

CLAIMS

What is claimed herein is:

1. A single chain polypeptide comprising, from N-terminal to C-terminal:
 - a. an N-terminal fragment of a reporter protein;
 - b. a linker comprising a neurotoxin cleavage site; and
 - c. a C-terminal fragment of the reporter protein;wherein the N-terminal and C-terminal fragments collectively comprise a functional reporter protein sequence; and wherein the linker functionally joins the N-terminal fragment and C-terminal fragment to generate a functional reporter fusion protein.
2. The single chain polypeptide of claim 1, wherein the reporter protein is a luciferase protein.
3. A single chain polypeptide comprising:
 - a. a N-terminal domain comprising a sequence with at least 90% sequence identity to amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉);
 - b. a linker comprising a neurotoxin cleavage site located C-terminal to the N-nano₁₋₁₅₉; and
 - c. a C-terminal domain having a sequence with at least 90% sequence identity amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀) located C-terminal to the linker;wherein the linker functionally joins the N-nano₁₋₁₅₉ and the C-nano₁₆₀₋₁₇₀ to generate a functional luciferase fusion protein.
4. A single chain polypeptide comprising:
 - a. a N-terminal domain comprising a sequence having less than 10 amino acid residues substitutions, deletions, or additions relative to amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉);
 - b. a linker comprising a neurotoxin cleavage site located C-terminal to the N-nano₁₋₁₅₉; and

- c. a C-terminal domain having a sequence having less than 3 amino acid residues substitutions, deletions, or additions relative to amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀) located C-terminal to the linker; wherein the linker functionally joins the N-nano₁₋₁₅₉ and the C-nano₁₆₀₋₁₇₀ to generate a functional luciferase fusion protein.
5. The single chain polypeptide of any of claims 3-4, wherein the N-terminal domain has the sequence of amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉) and the C-terminal domain has the sequence of amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀).
6. The single chain polypeptide of any of claims 1-5, wherein the neurotoxin cleavage site is a *C. botulinum* neurotoxin (BoNT) and/or a Tetanus neurotoxin cleavage site.
7. The single chain polypeptide of any of claims 1-6, wherein the linker further comprises one or more spacers located between the BoNT cleavage site and the N-terminal fragment or domain, between the neurotoxin cleavage site and the C-terminal fragment or domain, or a combination thereof.
8. The single chain polypeptide of any of claims 1-7, wherein the linker further comprises a binding fragment of a receptor for the BoNT and/or a tetanus neurotoxin.
9. The single chain polypeptide of claim 8, wherein the binding fragment is located between the N-terminal fragment or domain and the BoNT cleavage site.
10. The single chain polypeptide of any of claims 8-9, wherein the linker further comprises a spacer located between the binding fragment and the BoNT cleavage site.
11. The single chain polypeptide of any of claims 1-10, wherein at least one spacer is comprised of glycine and serine.
12. The single chain polypeptide of any of claims 1-11, wherein at least one spacer is 5 to 15 amino acids.
13. The single chain polypeptide of any of claims 1-12, wherein at least one spacer is selected from SEQ ID NOs: 4-9.
14. The single chain polypeptide of any of claims 6-13, wherein the BoNT is A, E, C, B, D, F or G.

