL
array2.32	VTGYRLFKQAL
array2.33	VTGYRLFKQEL
array2.34	VTGYRLFKEIL
array2.35	VTGYRLFKEAL
array2.36	VTGYRLFKEEL
array2.37	VTGYRLFQKIL
array2.38	VTGYRLFQKAL
array2.39	VTGYRLFQKEL
array2.40	VTGYRLFQQIL
array2.41	VTGYRLFQQAL
array2.42	VTGYRLFQQEL
array2.43	VTGYRLFQEIL
array2.44	VTGYRLFQEAL
array2.45	VTGYRLFQEEL
array2.46	VTGYRLFEKIL
array2.47	VTGYRLFEKAL
array2.48	VTGYRLFEKEL
array2.49	VTGYRLFEQIL
array2.50	VTGYRLFEQAL
array2.51	VTGYRLFEQEL
array2.52	VTGYRLFEEIL
array2.53	VTGYRLFEEAL
array2.54	VTGYRLFEEEL
array2.55	VEGYRLFKKIS
array2.56	VEGYRLFKKAS
array2.57	VEGYRLFKKES

array2.58	VEGYRLFKQIS
array2.59	VEGYRLFKQAS
array2.60	VEGYRLFKQES
array2.61	VEGYRLFKEIS
array2.62	VEGYRLFKEAS
array2.63	VEGYRLFKEES
array2.64	VEGYRLFQKIS
array2.65	VEGYRLFQKAS
array2.66	VEGYRLFQKES
array2.67	VEGYRLFQQIS
array2.68	VEGYRLFQQAS
array2.69	VEGYRLFQQES
array2.70	VEGYRLFQEIS
array2.71	VEGYRLFQEAS
array2.72	VEGYRLFQEES
array2.73	VEGYRLFEKIS
array2.74	VEGYRLFEKAS
array2.75	VEGYRLFEKES
array2.76	VEGYRLFEQIS
array2.77	VEGYRLFEQAS
array2.78	VEGYRLFEQES
array2.79	VEGYRLFEEIS
array2.80	VEGYRLFEEAS
array2.81	VEGYRLFEEES
array2.82	VEGYRLFKKIL
array2.83	VEGYRLFKKAL
array2.84	VEGYRLFKKEL
array2.85	VEGYRLFKQIL
array2.86	VEGYRLFKQAL
array2.87	VEGYRLFKQEL
array2.88	VEGYRLFKEIL
array2.89	VEGYRLFKEAL
array2.90	VEGYRLFKEEL
array2.91	VEGYRLFQKIL
array2.92	VEGYRLFQKAL
array2.93	VEGYRLFQKEL
array2.94	VEGYRLFQQIL
array2.95	VEGYRLFQQAL
array2.96	VEGYRLFQQEL
array2.97	VEGYRLFQEIL
array2.98	VEGYRLFQEAL
array2.99	VEGYRLFQEEL
array2.100	VEGYRLFEKIL
array2.101	VEGYRLFEKAL
array2.102	VEGYRLFEKEL
array2.103	VEGYRLFEQIL
array2.104	VEGYRLFEQAL

array2.105	VEGYRLFEQEL
array2.106	VEGYRLFEEIL
array2.107	VEGYRLFEEAL
array2.108	VEGYRLFEEEL

Peptides were diluted to 400uM (4X) in PBS+0.1% Prionex and then diluted serially 7 times (8 concentrations total) in 0.5 log steps (3.162 fold dilution). NLpoly 11S was diluted 1:10^6 into PBS+0.1% Prionex. 25uL each NLpep + 25uL NLpoly 11S were mixed and incubated for 30min at RT. 50uL NanoGlo+100uM Fz was added and incubated for 30min at RT. Luminescence was measured on a GloMax Multi+ with 0.5sec integration. Kd/Bmax were determined using Graphpad Prism, One site-specific binding, best-fit values. Table 10 indicates the dissociation constant and Bmax values for NLpoly 11S and the indicated NLPep. The results indicate the affects of mutations on the binding to NLpoly 11S and the ability of the complex to produce luminescence.

