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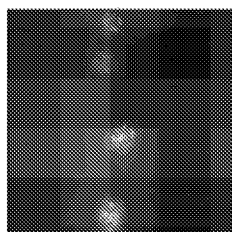
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(54) Title: PROGRAMMABLE IMAGING METHODS AND COMPOSITIONS

Fig. 1A



(57) Abstract: The invention encompasses methods and compounds for genetically targeted visualization using programmable nucleic acids to target expressed proteins in cells, tissues, and organisms.



5 PROGRAMMABLE IMAGING METHODS AND COMPOSITIONS

RELATED APPLICATIONS

 This application claims benefit under 35 U.S.C. § 119(e) of U.S. provisional
application serial number 62/824,521, filed March 27, 2019, the disclosure of which is
10 incorporated by reference herein in its entirety.

GOVERNMENT INTEREST

 This invention was made with government support under NIH 2388357, NIH
2732707, NIH 3843505, and 5R01-DA029639-07 each awarded by the National Institutes
15 of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

 The invention, in some aspects, relates to method and products for genetically
targeted detection of proteins in cells using programmable nucleic acids to target
20 expressed proteins in cells, tissues, and organisms.

BACKGROUND OF THE INVENTION

 Visualization of proteins in biological systems currently relies predominantly on
the use of antibodies or fluorescent proteins. With such methods, only 4 or 5 fluorescent
25 proteins can be used for visualization in a sample because of issues of spectral mixing.
Although it may be possible to visualize higher numbers of antibodies in a single sample if
the sample is subjected to multiple rounds of antibody staining, the process is laborious
and requires the development of different high-quality antibodies for each target. Each
approach is limited in its efficiency and usefulness for visualization.

30

SUMMARY OF THE INVENTION

 According to an aspect of the invention, methods of genetically targeted detection,
are provided, the methods including: delivering to at least one host cell one or more
preselected binding agents comprising an independently selected programmable binding
35 sequence, wherein each preselected binding agent is capable of selectively binding a
corresponding matched specific target agent; fixing the at least one host cell under
conditions wherein the one or more preselected binding agents in the fixed host cell is
capable of selectively binding the matched specific target agent to which it corresponds;

5 contacting the at least one fixed host cell with a composition comprising the matched specific target agents corresponding to the one or more preselected binding agents; and determining the binding of the one or more preselected binding agents and corresponding matched specific target agents in the at least one fixed host cell. In some embodiments, the preselected binding agent comprises one or more of a polypeptide molecule and a
10 nucleic acid molecule. In certain embodiments, the preselected binding agent comprises a polypeptide/nucleic acid complex. In some embodiments, each of the independently selected programmable binding sequences correspond to a nucleic acid sequence in a matched specific target agent. In some embodiments, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different independently selected programmable binding sequences are delivered into the
15 host cell. In certain embodiments, each of the programmable binding sequence corresponds to and is capable of selectively binding a matched specific target agent. In some embodiments, the preselected binding agent comprises at least one of: a nucleic acid-binding molecule and a nucleic acid analog. In some embodiments, the nucleic acid-binding molecule is one or more of: a nucleic acid-binding polypeptide, a DNA-binding
20 molecule, and a DNA-binding polypeptide. In some embodiments, the preselected binding agent comprises a transcription activator-like effector (TALE) sequence. In certain embodiments, the preselected binding agent comprises a megaTAL effector sequence. In some embodiments, the preselected binding agent comprises a CRISPR/Cas9 sequence or Cas9 analog sequence effector sequence. In some embodiments, the Cas9 analog sequence
25 comprises a Cas13a (c2c2), Cpf1, or MIMIVIRE sequence. In certain embodiments, the preselected binding agent comprises a zinc finger (ZF) effector sequence. In some embodiments, the specific target agent comprises at least one double-stranded nucleic acid molecule. In some embodiments, the specific target agent comprises at least one single-stranded nucleic acid molecule. In certain embodiments, the specific target agent
30 comprises one or more independently selected detectable labels. In some embodiments, the preselected programmable binding agent comprises a detectable label. In some embodiments, the detectable label comprises a fluorescent label. In some embodiments, fixing the at least one host cell comprises contacting the host cell with at least one cell fixative. In certain embodiments, the cell fixative comprises at least one of: MeOH, EtOH, acetone, an amine, a cleavable molecule, a cleavable polypeptide, a cleavable cross-linker
35 molecule, and an aldehyde. In some embodiments, fixing comprises contacting the at least one host cell with an alcohol solution. In some embodiments, the alcohol solution comprises at least one of MeOH and EtOH. In certain embodiments, the at least one host

5 cell is a plurality of host cells comprising one or more cell types. In some embodiments, a means for determining a characteristic of the one or more preselected binding agents in the fixed host cell, comprises detecting the one or more specific target agents bound to the matched preselected binding agent in the contacted fixed host cell. In some embodiments, the characteristic is one or more of: localization, presence, absence, and amount. In some
10 embodiments, the at least one host cell is obtained from a subject. In certain embodiments, the at least one host cell is obtained from culture. In some embodiments, the at least one host cell comprises a neuron. In some embodiments, the one or more preselected binding agent is a plurality of independently preselected binding agents. In some embodiments, the preselected binding agent is delivered to the at least one host cell using a physical
15 delivery means. In certain embodiments, the physical delivery means comprises at least one of microinjection, electroporation, ballistic delivery, and laser delivery. In some embodiments, the preselected binding agent is delivered to the at least one host cell by means of a vector. In certain embodiments, the vector is a viral vector or a non-viral vector. In some embodiments, the viral vector is a lentivirus vector (LV), an adenovirus
20 vectors (AdV), an adeno-associated virus vectors (AAV), or a herpes simplex-1 virus vectors (HSV-1s). In some embodiments, the non-viral vector is a lipid nanoparticle (LNP), a liposome, a polymer, or a cell-derived membrane vesicle (CMV). In certain embodiments, the preselected binding agent is delivered to the at least one host cell by a means comprising a cell-penetrating molecule. In some embodiments, the cell-penetrating
25 molecule comprises a cell-penetrating polypeptide. In some embodiments, the host cell is sectioned after the fixing. In certain embodiments, determining the binding of the one or more preselected programmable binding agents and corresponding matched specific target agents comprises: contacting the corresponding matched specific target agents with two or more independently selected detectable labels; imaging the detectable labels; quenching
30 the detectable labels; and repeating steps (i)-(iii) one or more times. In some embodiments, determining the binding of the one or more preselected binding agents and corresponding matched specific target agents comprises simultaneously contacting one or more of the corresponding matched specific target agents with two or more independently selected detectable labels and sequentially detecting the detectable labels. In some
35 embodiments, the detectable labels are detected with a means comprising a fluorescent in situ hybridization (FISH) method. In certain embodiments, the cell is a vertebrate cell, optionally a mammalian cell, optionally a human cell.