15. The single chain polypeptide of any of claims 6-14, wherein the BoNT cleavage site is from a SNARE protein.
16. The single chain polypeptide of claim 15, wherein the SNARE protein is SNAP-25, synaptobrevin (VAMP), or syntaxin.
17. The single chain polypeptide of any of claims 6-16, wherein the BoNT cleavage site is amino acids 141-206 of human SNAP-25b.
18. The single chain polypeptide of any of claims 6-16, wherein the BoNT cleavage site is amino acid 35-96 of human VAMP1.
19. The single chain polypeptide of any of claims 8-17, wherein the receptor is human SV2C.
20. The single chain polypeptide of claim 19, wherein the binding fragment is amino acids 529-566 of human SV2C, or a sequence having at least 90% identity with amino acids 529-566 of human SV2C, or a sequence with no more than 10 amino acid residues substitutions, deletions, or additions relative to amino acids 529-566 of human SV2C.
21. The single chain polypeptide of any one of claims 1-20, further comprising a polyhistidine affinity tag.
22. The single chain polypeptide of claim 21, wherein the polyhistidine affinity tag is located at the C-terminus of the polypeptide.
23. The single chain polypeptide of any of claims 1-22, wherein the linker further comprises an intact second luciferase polypeptide located between the N-terminal fragment or domain and the BoNT cleavage site.
24. The single chain polypeptide of claim 23, wherein the second luciferase polypeptide is firefly luciferase (e.g., *Photinus pyralis*), bacterial luciferase (e.g., *Vibrio fischeri*, *Vibrio harveyi*), sea pansy luciferase (*Renilla reniformis*), dinoflagellate luciferase, *Gaussia* luciferase, or copepod luciferase.
25. The single chain polypeptide of claim 23 or 24, wherein the linker further comprises a spacer located between the second luciferase polypeptide and the BoNT cleavage site.
26. The single chain polypeptide of claim 25, wherein the spacer is from 5 to 15 amino acids.
27. The single chain polypeptide of claim 25 or 26, wherein the spacer is GSSGGGGSGGGGSSG (SEQ ID NO: 4), GSSGGGGSGGGSSG (SEQ ID NO: 5), or GGGGS (SEQ ID NO: 6).
28. A single chain polypeptide comprising:

- a. amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉);
- b. a linker located C-terminal to the N-nano₁₋₁₅₉ comprising:
 - i. a first spacer of 5 to 15 amino acids;
 - ii. a binding fragment comprised of amino acids 529-566 of SV2C located C-terminal to the first spacer;
 - iii. a second spacer located C-terminal to the binding fragment;
 - iv. a BoNT cleavage site comprising amino acids 141-206 of human SNAP25b located C-terminal to the second spacer;
 - v. a third spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and
- c. amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀) located C-terminal to the linker;

wherein the linker functionally joins the N-nano₁₋₁₅₉ and the C-nano₁₆₀₋₁₇₀ to generate a functional luciferase fusion protein.

29. A single chain polypeptide comprising:

- a. amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉);
- b. a linker located C-terminal to the N-nano₁₋₁₅₉ comprising:
 - i. a first spacer from 5 to 15 amino acids;
 - ii. a BoNT cleavage site comprising amino acids 141-206 of human SNAP25b located C-terminal to the first spacer;
 - iii. a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and
- c. amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀) located C-terminal to the linker;

wherein the linker functionally joins the N-nano₁₋₁₅₉ and the C-nano₁₆₀₋₁₇₀ to generate a functional luciferase fusion protein.

30. A single chain polypeptide comprising:

- a. amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉);
- b. a linker located C-terminal to the N-nano₁₋₁₅₉ comprising:
 - i. a first spacer of 5 to 15 amino acids;

- ii. a BoNT cleavage site comprising amino acids 35-96 of human VAMP1 located C-terminal to the first spacer;
 - iii. a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and
 - c. amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀) located C-terminal to the linker;
- wherein the linker functionally joins the N-nano₁₋₁₅₉ and the C-nano₁₆₀₋₁₇₀ to generate a functional luciferase fusion protein.
31. A single chain polypeptide comprising:
- a. amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉);
 - b. a linker located C-terminal to the N-nano₁₋₁₅₉ comprising:
 - i. a second functional luciferase polypeptide;
 - ii. a first spacer of 5 to 15 amino acids located C-terminal of the second luciferase polypeptide;
 - iii. a BoNT cleavage site comprising amino acids 1-206 of human SNAP25b located C-terminal to the first spacer;
 - iv. a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and
 - c. amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀) located C-terminal to the linker;
- wherein the linker functionally joins the N-nano₁₋₁₅₉ and the C-nano₁₆₀₋₁₇₀ to generate a functional luciferase fusion protein.
32. The single chain polypeptide of any of claims 28-31, wherein one or more spacers are GSSGGGGSGGGGSSG (SEQ ID NO: 4), GSSGGGGSGGGGSSG (SEQ ID NO: 5), or GGGGS (SEQ ID NO: 6).
33. The single chain polypeptide of any of claims 28-32, further comprising a His₆ sequence (SEQ ID NO: 12) located at the C-terminus.
34. A nucleic acid comprising a nucleotide sequence that encodes the single chain polypeptide of any one of claims 1-33.
35. A nucleic acid vector comprising the nucleic acid of claim 34.