Table 10

	Peptide Sequence	Bmax	Kd	Bmax	Kd
array2.1	VTGYRLFKKIS	134567	0.01334	4936	0.003695
array2.2	VTGYRLFKKAS	103904	0.2411	711.8	0.006084
array2.3	VTGYRLFKKES	55963	0.773	1705	0.06499
array2.4	VTGYRLFKQIS	104275	0.7462	4670	0.09318
array2.5	VTGYRLFKQAS	31031	1.953	436.4	0.05649
array2.6	VTGYRLFKQES	5006	1.348	182	0.1583
array2.7	VTGYRLFKEIS	32026	4.438	1173	0.5196
array2.8	VTGYRLFKEAS	3929	2.568	200.6	0.3566
array2.9	VTGYRLFKEES	1453	3.863	118.6	1.044
array2.10	VTGYRLFQKIS	112540	0.08118	4037	0.01352
array2.11	VTGYRLFQKAS	80943	0.7485	4035	0.1039
array2.12	VTGYRLFQKES	17237	0.4173	3190	0.3233
array2.13	VTGYRLFQQIS	19401	0.876	2357	0.47
array2.14	VTGYRLFQQAS	4351	1.111	311.1	0.3392
array2.15	VTGYRLFQQES	5197	7.486	198.7	0.797
array2.16	VTGYRLFQEIS	1321	2.561	112.6	0.5939
array2.17	VTGYRLFQEAS	ND	ND	ND	ND
array2.18	VTGYRLFQEES	5112	67.32	426.5	11.22
array2.19	VTGYRLFEKIS	122961	0.6047	11827	0.2689
array2.20	VTGYRLFEKAS	36284	1.794	935.8	0.09793
array2.21	VTGYRLFEKES	8622	1.491	599.7	0.3267
array2.22	VTGYRLFEQIS	121402	10.78	3711	1.121
array2.23	VTGYRLFEQAS	3824	4.174	243.4	0.8621
array2.24	VTGYRLFEQES	1829	7.832	24.45	0.2891
array2.25	VTGYRLFEEIS	ND	ND	ND	ND
array2.26	VTGYRLFEEAS	ND	ND	ND	ND
array2.27	VTGYRLFEEES	ND	ND	ND	ND
array2.28	VTGYRLFKKIL	140640	0.07664	6033	0.02
array2.29	VTGYRLFKKAL	98575	0.2755	1679	0.0168
array2.30	VTGYRLFKKEL	51143	0.6714	2000	0.07542
array2.31	VTGYRLFKQIL	115248	2.989	2995	0.3361
array2.32	VTGYRLFKQAL	34875	3.561	496	0.1247
array2.33	VTGYRLFKQEL	8548	1.953	581.1	0.5209
array2.34	VTGYRLFKEIL	21933	4.405	867.2	0.7072

array2.35	VTGYRLFKEAL	5547	5.153	180.1	0.6609
array2.36	VTGYRLFKEEL	1720	7.785	75.68	1.256
array2.37	VTGYRLFQKIL	127404	0.3625	7870	0.1108
array2.38	VTGYRLFQKAL	72788	0.9748	3853	0.1796
array2.39	VTGYRLFQKEL	33109	2.477	687.6	0.1414
array2.40	VTGYRLFQQIL	66256	122.3	13366	40.42
array2.41	VTGYRLFQQAL	3472	3.97	314	1.484
array2.42	VTGYRLFQQEL	14230	18.99	180.8	0.714
array2.43	VTGYRLFQEIL	9406	17.25	544.2	2.141
array2.44	VTGYRLFQEAL	4233	15.99	426.5	4.994
array2.45	VTGYRLFQEEL	14254	35.43	614.2	3.766
array2.46	VTGYRLFEKIL	219381	1.917	7349	0.2965
array2.47	VTGYRLFEKAL	34526	1.807	1377	0.216
array2.48	VTGYRLFEKEL	10865	2.437	823.9	0.5103
array2.49	VTGYRLFEQIL	99205	124.3	2780	5.68
array2.50	VTGYRLFEQAL	17117	40.4	294	1.642
array2.51	VTGYRLFEQEL	46162	85	1436	4.881
array2.52	VTGYRLFEEIL	15703	104.1	560	6.409
array2.53	VTGYRLFEEAL	ND	ND	ND	ND
array2.54	VTGYRLFEEEL	251166	68.27	15593	5.901
array2.55	VEGYRLFKKIS	42384	0.07805	3011	0.02593
array2.56	VEGYRLFKKAS	15920	0.6409	510.2	0.05975
array2.57	VEGYRLFKKES	3374	0.891	142.5	0.1335
array2.58	VEGYRLFKQIS	21512	2.091	665.9	0.244
array2.59	VEGYRLFKQAS	2300	2.088	74.01	0.1938
array2.60	VEGYRLFKQES	4346	10.64	91.51	0.7646
array2.61	VEGYRLFKEIS	5459	14.43	116.8	0.7024
array2.62	VEGYRLFKEAS	2375	22.05	112.3	2.964
array2.63	VEGYRLFKEES	17264	220.3	3074	54.34
array2.64	VEGYRLFQKIS	36517	0.5863	781.4	0.05853
array2.65	VEGYRLFQKAS	10620	1.929	271.7	0.1454
array2.66	VEGYRLFQKES	3489	2.87	132.3	0.3846
array2.67	VEGYRLFQQIS	5223	8.143	199.6	0.8457
array2.68	VEGYRLFQQAS	3753	20.01	117.8	1.833
array2.69	VEGYRLFQQES	ND	ND	ND	ND
array2.70	VEGYRLFQEIS	29161	230.2	560.4	6.062
array2.71	VEGYRLFQEAS	44893	24.03	1778	1.825
array2.72	VEGYRLFQEES	ND	ND	ND	ND
array2.73	VEGYRLFEKIS	22544	2.148	641.3	0.2291
array2.74	VEGYRLFEKAS	3808	4.138	122.2	0.3119
array2.75	VEGYRLFEKES	1170	2.969	136.7	1.282
array2.76	VEGYRLFEQIS	17957	52.79	724	4.614
array2.77	VEGYRLFEQAS	26862	48.29	436.5	1.752
array2.78	VEGYRLFEQES	39375	252.3	4842	41.61
array2.79	VEGYRLFEEIS	ND	ND	ND	ND
array2.80	VEGYRLFEEAS	383183	1419	572696	2258
array2.81	VEGYRLFEEES	ND	ND	ND	ND
array2.82	VEGYRLFKKIL	43371	0.563	2640	0.16
array2.83	VEGYRLFKKAL	20849	1.588	591.2	0.1396
array2.84	VEGYRLFKKEL	7828	1.413	ND	ND
array2.85	VEGYRLFKQIL	31425	10.34	358.7	0.2986
array2.86	VEGYRLFKQAL	2304	2.274	54.26	0.2428
array2.87	VEGYRLFKQEL	1790	12.59	113	2.614
array2.88	VEGYRLFKEIL	9831	17.75	551.8	3.002
array2.89	VEGYRLFKEAL	5574	42.42	435.1	7.715
array2.90	VEGYRLFKEEL	12241	100.9	458.5	6.589
array2.91	VEGYRLFQKIL	50503	2.077	2173	0.3373
array2.92	VEGYRLFQKAL	12294	2.023	430.5	0.206