5 According to another aspect of the invention, compositions are provided, the compositions including a fixed cell or cell section that includes at least one preselected programmable binding agent bound to its matched specific target agent, forming a bound complex, wherein the preselected binding agent comprises an independently selected programmable binding sequence, and wherein the preselected binding agent is selected, in
10 part, based on its capability to selectively bind its matched specific target agent. In some embodiments, the cell or cell section comprises two or more different independently preselected programmable binding agents bound to their matched specific target agents each forming a bound complex. In certain embodiments, the bound complex comprises a detectable label. In some embodiments, the preselected binding agent comprises one or
15 more of a polypeptide molecule and a nucleic acid molecule. In some embodiments, the preselected binding agent comprises a polypeptide/nucleic acid complex. In some embodiments, each of the independently selected programmable binding sequences correspond to a nucleic acid sequence in a matched specific target agent. In certain embodiments, the preselected binding agent includes at least one of: a nucleic acid-binding
20 molecule and a nucleic acid analog. In some embodiments, the nucleic acid-binding molecule is one or more of: a nucleic acid-binding polypeptide, a DNA-binding molecule, and a DNA-binding polypeptide. In some embodiments, the preselected binding agent comprises a transcription activator-like effector (TALE) sequence. In certain
25 embodiments, the preselected binding agent comprises a megaTAL effector sequence. In some embodiments, the preselected binding agent comprises a CRISPR/Cas9 sequence or Cas9 analog sequence effector sequence. In some embodiments, the Cas9 analog sequence comprises a Cas13a (c2c2), Cpf1, or MIMIVIRE sequence. In some embodiments, the preselected binding agent comprises a zinc finger (ZF) effector sequence. In certain
30 embodiments, the specific target agent comprises at least one double-stranded nucleic acid molecule. In some embodiments, the specific target agent comprises at least one single-stranded nucleic acid molecule. In certain embodiments, the detectable label comprises a fluorescent label. In some embodiments, the fixed cell is a neuron. In some embodiments, the fixed cell section is a section of a neuron. In some embodiments, the fixed cell is a vertebrate cell, optionally is a mammalian cell, and optionally is a human cell. In certain
35 embodiments, the fixed cell section is a section of a vertebrate cell, optionally a section of a mammalian cell, and optionally is a section of a human cell. In some embodiments, the preselected binding agent is part of a fusion protein. In some embodiments, the at least a

5 portion of the fixed cell includes all of the fixed cell. In certain embodiments, the at least a portion of the fixed cell comprises a section obtained from the fixed cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-H shows photomicrographic images of cells visualized with an embodiment of a method of the invention. Fig. 1A & B show HEK cells expressing PL61 that were fixed with the ethanol protocol described in Example 1 and stained with Hairpin 13. Fig. 1A shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 1B shows detected fluorescence of Cy5 (attached to hairpin 13), which indicates co-localization of the mRuby and Cy5 signals. Fig. 1C & D show HEK cells expressing PL62 that were fixed as above and were stained with Hairpin 13. Fig. 1C shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 1D shows detected fluorescence of Cy5 (attached to hairpin 13), which indicates there was no co-localization of the mRuby and Cy5 signals. Fig. 1E & F show HEK cells expressing PL62 that were fixed as above and were stained with Hairpin 14. Fig. 1E shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 1F shows detected fluorescence of Cy5 (attached to hairpin 14), which indicates there was no co-localization of the mRuby and Cy5 signals. Fig. 1G & H show HEK cells expressing PL62 that were fixed as above and stained with Hairpin 14. Fig. 1G shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 1H shows detected fluorescence of Cy5 (attached to hairpin 14), which indicates strong co-localization of the mRuby and Cy5 signals.

Figure 2A-H shows photomicrographic images of cells visualized with an embodiment of a method of the invention. Fig. 2A & B show HEK cells expressing PL61 that were fixed with the methanol protocol described in Example 1 and stained with Hairpin 13. Fig. 2A shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 2B shows detected fluorescence of Cy5 (attached to hairpin 13), which indicates co-localization of the mRuby and Cy5 signals. Fig. 2C & D show HEK cells expressing PL62 that were fixed as above and were stained with Hairpin 13. Fig. 2C shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 2D shows detected fluorescence of Cy5 (attached to hairpin 13), which indicates there was no co-localization of the mRuby and Cy5 signals. Fig. 2E & F show HEK cells expressing PL62 that were fixed as above and were stained with Hairpin 14. Fig. 2E shows detected

5 fluorescence of mRuby, which indicates the location of the TALEs. Fig. 2F shows detected fluorescence of Cy5 (attached to hairpin 14), which indicates there was no co-localization of the mRuby and Cy5 signals. Fig. 2G & H shows HEK cells expressing PL62 that were fixed as above and stained with Hairpin 14. Fig. 2G shows detected fluorescence of mRuby, which indicates the location of the TALEs. Fig. 2H shows
 10 detected fluorescence of Cy5 (attached to hairpin 14), which indicates strong co-localization of the mRuby and Cy5 signals.

Figure 3A-H provides photomicrographic images showing HEK cells transfected with ZF 140-CODA-3-GFP fusion protein constructs, fixed with either 95% MeOH + 5% Acetic
 15 acid (Fig. 3A-D) or 4% paraformaldehyde (Fig. 3E-H), then blocked in zinc binding buffer and stained with either cognate (Fig. 3A, 3C, 3E, and 3G) or non-cognate (Fig. 2B, 3D, 3F, and 3H) fluorescent DNA hairpins. Staining detected in Fig. 3A, 3B, 3E, and 3F is (green) indicated native GFP signal. Staining detected in Fig. 3C, 3D, 3G, and 3H is (blue or magenta) indicated fluorescent DNA hairpin signal.

20 Figure 4A-B shows a photomicrographic image of the hippocampus of mouse brain injected intracranially with an AAV PHP.eB coding for a zinc finger protein. Two weeks post-injection, the mouse was perfused with ice-cold 95% methanol, 5% acetic acid and the brain sliced into 50µm coronal sections on a vibratome. Following blocking in zinc
 25 binding buffer, sections were stained with their fluorescent cognate oligonucleotide hairpins (Fig. 4A) and DAPI (Fig. 4B).

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 is a target DNA sequence of TALE(13): tgctacgactaggtatacct.

30 SEQ ID NO: 2 is a target DNA sequence of TALE(14) tcggacggcccttcgccatc.

SEQ ID NO: 3 is DNA sequence referred to herein as Hairpin 13:
 /5Cy5/agatatgctacgactaggtatacctccgttaaaaaaacggaggtatacctagtcgtagcatatct.

35 SEQ ID NO: 4 is DNA sequence referred to herein as Hairpin 14:
 /5Cy5/agatatcggaaggcccttcgccatcccggttaaaaaaacgggatggcgaaggccgtccgatatct.

5 SEQ ID NO: 5 is DNA sequence referred to herein as SRC-60B:

atgggaaaacctattcctaactctctgctgggcctggattctaccggaggcatggccctggctctgaagctggctggcctggacct
ggtggacggcgagtggaactgagaactgggatattctcagcagcagcaggagaagatcaagcccaagtgagatccaca
gtggcccagcaccacgaagccctggtgggacacggatttacacacgccacattgtggccctgtctcagcacctgccgcctg
ggaacagtggcctgaaatcagggatgattgccgcctgctgaggccacacgaagccattgtgggagtgaggaaaaca
10 gtggtctggagccagagccctggaagccctgctgacagtggccggagaactgagaggacctcctctgcagctggatacaggac
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ggaaacagtgcagcggctgctgtgctgtgtcaggctcacggcttactccagaacaggtggtggctattgcttcaacaac
15 ggggggaaacaggccctggaaactgtgcagcgctgctgcccagtgtgtccaggctcacggactgacccccgaacaggtgg
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30 gcagcgctgctgcccagtgtgtgccaggctcacggctgacccccgaacaggtggtggccattgccagcaacaacggcggc
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cgctctaataatcgaggaaaacaggctctggaacagtgcagcggctgctgctgtgtgtcaggctcacggcttactcca
cagcaggctgtggcaattgctagccacgacggcgacggcccgccctggagagcattgtggcccagctgtctagacctgatcct
gcccctggcgccctgacaaatgatcacctggtggccctggcctgtctgggaggcagacctgccttgatgccgtgaaaaaagg
35 actgctcacgcccctgcccgatcaagagaacaaatagaagaatccccgagcggacctctcacagatggccgatcacgccc
aggtggtgagagtgtgggattttcagtgctactctcacctgccaggcccttgatgatccatgacacagttggcatgagcag
acacggactgctgcagctgttagaagagtgggagtgacagaactggaggccagatccggaacctgcctcctgcctctcagag

5 atgggataggattctgcagggttcccggttaacaagctgtcgacgggtaccgaattcagcttatcgataatcaacctctggattaca
aaatttgtgaaagattga.