36. The nucleic acid vector of claim 35 that is an expression vector and comprises the nucleic acid sequence in expressible form.
37. The nucleic acid vector of claim 36 that is selected from the group consisting of a viral expression vector, a prokaryotic expression vector, a yeast expression vector, an insect expression vector, or a mammalian expression vector.
38. A cell comprising the nucleic acid of claim 34 or the nucleic acid vector of claims 35-37.
39. The cell of claim 38, that expresses the single chain polypeptide of any one of claims 1-33.
40. The cell of claim 39, that is a prokaryotic cell, a yeast cell, an insect cell, or an animal cell.
41. A method for determining the potency of a botulinum neurotoxin, comprising:
- a) contacting the neurotoxin to a single chain polypeptide of any of claims 1-33 under conditions appropriate for BoNT activity; and
 - b) determining the luciferase activity of the polypeptide, as compared to a reference, thereby determining the potency.
42. A method for detecting C. botulinum neurotoxin (BoNT) activity in a sample, comprising:
- a) contacting the sample to a single chain polypeptide of any of claims 1-33, under conditions appropriate for BoNT activity; and
 - b) determining luciferase activity of the polypeptide, as compared to luciferase activity of the polypeptide in the absence of the sample, wherein a decrease of luciferase activity indicates BoNT activity in the sample.
43. The method of any of claims 41-42 that is performed *in vitro*.
44. The method of any of claims 41-43, wherein contacting occurs in a buffer of 50mM HEPES, 20 μ M ZnCl₂, 2 mM DTT, 1 mg/ml BSA, pH 7.1.
45. The method of claims 41-44, wherein the concentration of the single chain polypeptide contacted to the sample is from about 30 nM to 300 nM.
46. The method of claim 45, wherein the concentration of single chain polypeptide contacted to the sample is about 30 nM.

47. The method of any of claims 41-46, wherein the luciferase activity is determined by addition of luciferase substrate to the single chain polypeptide and quantitative measurement of a luminescent signal produced.
48. The method of any of claims 41-47, wherein the conditions comprise incubation at about 37°C for a period of from about 1 hour to about 36 hours.
49. The method of any of claims 41-48, wherein the conditions comprise incubation at about 37°C for a period of from about 1 hour to about 24 hours.
50. The method of any of claims 41-49, wherein the conditions comprise incubation at about 37°C for a period of from about 4 hours to about 24 hours.
51. The method of any of claims 41-50, wherein the linker comprises a first spacer of 5 to 15 amino acids, a binding fragment of amino acids 529-566 of human SV2C located C-terminal to the first spacer and N-terminal to the BoNT cleavage site, and a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 141-206 of human SNAP25.
52. The method of any of claims 41-50, wherein the linker comprises a first spacer of 5 to 15 amino acids located N-terminal to the BoNT cleavage site, and a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 141-206 of human SNAP25.
53. The method of any of claims 41-50, wherein the linker comprises a first spacer of 5 to 15 amino acids located N-terminal to the BoNT cleavage site, a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 35-96 of human VAMP1.
54. The method of any of claims 41-53, wherein the single chain polypeptide is expressed by a neuronal cell and the method further comprises, after the contacting step:
incubating the neuronal cells for a period of from about 12 hours to about 60 hours and harvesting lysate from the neuronal cells.
55. A method for detecting *C. botulinum* neurotoxin (BoNT) activity in a sample, comprising:
a) contacting the sample to neuronal cells expressing a single chain polypeptide comprising:

amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano₁₋₁₅₉), amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano₁₆₀₋₁₇₀), separated and functionally joined by a linker comprising a BoNT cleavage site located C-terminal to the N-nano₁₋₁₅₉ and N-terminal to the C-nano₁₆₀₋₁₇₀;