array2.93	VEGYRLFQKEL	4090	1.691	278.5	0.4617
array2.94	VEGYRLFQQIL	2281	9.39	112	1.201
array2.95	VEGYRLFQQAL	38229	18.81	1578	1.617
array2.96	VEGYRLFQQEL	104621	99.4	6265	10.43
array2.97	VEGYRLFQEIL	ND	ND	ND	ND
array2.98	VEGYRLFQEAL	2696	99.9	238.8	15.53
array2.99	VEGYRLFQEEL	ND	ND	ND	ND
array2.100	VEGYRLFEKIL	34989	10.56	1747	1.801
array2.101	VEGYRLFEKAL	6372	12.62	186	0.8756
array2.102	VEGYRLFEKEL	961.5	5.786	67.06	1.216
array2.103	VEGYRLFEQIL	ND	ND	ND	ND
array2.104	VEGYRLFEQAL	9882	335.8	544.7	23.35
array2.105	VEGYRLFEQEL	ND	ND	ND	ND
array2.106	VEGYRLFEEIL	ND	ND	ND	ND
array2.107	VEGYRLFEEAL	ND	ND	ND	ND
array2.108	VEGYRLFEEEL	ND	ND	ND	ND

Example 153

Dark peptides and quencher peptides for reducing background signal from NLpoly11S

A purified sample of NLpoly11S was diluted into NanoGlo reagent to give a final concentration of 2 uM. Pep86 is a high affinity luminogenic peptide and was used to induce maximum signal for NLpoly11S. Pep86 was prepared at 1 nM in PBS (pH 7.2) for a working solution. Dark peptide and quencher peptides (Figure 180) were dissolved to 1 mM (or lower) in either PBS pH 7.2 or 150 mM NH_4HCO_3 and added in equal volume to the NanoGlo/NLpoly11S and then samples were read on a Tecan Infinite F500 reader using a 5 min time point.

Figure 202A shows that both GWALFKK and Dabcyl-GWALFKK reduce the background luminescence generated by NLpoly11S in the absence of any other luminogenic peptide. Figure 202B shows that Pep86 is able to induce luminescence even in the presence of GWALFKK and Dabcyl-GWALFKK.

Figure 203A shows that VTGWALFEEIL (Trp 11mer) and VTGYALFEEIL (Tyr 11mer) induce luminescence over background (NLpoly11S alone; no peptide control), but that the N-terminal Dabcyl versions of each provide significant quenching of this signal. Figure 203B shows that Pep86 is able to induce luminescence even in the presence of the Dabcyl versions of Trp 11mer and Tyr 11mer.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying

out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the present invention.

CLAIMS

1. A bioluminescent complex comprising:

(a) a first fusion polypeptide comprising:

(i) a polypeptide comprising an amino acid sequence having less than 100% and greater than 90% sequence identity with SEQ ID NO: 440; and

(ii) a first interaction polypeptide that is configured to form a complex with a second interaction polypeptide upon contact of the first interaction polypeptide and the second interaction polypeptide;

(b) a second fusion polypeptide comprising:

(i) a complement peptide comprising an amino acid sequence having less than 100% and greater than 70% sequence identity with SEQ ID NO: 2, wherein a detectable bioluminescent signal is produced in the presence of a substrate when the polypeptide contacts the complement peptide that is greater than the bioluminescent signal produced by the polypeptide and substrate in the absence of the complement peptide; and

(i) the second interaction polypeptide.