SEQ ID NO: 6 comprises DNA sequence that binds SRC-60B (SEQ ID NO: 5):

/5Atto488N/aggaattcatactgtctcgacgtaactgacccgttaaaaaaacgggtcagttacgtcgagcagatatgaattcc
10 t.

SEQ ID NO: 7 is DNA sequence referred to herein as SRC-140-4:

atggtgagcaaggcgaggagctgtcaccggggtggtgccatcctggtcgagctggacggcgacgtaaaccggccacaagtt
cagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccggcaagctgcc
15 gtgccctggcccaccctcgtgaccaccttcggctacggcctgcagtgcttcgcccgtaccccgaccacatgaagcagcacgac
ttcttcaagtccgccatgcccgaaggctacgtccaggagcgaccatcttctcaaggacgacggcaactacaagaccgcgcc
gaggtgaagttcgaggcgacacctggtgaaccgcatcgagctgaagggtcagcttcaaggaggacggcaacatcctgg
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20 tgctgctgcccgacaaccactacgtgagctaccagtcggccctgagcaaaagacccaacgagaagcgcgatcacatggtcctgc
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gaggtacgggaggtagcgggggcacaatgggttctagaccggggagcggcccttcagtgctgcatttgcagtcggaactttt
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gacgagtccaacctgcccggcatctacgtacgcacaccggcgagaagccattccaatgccgaatatgcatgcgcaacttcagt
25 cgggttgacaacctgccccggcacctaaaaaccacctgagggggtcccaactagtcaaaagtgaactgccgcggctgcaggt
cgacacgcgtgaacaaaaactcatctcagaagaggatctgaatctggctctgaagctggctggcctggacctgtagaagctttaa
atgatatcagcttatcgataa.

SEQ ID NO: 8 is DNA sequence referred to herein as SRC-140-5:

atggtgagcaaggcgaggagctgtcaccggggtggtgccatcctggtcgagctggacggcgacgtaaaccggccacaagtt
30 cagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccggcaagctgcc
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ttcttcaagtccgccatgcccgaaggctacgtccaggagcgaccatcttctcaaggacgacggcaactacaagaccgcgcc
gaggtgaagttcgaggcgacacctggtgaaccgcatcgagctgaagggtcagcttcaaggaggacggcaacatcctgg
35 ggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttca
agatccgccacaacatcgaggacggcagcgtgcagctcgcgaccactaccagcagaacacccccatcggcgacggccccg
tgctgctgcccgacaaccactacgtgagctaccagtcggccctgagcaaaagacccaacgagaagcgcgatcacatggtcctgc
tggagttcgtgaccgccgcccggatcactctcggcatggacgagctgtacaagtccggactcagatctcgagctggggggtcag

5 gaggtacgggaggttagcgggggcacaatgggtttagaccggggagcgcccttcagtgctgcatttgcagcgaactttt
cgaccaaccagaagctggaggtgcataccgtactcataccggtgaaaaaccgtttcagtgctggatctgtatgcgaaatttctcc
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cagcaccccaacctgaccggcacctaaaaaccacctgaggggatcccaactagtcaaaagtgaactgccgaggctgcaggt
cgacacgcgtgaacaaaaactcatctcagaagaggatctgaatctggctctgaagctggctggcctggacctgtag.

10

SEQ ID NO: 9 is DNA sequence referred to herein as SRC-140-CODA-3:

atggtgagcaagggcgaggagctgttcaccgggggtggtgcccactctggtcgagctggacggcgacgtaaacggccacaagtt
cagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccggcaagctgccc
gtgccctggcccaccctcgtgaccaccttcgggtacggcctgcagtgcttcgccgctaccccgaccacatgaagcagcacgac
15 ttcttaagtcgccatgccgaaggctacgtccaggagcgcaccatcttcttaaggacgacggcaactacaagaccgcgcc
gaggtgaagttcgagggcgacacctgggtgaaccgcatcgagctgaaggcgatcgacttcaaggaggacggcaacatcctgg
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tgctgctgcccgacaaccactacctgagctaccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgc
20 tggagttcgtgaccgccgcccggatcactctcgcatggacgagctgtacaagtccggactcagatctcgagctggggggtcag
gaggtacgggaggttagcgggggcacaatgggtttagaccgggtgaacggccctttcaatgtcgaatttgcagagaacttctc
ccagcgcagtaacctggctcgccacacaagaactcacacaggtgagaagccttttcagtgctgcatttgcgaaactttccga
ccggggtaaccttacacggcatctgcggaccataccggagagaagccatttcagtgagaatatgtatgcgaaatttttagccag
agtaacaatttgaaccgacacctcaaaacccatctccgaggtcacaaactcgtaaagtcaggctccccgacttcaggtcgatac
25 gcgtgaacaaaaactcatctcagaagaggatctgaatctggctctgaagctggctggcctggacctgtag.

SEQ ID NO: 10 is DNA sequence: acatcGAGGACGGCagcgt.

SEQ ID NO: 11 is DNA sequence:

30 acgctGCCGTCCTCgatgTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/.

SEQ ID NO: 12 is DNA sequence: ttgaaGAAGATGGTgcgct.

SEQ ID NO: 13 is DNA sequence:

35 agcgcACCATCTTctcaaTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/.

SEQ ID NO: 14 is DNA sequence:

gccgaGAAGACGAAtacatTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/.

5

SEQ ID NO: 15 DNA sequence: atgtaTTCGTCTTCtcggc.

SEQ ID NO: 16 is the DNA sequence for the complete plasmid encoding 140-CODA-3:

cttccgcttcctcgctcactgactcgtcgctcggtcgttcggctcggcgagcgggtatcagctcactcaaaggcggtaatacgg
10 ttatccacagaatcaggggataaacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccg
cgttgctggcggttttccataggctccgccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgac
aggactataaagataaccaggcggtttccccctggaagctccctcgtcgctctcctgttcggacctgcccgttaccggatacctgtc
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gatcttcacctagatccttttaataaaaaatgaagtttaaatcaatctaaagtatatatagtaaacttggctgacagttaccaatgct
20 taatcagtgaggcacctatctcagcgatctgtctatttctgctcatccatagttgcctgactccccgtcgtgtagataactacgatacgg
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35 gtggcgagaaaggaagggaagaaagcgaaggagcggcgctaggcgctggcaagtgtagcggtcacgctgcgcgtaac
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5 gattaacccgccatgctacttatctacgtagccatgctctaggaagatcgtaggtaattaaggctccggtgccggtcagtgggcag
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10 cgaggccttgcccttaaggagcccccttcgcctcgtgcttgagttgaggcctggcctggcgctggggccgcccgtgcgaatct
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25 atcgagctgaaggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgt
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35 gaaagattgactggtattcttaactatgtgtccttttacgctatgttgatacgtgctttaatgcctttgtatcatgctattgttcccgt
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tattgccacggcggaactcatcgccgctgccttggccgctgctggacaggggctcggctgttgggcactgacaattccgtggtg

5 ttgtcggggaaatcatcgtccttcttggctgctcgctgtgttggccacctggattctgcgcgggacgtccttctgctacgtcccttc
 ggcctcaatccagcggaccttcttcccggcctgctgccggctctgcggccttccgcgtcttcgccttcgccctcagacga
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 attgcatcgcattgtctgagtaggtgtcattctattctggggggtgggggtggggcaggacagcaagggggaggattgggaagac
 10 aatagcaggcatgctggggatgcgggccgactgtacaattgcattcatttatgtttcaggttcagggggagatgtgggaggttttt
 aaagcaagtaaaacctctacaaatgtggtaaaatcgataaggatcttctagagcatggctacgtagataagtagcatggcgggtt
 aatcattaactacaaggaacccctagtgtgaggtggccactccctctctgcgcgctcgctcgctcactgaggccggggcgacca
 aaggctgcccgcacggggcttggccggggcgcctcagtgcgagcgcgcgcagctgcattaatgaatcgccaacg
 cgcggggagaggcggttgcgtattgggcgct.