- b) incubating the neuronal cells for a period of from about 12 hours to about 60 hours;
 - c) harvesting lysate from the neuronal cells;
 - d) measuring luciferase activity in the lysate, as compared to luciferase activity in identically treated neuronal cells in the absence of the sample, wherein a decrease in the luciferase activity indicates BoNT activity in the sample.
56. The method of any of claims 41-55, wherein the linker further comprises an intact second luciferase polypeptide located between the N-nano₁₋₁₅₉ and the cleavage site.
57. The method of claim 56, wherein the second luciferase polypeptide firefly luciferase (*photinus pyralis*), bacterial luciferase (*vibrio fischiri*, *vibrio harveyi*), sea pansy luciferase (*renilla reniformis*), dinoflagellate luciferase, *gaussia* luciferase, or copepod luciferase.
58. The method of any of claims 56-57, wherein the luciferase activity of the second luciferase polypeptide in the sample is determined and used as an indicator of total single chain polypeptide present in the harvested lysate.
59. The method of any of claims 54-58, wherein the neuronal cells express the single chain polypeptide from a viral expression vector.
60. The method of claim 59, wherein the single chain polypeptide is expressed for 6 days prior to step a).
61. The method of claim 59, wherein the viral expression system is a lentivirus expression system.
62. The method of any of claims 54-61, wherein the incubating step is about 48 hours.
63. The method of any of claims 54-62, wherein the harvesting step is by addition of a lysis buffer.
64. The method of any one of claims 41-63, wherein the BoNT cleavage site is from SNAP-25, synaptobrevin (VAMP), or syntaxin.
65. The method of any one of claims 41-64, wherein the BoNT cleavage site is specifically recognized by BoNT A, E and C, or is specifically recognized by BoNT B, D, F and G.

66. The method of any one of claims 41-65, wherein a combination of single chain polypeptides having different BoNT cleavage sites are used.
67. The method of claims 41-66, wherein the BoNT cleavage site is a.a.141-206 of human SNAP-25, or a.a. 35-96 of human VAMP1, a.a. 1-206 of human SNAP35, or a.a. 1-96 of VAMP1.
68. The method of any one of claims 41-67, wherein the linker further comprises a binding fragment of a BoNT receptor.
69. The method of claim 68, wherein the receptor is SV2C.
70. The method of any of claims 68-69, wherein the binding fragment comprises amino acids 529-566 of human SV2C.
71. The method of any of claims 41-70, wherein the linker comprises a second luciferase polypeptide, a first spacer of 5 to 15 amino acids located C-terminal of the second luciferase polypeptide and N-terminal of the BoNT cleavage site, a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acid 1-206 of human SNAP25 and is located C-terminal to the first spacer.
72. The method of any of claims 41-71, wherein measuring luciferase activity is by addition of a substrate specific for the luciferase and quantitative detection of the resulting luminescent signal.
73. The method of claim 72, wherein the substrate for the luciferase of SEQ ID NO: 1 is furimazine (2-furanylmethyl-deoxy-coelenterazine).
74. A kit comprising:
- one or more single chain polypeptides described in claims 1-33, with each or a combination of the single chain polypeptides packaged into a separate container;
 - one or more nucleic acids or nucleic acid vectors described in claims 34 -37, with each or a combination of the nucleic acids or nucleic acid vectors packaged into a separate container; and/or
 - one or more cells described in claims 38-40 with each or a combination of the cells packaged into a separate container.
75. A kit according to claim 74, further comprising a luciferase substrate packaged into a separate container.