15

SEQ ID NO: 17 is DNA sequence set forth as SEQ ID NO: 11, without the 3AlexF546N
 label: acgctGCCGTCCTCgatGTATAAGCGGGAGATTCGTCCTCATA.

20 SEQ ID NO: 18 is DNA sequence set forth as SEQ ID NO: 11, without the 3AlexF546N
 label: agcgcACCATCTTCttcaaTATAAGCGGGAGATTCGTCCTCATA.

SEQ ID NO: 19 is DNA sequence set forth as SEQ ID NO: 14, without the 3AlexF546N
 label: gccgaGAAGACGAAtacatTATAAGCGGGAGATTCGTCCTCATA.

25 SEQ ID NO: 20 is DNA sequence set forth as SEQ ID NO: 3 without the 5Cy5 label:
 agatatgctacgactaggtatacctccgttaaaaaaacggaggtatacctagtcgtagcatatct.

SEQ ID NO: 21 is DNA sequence set forth as SEQ ID NO: 4 without the 5Cy5 label:
 agatatcggacggcccttcgccatcccgttaaaaaaacgggatggcgaagggccgtccgatatct.

30

SEQ ID NO: 22 is DNA sequence set forth as SEQ ID NO: 6 without the 5Atto488N
 label: aggaattcatatctgctcgacgtaactgacccgttaaaaaaacgggtcagttacgtcgagcagatatgaattcct.

DETAILED DESCRIPTION

35 The invention, in part, relates to multiplex methods to determine characteristics of
 one or a plurality of different polypeptides in a single preparation. Certain aspects of the
 invention include genetic targeting to permit detection of polypeptides by utilizing
 programmability of nucleic acids for use in methods to detect the presence, absence,

5 and/or location of preselected binding agents in fixed biological cells and tissues. Methods of the invention comprise preselecting binding agents that each selectively binds its own matched specific target agent, delivering the preselected binding agents into host cells, fixing the host cells under conditions in which the preselected binding agents retain capability to selectively bind their specific target agents in the fixed host cells, contacting
10 the fixed host cells with the specific matched target agents, and detecting target agents bound to their matched binding agent in the fixed host cells using suitable detection methods.

One advantage of methods of the invention compared to prior visualization methods is that methods of the invention are based, in part, on the ability to include
15 different detectable labels on specific target agents that are each able to selectively bind a matched preselected binding agent when it is present in a fixed host cell. This permits the use and detection of different detectable labels in a preparation of single host cell or plurality of host cells, thereby permitting detectable labels to be used to determine one or more characteristics of preselected binding agents in host cells.

20 Certain aspects of the invention comprise contacting a fixed host cell with a matched specific target agent that comprises a detectable label, and some aspects of the invention comprise contacting a fixed host cell with a matched specific target agent that does not, at that time, comprise a detectable label. Methods of the invention may be carried out in a single host cell or a plurality of host cells, and a plurality of cells may
25 include one or more than one different cell types. In a plurality of cells, some embodiments of the invention may comprise delivering a one preselected binding agent to the cells and certain embodiments of the invention may comprise delivering two or more different preselected binding agents to the cells. The different combinations of host cell types, preselected binding agents, and flexibility in detectable labeling of matched target
30 agents permits methods of the invention, unlike previous methods, to be utilized to detect characteristics of numerous different preselected binding agents using essentially infinite color imaging.

In addition to the combinations of methods described herein, inclusion of host cell fixation methods that permit the preselected binding agents to retain the capability to
35 selectively bind their own matched specific target agents greatly expands the number of different selective bindings that can be determined in a sample that includes a single host cell or a sample comprising a plurality of host cells using methods of the invention.

5 As used herein the term “preselected” used in reference to a binding agent or a target agent means that the agent, compound, or a component thereof was chosen at least in part based on one or more of characteristics, such as but not limited to: amino acid sequence, nucleic acid sequence, binding specificity, binding selectively, binding capability to bind and/or interact with a detectable label agent, stability, localization of
10 expression, efficiency of expression, suitable delivery means, etc. As used herein the term “independently selected” used in reference to one or more of: agents, components, detectable labels, and molecules means that the agents, components, respectively may be selected that are the same as others selected, may be different from all others selected, or may be different from a portion of those selected.

15 As used herein the term “agent” is used to refer to a single molecule or a complex of two or more molecules. For example, a binding agent or target agent used in methods of the invention may include: one polypeptide, one nucleic acid, two or more polypeptides, two or more nucleic acid, one polypeptide and one nucleic acid, one or more polypeptides and one or more nucleic acids, and any of the foregoing that also includes
20 one or more additional components. Non-limiting examples of an additional component are: a detectable label, a trafficking molecule, and a delivery molecule.

 The terms “polypeptide” and “protein” are used interchangeably herein and the term polypeptide may be used to refer to a full-length protein and may also be used to refer to a fragment of a full-length protein, and/or functional variants thereof. As used
25 herein, the terms “polynucleotide” and “nucleic acid sequence” may be used interchangeably and may comprise genetic material including, but not limited to: RNA, DNA, mRNA, cDNA, etc., which may include full length sequences, functional variants, and/or fragments thereof.

30 *Binding Agents*

 A preselected binding agent included in an embodiment of the invention may be a single molecule or may include two or more independently selected molecules. A preselected binding agent used in certain methods of the invention includes one or more of a polypeptide and a nucleic acid. A binding agent may include 1, 2, 3, 4 or more
35 molecules, each independently selected from polypeptides, nucleic acids, chemical entities, and other molecules. A non-limiting example of a binding agent that may be used in methods of the invention comprises a polypeptide and a nucleic acid molecule.

5 A binding agent or one or more components thereof that are delivered into a host cell are considered herein as “exogenous” to the host cell. Similarly, a target agent or one or more components thereof that are contacted to a host cell are considered to be exogenous. It will be understood that if an agent or component thereof is naturally present in a host cell, and the agent or component thereof is also delivered into the host cell
10 according to a method of the invention, the delivered agent or component thereof is considered to be, and is referred to herein as “exogenous”. A cell into which an exogenous protein, nucleic acid or other component is delivered is referred to herein as a “host” cell.

 Embodiments of the invention utilize preselected binding agents, such as but not
15 limited to programmable DNA-binding proteins, non-limiting examples of which are TAL effectors and zinc fingers as programmable DNA binding proteins in cells, and CRISPR/Cas9 effectors. The use of such programmable agents in certain embodiments of the invention permits inclusion within a single cell of a plurality of binding agents that each specifically binds its target sequence, permitting precise detection of a plurality of
20 preselected polypeptides. In some aspects of the invention, methods comprise delivering one or a plurality of preselected DNA-binding protein agents into a host cell, fixing the host cell under conditions that permit selective binding of the preselected DNA-binding protein agents with their specific target agents when the fixed cells are contacted with the specific target agents under suitable binding conditions. The methods permit detection of
25 detectable labels on the bound target agents, thereby providing information on one or more characteristics of the preselected DNA-binding proteins in the fixed host cells. Non-limiting examples of characteristics that may be determined are: presence, absence, quantity, cellular localization, and localization in a tissue or subject.

 Aspects of the invention may include preselecting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,
30 12, 13, 14, 15, 16, 17, 18, 19, 20, or more binding agents each having the capability to selectively bind its matched specific target agent. Preselected binding agents may be delivered into 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more host cells. Preselected binding agents delivered into a plurality of host cells may be independently selected. Independent selection can be used such that in some embodiments
35 of the invention at least one preselected binding agent is delivered to each host cell in a plurality of host cells. In other embodiments of the invention, independent selection may result in a different preselected binding agent delivered into different host cells in a plurality of host cells. In still other embodiments of the invention independent selection

5 can result in one or more preselected binding agents delivered to some but not all host cells in a plurality of host cells.

It will be understood that because certain embodiments of the invention include delivering two or more preselected binding agents into host cells in a plurality of host cells, independent selection can be used to prepare a plurality of host cells in which
10 different host cells comprise different combinations of preselected binding agents. Another aspect of the invention includes methods of timing delivery of one or more preselected binding agents into one or more host cells. Non-limiting examples of means that may be used to impact timing of delivery of a preselected binding agent to a host cell are: inclusion of an inducible promoter, use of pharmacological regulation, temperature
15 regulation. In some aspects of the invention, two or more preselected binding agents may be delivered to a host cell or to host cells in a plurality of host cells at different times. Other art-known means may also be included in methods of the invention to control delivery and/or modulate expression of one or more preselected binding agent in a host cell.

20 Certain methods of the invention include preselecting a binding agent sequence that has the capability to selectively bind a specific “matched” target agent. As used herein the terms “match” and “matched” when used in reference to a binding agent and target agent, mean that under suitable conditions the binding agent and target agent selectively bind to each other, forming what may be referred to herein as a “bound
25 complex”. In certain aspects of the invention a bound complex includes one preselected binding agent bound to its target agent, and in some aspects of the invention a bound complex comprises a preselected binding agent, its matched target agent, and one or more additional molecules. Non-limiting examples of additional molecules that may be included in a bound complex are: polypeptides, nucleic acids, and detectable agents.

30 A binding agent useful in a method of the invention may include one or more of a binding domain, a nucleic acid binding domain, a single-stranded binding domain, a double-stranded binding domain, a nucleic acid sequence that is complementary to a nucleic acid sequence present in its matched target agent, etc. Specific protein-nucleic acid binding sites/sequences; nucleic acid/nucleic acid binding sites/sequences;
35 protein/protein binding sites/sequences; and conditions under which their selective binding occurs are used in methods of the invention. Methods to select and confirm matched sequence pairs that selectively bind each other are well known and routinely practiced in the art.

5 Non-limiting examples of components that may be included in preselected binding agents of the invention are: TAL sequences, CRISPR/Cas9 sequences, zinc finger (ZF) effector sequences, and Cas9 analog sequence effector sequences. Examples of Cas9 analog sequences include, but are not limited to: Cas13a (c2c2), Cpf1, and MIMIVIRE sequences. It will be understood that routine methods can be utilized to identify and use additional art-
10 known binding agents in certain embodiments of methods of the invention. Some methods of the invention include use of programmable binding sequences. A programmable binding sequence may be a sequence that binds a nucleic acid, such as but not limited to DNA. In certain aspects of the invention, a preselected binding agent comprises a programmable nucleic acid recognition component.

15 Methods for selecting and delivering one or more programmable nucleic acid recognition components to cells, tissues, and organisms are known in the art and can be used in combination with the teaching provided herein. Non-limiting examples of art-known methods for preparing and using programmable nucleic acid recognition systems, recognition sequences, binding sequences, and components thereof described in the art, see for example: Ghaemmaghaml, S. (2003) *Nature* Vol. 425, 737-741; Deng, W. et al., (2015) *PNAS*, Vol. 112, No. 38, 11870-11875; and Shechner, D. M., et al., (2015) *Nature Methods* Vol. 12(7):664-670; each of which is incorporated herein by reference in its entirety. Art-known methods and compounds for selecting programmable recognition
20 sequences and binding sequences may be used in combination with the teaching provided in certain embodiments of methods of the invention.

Certain methods of the invention include delivering one or a plurality of preselected binding agents into a host cell, wherein each preselected binding agent comprises a nucleic acid binding sequence or nucleic acid analog that when contacted with a target agent comprising a matched nucleic acid sequence, selectively binds that specific
30 target agent. Non-limiting examples of nucleic acid-binding molecules that may be used in methods of the invention are: nucleic acid-binding polypeptides, DNA-binding molecules, DNA-binding polypeptides, and other art-known nucleic acid-binding molecules.

In certain aspects of the invention a transcription activator-like (TAL) effector, also referred to herein as a transcription activator-like effector (TALE) sequence, is
35 included in a binding agent of the invention. In addition to those disclosed herein, other art-known TAL effector sequences that selectively bind specific nucleic acid sequences are suitable for use in embodiments of the invention in which a preselected binding agent is a preselected TAL effector sequence that has the capability to selectively bind its specific

5 target nucleic acid sequence and can be preselected with routine methods and delivered to one or more host cells.

In some aspects of the invention, methods include use of a TAL labeling scheme, wherein a subset of a large number of TALEs are the preselected binding agents that are expressed randomly in host cells, the host cells are fixed under appropriate conditions to
10 permit the TALEs to selectively bind their matched target agents, and the bound target agents are detected. Detection of the bound target agents can be used to identify single host cells on the basis of the specific preselected TALEs expressed in the host cells prior to the host cell fixation.

Non-limiting examples of preselected binding agents and matched target agents
15 include a PL62 TALE sequence as a preselected binding agent and a target agent comprising a Hairpin 14 sequence. Another non-limiting example includes a PL61 TALE sequence as a preselected binding agent and a target agent comprising Hairpin 13 sequence. Additional non-limiting examples are provided herein.

In certain aspects of the invention a zinc finger (ZF) effector sequence is included
20 in a binding agent of the invention. Art-known ZF effector sequences that selectively bind specific nucleic acid sequences are suitable for use in embodiments of the invention in which a preselected binding agent is a preselected ZF effector sequence that has the capability to selectively bind its specific target nucleic acid sequence. ZF effector sequences and binding partners are suitable for use in certain embodiments of the
25 invention are known in the art and can be preselected with routine methods and delivered to one or more host cells.

In some aspects of the invention, methods include use of a ZF labeling scheme, wherein a subset of a large number of ZFs are the preselected binding agents that are expressed randomly in host cells, the host cells are fixed under appropriate conditions to
30 permit the ZFs to selectively bind their matched target agents, and the bound target agents are detected. Detection of the bound target agents can be used to identify single host cells on the basis of the specific preselected ZFs expressed in the host cells prior to the host cell fixation.

Non-limiting examples of preselected binding agents and matched target agents include:
35 SRC-140-4 set forth herein as SEQ ID NO: 7; SRC-140-5 set forth herein as SEQ ID NO: 8; and SRC-140-CODA-3 set forth herein as SEQ ID NO: 9. A non-limiting example of a double-stranded probe used as a target agent for SRC-140-4 may be prepared by annealing: acatcGAGGACGGCagcgt (SEQ ID NO: 10) and

5 acgctGCCGTCCTCgatgTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/ (SEQ ID NO: 11). A non-limiting example of a double-stranded probe used as a target agent for SRC-140-5 may be prepared by annealing: ttgaaGAAGATGGTgcgct (SEQ ID NO: 12) and agcgcACCATCTTctcaaTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/ (SEQ ID NO: 13). As non-limiting example of target agents for SRC-140-CODA-3, two
 10 probe sequences including:
 gccgaGAAGACGAAtacatTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/ (SEQ ID NO: 14) and atgtaTTCGTCTTctcggc (SEQ ID NO: 15) may be used in certain embodiments of the invention. Probe sequences set forth herein comprise:
 acgctGCCGTCCTCgatgTATAAGCGGGAGATTCGTCCTCATA (SEQ ID NO: 17);
 15 agcgcACCATCTTctcaaTATAAGCGGGAGATTCGTCCTCATA (SEQ ID NO: 18);
 and gccgaGAAGACGAAtacatTATAAGCGGGAGATTCGTCCTCATA (SEQ ID NO: 19), which are the DNA sequences to which detectable label may be attached. Non-limiting examples of a detectable label that can be used in certain embodiments of methods and compositions of the invention are: AlexF546N and Atto488N. It will be
 20 understood that other art-known detectable labels are suitable for use in methods and compositions of the invention.

Numerous other examples of binding agents and their matched target agents are known in the art and suitable for use in methods and compositions of the invention. Another non-limiting example of a type of preselected binding agent suitable for use in
 25 embodiments of the invention is a megaTAL effector sequence. Specific art-known megaTAL effector sequences are suitable for use in certain embodiments of the invention and can be preselected with routine methods.

Binding Agent Delivery

30 Embodiments of methods of the invention may comprise delivering one or more preselected binding agents into a host cell, wherein the binding agent comprises a sequence that selectively binds a specific target agent. As used herein, the term “delivery” is used in reference to direct and indirect delivery. A non-limiting example of indirectly delivering a preselected binding agent into a host cell in a method of the invention is delivering a
 35 vector that encodes the preselected binding agent or component to the host cell, with subsequent expression of the preselected binding agent in the host cell. Numerous art-known means are suitable for use in methods of the invention to indirectly deliver one or more binding agents, polypeptides, nucleic acids, and other molecules into a host cell. In

5 certain aspects of the invention, a preselected binding agent is delivered as part of a fusion protein.

Non-limiting examples of direct delivery methods that can be used in certain embodiments of methods of the invention are: physical delivery means, non-viral vector delivery means, and use of a carrier-molecule for delivery into the host cell. Non-limiting
10 examples of direct delivery means used in certain embodiments of the invention are preselected binding agent delivery using: nanoparticles, liposomes, polymers, cell-penetrating molecules, and cell-derived membrane vesicles (CMV). Additional non-limiting methods to directly deliver a binding agent in a method of the invention are: electroporation, microinjection, laser delivery, and ballistic delivery. Additional direct
15 and indirect delivery means that are suitable for use in methods of the invention are known and routinely practiced in the art.

In some embodiments of the invention a preselected binding agent that is delivered into a host cell is part of a fusion protein. It is well known in the art how to prepare and utilize fusion proteins that comprise one or more polypeptide sequences. In certain
20 embodiments of the invention, a fusion protein can be used to deliver a binding agent, such as a TAL effector sequence, a CRISPR/Cas9 sequence, a zinc finger (ZF) effector sequence, and a Cas9 analog effector sequence, or other preselected binding agent into a host cell. In some aspects of the invention, a fusion protein comprising a preselected binding agent includes one or more additional polypeptides. Non-limiting examples of
25 types of additional polypeptides that may be included are opsins; regulatory proteins; enzymes; structural proteins such as, but not limited to tubulin and actin; synaptic proteins such as, but not limited to Homer and Bassoon; detectable polypeptides, etc.

A fusion protein comprising a preselected binding agent for use in a method of the invention may be delivered to (expressed) in a specific cell type, tissue type, organ type, and/or region in a subject, or *in vitro*, for example in culture, or in a slice preparation, etc.
30 Methods for preparing and/or delivering a fusion protein or its encoding nucleic acid sequence are well known in the art. Routine methods can be used in conjunction with teaching provided herein to express a preselected binding agent in a desired host cell, tissue, or region in a subject or *in vitro* and to process the host cell according to further
35 methods of the invention in order to permit detection of characteristics of the preselected binding agent such as presence, absence, expression level, localization, etc. as described elsewhere herein.

5 Routine methods for fusion protein preparation and delivery can be used in methods of the invention to deliver a preselected binding agent into at least one of: a specific cell type, a specific cell subtype, a specific spatial region within an organism, and a sub-cellular region within a cell. Routine genetic procedures can also be used to control parameters of expression, such as but not limited to: the amount of a preselected binding agent expressed, the timing of its expression, targeting of the binding agent to a specific location in a host cell, etc.

 In some embodiments of the invention a reagent for genetically targeted expression of a preselected binding agent is a vector comprising a gene encoding the preselected binding agent. As used herein in reference to indirect delivery means, the term “vector” refers to a nucleic acid molecule capable of transporting between different genetic environments another nucleic acid to which it has been operatively linked. The term “vector” may also be used to refer to a virus or organism that is capable of transporting the nucleic acid molecule. One type of vector is an episome, i.e., a nucleic acid molecule capable of extra-chromosomal replication. Some vectors are capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as “expression vectors”. Other vectors that may be used in certain methods of the invention include, but are not limited to viruses such as lentiviruses, retroviruses, adenoviruses, and phages. Vectors useful for indirect delivery in certain embodiments of the invention can genetically insert a preselected binding agent into dividing and/or non-dividing cells and can insert the preselected binding agent into an *in vivo*, *in vitro*, or *ex vivo* host cell.

 Vectors useful in certain indirect delivery methods of the invention may include additional sequences such as, but not limited to, one or more signal sequences, trafficking sequences, promoter sequences, or combinations thereof. In certain embodiments of the invention, a vector may be a lentivirus, adenovirus, adeno-associated virus, or other vector that comprises a gene encoding a preselected binding agent (also referred to herein as an “effector”), a non-limiting example of which is a TAL effector. In some embodiments of the invention a TAL effector encoded in a vector is expressed in a cell, the cell is fixed, and then the cell is contacted with a specific target agent matched to the TAL effector. Non-limiting examples of target sequences that are matched to specific TAL effectors are: a TALES(13) sequence (SEQ ID NO: 1) and a TALES(14) sequence (SEQ ID NO: 2). In certain embodiments of the invention, a vector comprising a nucleic acid encoding a TAL

5 binding agent, SRC-60B (SEQ ID NO: 5), is delivered into a cell, the TAL binding agent is expressed in the cell, the cell contacted with a fixation means, and after fixation the cell is contacted with a specific target agent for SRC-60B, a non-limiting example of which is the sequence set forth herein as SEQ ID NO: 6. SEQ ID NO: 22 is DNA sequence set forth as SEQ ID NO: 6 without the 5Atto488N label. It will be understood that the
10 identification of certain detectable labels herein may optionally be shown with a number “3” or “5” indicating their attachment position on the probe nucleic acid molecule.

Some embodiments of the invention include a vector comprising a gene encoding a preselected Zinc Finger (ZF) binding agent. Non-limiting examples of the encoding sequences are: SRC-140-4 (SEQ ID NO: 7), SRC-140-5 (SEQ ID NO: 8), and SRC-140-
15 CODA-3 (SEQ ID NO: 9). The sequence set forth herein as SEQ ID NO: 16 is a non-limiting example of a plasmid vector sequence that comprises a sequence encoding a preselected binding agent, in this instance, SRC-140-CODA-3 (SEQ ID NO: 9).

It will be understood that other vectors and other sequences encoding a preselected binding agent can be used and included in methods of the invention. Adeno-associated
20 viruses (AAVs) such as AAV8, AAV1, AAV2, AAV4, AAV5, AAV9, are non-limiting examples of vectors that may be used in embodiments of methods of the invention to express in a cell, a fusion protein comprising a preselected binding agent. Expression vectors and methods of their preparation and use are well known in the art. Non-limiting examples of suitable expression vectors and methods for their use to deliver a preselected
25 binding agent to a host cell are provided herein.

Promoters that may be used in methods and vectors for indirect delivery of preselected binding agents in methods of the invention include, but are not limited to, cell-specific promoters or general promoters. Non-limiting examples promoters that can be used in vectors of the invention are: ubiquitous promoters, such as, but not limited to:
30 CMV, CAG, CBA, and EF1a promoters; and tissue-specific promoters, such as but not limited to: Synapsin, CamKIIa, GFAP, RPE, ALB, TBG, MBP, MCK, TNT, and aMHC promoters. Methods to select and use ubiquitous promoters and tissue-specific promoters are well known in the art. A non-limiting example of a tissue-specific promoter that can be used in certain methods of the invention to express a preselected binding agent in a host
35 cell such as a neuron is a synapsin promoter. Additional tissue-specific promoters and general promoters are well known in the art and may be suitable for use in methods of the invention.

5 Additional sequences that may be included in a fusion protein that comprises a preselected binding agent and is suitable for use in embodiments of the invention are trafficking sequences, including, but not limited to: Kir2.1 sequences and functional variants thereof, KGC sequences, ER2 sequences, etc. Trafficking polypeptides and their encoding nucleic acid sequences that are suitable for use in embodiments of the invention
10 are known in the art and routine methods can be used to include and use such sequences in fusion proteins and vectors, respectively, for use in methods of the invention. Other art-known molecules and sequences may be included in a fusion protein used in the invention and routine procedures, including, but not limited to: fluorescent proteins, for example see: Addgene Plasmid Depository (Watertown, MA), ([addgene.org/fluorescent-](https://addgene.org/fluorescent-proteins/localization/)
15 [proteins/localization/](https://addgene.org/fluorescent-proteins/localization/)). Another non-limiting example of a sequences that may be included in a vector in some embodiments of the invention is an actin fusion, which may be used in membrane localization. See for example Murakoshi, H. et al. *Brain Cell Biol.* 2008 August; 36(1-4): 31–42, the content of which is incorporated by reference herein in its entirety. Another non-limiting example of a sequences that may be included in a vector in
20 some embodiments of the invention is a palmitoylation domain, which may be used in membrane localization. See for example Goedhart, J. et al., *Nat Commun.* 2012 Mar 20; 3: 751, the content of which is incorporated by reference herein in its entirety.

The use of fusion proteins to deliver different preselected binding agents and their use in certain methods of the invention has now been demonstrated. As a non-limiting
25 example, fusions of TALs to fluorescent polypeptides were expressed in cultured HEK host cell samples. The expression was validated by the presence of fluorescence. Host cell samples were fixed using a variety of fixation protocols, including aldehyde, alcohol, and acetone fixations. After fixation, target agents comprising (1) DNA hairpins that included the binding sequence of the TALs and a fluorophore or (2) different sequence
30 (not including TAL binding sequences) and a fluorophore were incubated with the fixed host cell samples. In (1), excess hairpin target agents were removed from solution and fluorescence detected. In host cell samples fixed with an alcohol solution only, a fluorescent signal from the specific target agent DNA hairpin was detected and determined to co-localized with the fluorescent signal from the TAL fusion protein, and no fluorescent
35 signal was detected from the non-specific DNA hairpin, and there was no signal detected in host cell samples fixed with an aldehyde solution. In other embodiments of the invention comprising fixation of host cell samples with a methanol solution, specific target agent DNA hairpins were detected and determined to co-localize with the fluorescent

5 signal from the TAL fusion protein, with no fluorescent signal detected from host samples contacted with the non-specific DNA hairpin and no specific target agent comprising the matched DNA hairpin. In some embodiments of the invention comprising fixation of host cell samples with ethanol, specific target agent DNA hairpins were detected and determined to co-localize with the fluorescent signal from the TAL fusion protein, with no
10 fluorescent signal detected from host samples contacted with the non-specific DNA hairpin and no specific target agent comprising the matched DNA hairpin.

As another non-limiting example, fusions of Zinc Fingers to fluorescent polypeptides were expressed in cultured HEK host cell samples. The expression was validated by the presence of fluorescence. Host cell samples were fixed using a variety of
15 fixation protocols, including aldehyde, alcohol, and acetone fixations. After fixation, target agents comprising (1) nucleic acid sequences (target agents) comprising the binding sequence of the ZFs and a fluorophore or (2) different sequence (not including ZF binding sequences) and a fluorophore were incubated with the fixed host cell samples. In (1), excess ZF target agents were removed from solution and fluorescence detected. The
20 binding was found to co-localize with the fluorescent signal from the ZF fusion protein, with no fluorescent signal detected from host samples contacted with the non-specific DNA sequences not comprising ZF target agent DNA sequences.

Certain embodiments of the invention include use of fixation protocols prior to contacting host cells with a target agent DNA, for example that binds to the TAL effector, ZF effector, or other preselected binding polypeptide. Certain examples of fixatives and
25 fixative protocols that may be used in methods of the invention are provided herein, and include but are not limited to: 10% formalin, 4% paraformaldehyde, methanol-containing fixatives, ethanol-containing fixatives, and procedures described elsewhere herein. These and other fixative methods of the invention may be used in certain embodiments of the
30 invention to fix host cell samples in which one or more preselected binding proteins have been expressed. The fixation methods of the invention are suitable to permit the fixed host cell samples to be contacted with target agent DNAs specific for their expressed preselected binding proteins, and the binding detected. Detection may include identifying localization in the host cell, quantification, and other parameters. In some aspects of the
35 invention, more than one preselected binding protein may be expressed in a host cell and after binding and assessment of one target agent DNA, the bound target agent DNAs may be removed and a second target agent DNA may be contacted with the fixed host cells. This process may be repeated 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times permitting assessment

5 of 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different preselected binding proteins expressed in a host cell.

Target agents

After delivery of one or more preselected binding agents to a host cell, the host
10 cell, or a plurality of host cells may be fixed and following fixation may be contacted with one or more independently selected matched target agents. Independent selection of target agents permits either a single specific target agent or two or more different target agents to be included in certain embodiments methods of the invention. For example, if in an embodiment of a method of the invention one preselected binding agent is delivered to a
15 single host cell, after suitable fixation, the host cell may be contacted with the single matched target agent. If in another embodiment of a method of the invention two or more preselected binding agents are delivered to a single host cell, after suitable fixation, the host cell may be contacted with the two or more matched target agents. In a situation in which an embodiment of the invention includes delivering one or more preselected
20 binding agents to two or more host cells, the target agents that contact the host cells may be independently selected such that after suitable fixation: all of the host cells are contacted with the same target agent; a subset of the host cells are contacted with the same target agent; or each of the host cells are contacted with a different target agent.

As described herein, a target agent used in a method of the invention may include a
25 region or sequence that matches and selectively binds a preselected binding agent that has been delivered to and/or expressed in a host cell. In a non-limiting example, an independently selected target agent comprises a specific double-stranded DNA (dsDNA) sequence that is selectively bound by a preselected TAL effector binding agent. The preselected TAL effector binding agent is delivered to a host cell and the host cell is fixed
30 under suitable conditions to permit binding of the preselected TAL effector binding agent and its matched target agent. After fixation, the host cell is contacted with the target agent and the target agent is detected, which indicates one or more characteristics of the preselected TAL effectors. Hairpin sequences may be used as matched target agents in certain methods of the invention. DNA binding sequences and matched hairpin target
35 agent sequences are known in the art, see for example: Praetorius, F. and Deitz, H. Science 24 Mar 2017: Vol. 355, Issue 6331, pp 54881, the content of which is incorporated herein by reference in its entirety.

5 Following fixation of a cell or cells comprising the expressed binding protein, the fixed cell or cells are contacted with the target polynucleotide under suitable binding conditions. The recognition of the target sequence by the expressed binding protein results in a binding complex comprising the target polynucleotide and the binding protein. In some embodiments of the invention, a target polynucleotide is detectable, thus
10 permitting detection of the binding complex. In some embodiments of the invention, a target polynucleotide includes and/or is attached to a detectable label.

 As another non-limiting example, a Zinc finger binding polypeptide sequence is selected and an expression vector is prepared that comprises the DNA encoding the binding protein, in this example, SRC-140-4, the sequence of which is set forth herein as
15 SEQ ID NO: 7. In this example, the vector is delivered into a cell and the selected zinc finger binding protein is expressed in the cell. The cell is processed using one or more methods of the invention such as, but not limited to: fixation, washing, permeabilization, etc. and the cell is contacted with its target polynucleotide, which in this example, comprises a double-strand probe prepared by annealing the sequences set forth therein as:
20 SEQ ID NO: 10 and SEQ ID NO: 11, which includes a detectable label: AlexF546N. The double-stranded probe binds the SRC-140-4 Zinc finger binding protein. After the cell is contacted, one or more bound complexes of the SRC-140-4 Zinc finger binding protein and its matched double-stranded target polynucleotide can be detected by determining the presence of the detectable label.

25 In some embodiments a specific target nucleic acid may comprise a detectable label and different target nucleic acids may comprise different detectable labels, thus permitting differential detection of target nucleic acids and the specific binding agent to which they are bound. In certain embodiments of the invention a specific target nucleic acid may not initially comprise a detectable label but subsequently comprise a detectable
30 label. Non-limiting examples of different steps that may be included in embodiments of methods of the invention: are: contact a fixed host cell with two or more target agents comprising the same type of detectable label; contacting a fixed host cell with two or more target agents comprising two or more different detectable labels; removing one or more detectably labeled target agent from a fixed host cell; and contacting fixed host cell with
35 one or more different detectably labeled target agents at one or more different times. It will be understood that certain embodiments of methods of the invention may utilize additional detectable labeling strategies suitable to permit detection of a plurality of polypeptides in a host cell, host cells, and/or tissues comprising one or a plurality of host

5 cells. Strategies may include variations in parameters such as, but not limited to: timing of including one or more detectable labeled target agents, type of detectable labels on one or more target agents, means to detect one or more detectable labels on target agents; and one or more of serial and sequential contact of a fixed host cell comprising a preselected binding agent with one or more detectably labeled matched target agents, etc., thus
10 permitting differential detection of target nucleic acids and the specific binding agent to which they are bound.

Serial detection methods of the invention may include contacting a fixed host cell or plurality of fixed host cells comprising one or more preselected binding agents, with two or more matched specific target agents comprising different detectable labels. After
15 binding and removal of unbound target agents, the fixed host cell(s) are exposed to a series of detection means, each suitable to detect at least one of the different detectable labels. Use of a series of two or more different detection means, each type of detectable label in the fixed host cell(s) may be individually detected, thereby permitting identification of one or more characteristics of each different preselected binding agent bound to its matched
20 detectably labeled target agent.

Certain embodiments of the invention may use methods for successive detection methods. Successive detection methods may include contacting a fixed host cell or plurality of fixed host cells comprising one or more preselected binding agents with a succession of their matched target agents and utilizing a suitable detection means
25 following contact with each type of matched target agent. For example, fixed host cell(s) may be contacted with a first matched specific target agent and the detectable label detected to assess the target agent bound to its matched binding agent in the fixed cell(s). After detection, the first matched specific target agent the fixed host cell or plurality of host cells may be contacted with a second (different) matched target agent and the
30 detectable label detected to assess the second target agent bound to its matched binding agent in the fixed host cell(s). These steps may be repeated 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 100, or more times, which enables detection of a corresponding number of bound target agents and the concomitant determination of one or more characteristics of the bound matched preselected binding agents in a single fixed host cell or a plurality of fixed
35 host cells.

It will be understood that in certain aspects of the invention, a matched target agent comprising a detectable label may be removed from its matched binding agent, at a time that may be subsequent to detection of the detectable label in the fixed host cell. It will be

5 understood that a fixed host cell or plurality of fixed host cells may be contacted with two or more different matched target agents at the same time and/or at different times, and that a type of matched target agent may be contacted with a fixed host cell or plurality of fixed host cells one or more times, either alone or with one or more different matched target agents.

10

Detection Agents and Imaging

As used herein the terms: “detect” and “detection” used in reference to determining binding mean identifying using any suitable means, a detectable label or other physical characteristic associated with a target agent. Non-limiting examples of detection means
15 that can be used in methods of the invention are: fluorescence detection, luminescence detection, chromogenic detection, autoradiography, fluorography, phosphor imaging, etc.

Certain embodiments of methods of the invention include contacting a fixed host cell with at least one target agent and detecting the target agent in the fixed host cell. A target agent used in methods of the invention may comprise a detectable label at the time
20 the fixed host cell is contacted with the target agent, or the target agent may not include a detectable label at the time of contact, but a detectable label may be subsequently attached to the target agent.

As used herein the term “detection agent” may be used interchangeably with the term “detectable label”. In instances where a target agent comprises a detectable label
25 when it contacts the host cell and binds its matched binding agent in the host cell, the detectable label may be detected as a determination of one or more characteristics of the preselected binding agent to which the matched target agent is bound in the fixed host cell. A target agent used in a method of the invention may comprise a detectable label or detectable agent, and when the target agent is bound to its matched preselected binding
30 agent in a fixed host cell, detecting the detectable label provides information about characteristics of the target agent, the preselected binding agent, and the target agent/preselected binding agent bound complex.

Non-limiting examples of means suitable to attach a detectable label to a target agent for use in embodiments of the invention are: a chemical bond, crosslinking, a covalent bond, a
35 non-covalent bond, and other art-known method for attaching a detectable label to a target molecule. Various labeling techniques and attachment methods suitable for use in combination with the teaching presented herein are routinely used in the art. See for

5 example, *Bioconjugate Techniques*, Hermanson, Greg T. Academic press, 2013, the content of which is incorporated herein by reference in its entirety.

Non-limiting examples of types of detectable agents that can be used in certain embodiments of the invention as part of a detectable label are: fluorophores, isotopes, and bioluminescent agents. In certain aspects of the invention, a detectable label comprises a
10 fluorophore. Non-limiting examples of fluorophore molecules and agents that are suitable for use in certain embodiments of the invention are synthetic fluorophores, fluorescent polypeptides and variants thereof that selectively bind a specific region or functional group on its target molecule, which may be a target agent as described herein.

Non-limiting examples of detectable labels that may be attached to one or more of
15 target agents and used in embodiments of methods of the invention are: green fluorescent protein (GFP); enhanced green fluorescent protein (EGFP), red fluorescent protein (RFP); yellow fluorescent protein (YFP), dtTomato, mCherry, DsRed, mRuby, cyan fluorescent protein (CFP); far red fluorescent proteins, etc. Numerous fluorescent proteins and their encoding nucleic acid sequences are known in the art and routine methods can be used to
20 include such sequences in molecules, agents, fusion proteins and vectors, respectively, for use in methods of the invention. Different detection means may be used in embodiments of the invention to detect different fluorophore detectable labels. The use of different detectable labels, a non-limiting example of which is the use of different fluorophore detectable labels, can permit serial and successive detection strategies as described
25 elsewhere herein.

Methods for detecting detectable labels used in methods of the invention include, but are not limited to one or more of: microscopy, autoradiography, fluorescence microscopy, bioluminescence imaging, MRI detection methods, radioisotope detection methods, etc. Art-known methods for detecting detectable labels in fixed cells may be
30 used in conjunction with the teaching provided herein for assessing, quantifying, localizing a detectable label in an embodiment of a method of the invention.

In a non-limiting example of a method of the invention, a fluorophore detectable label is covalently attached to a dsDNA target agent during synthesis of the target agent, the fluorophore is detected using FISH methods after the dsDNA target agent is bound by
35 its matched TALE binding agent that is present in a fixed host cell. In this example, the method of the invention includes a form of “*in situ* hybridization” using double-stranded DNA, wherein the hybridization of the preselected binding agent (the TAL protein) by the detectably labeled matched target agent is carried in a fixed system. Methods of the

5 invention permit delivering a preselected DNA-binding agent comprising a polypeptide into one or more host cells, fixing the host cells, binding the preselected DNA-binding agent to a matched target agent that comprises a detectable label, thereby essentially enabling labeling and detection of the polypeptide using the versatility and programmability of DNA within the environment of the host cells.

10

Fixation

Methods of the invention include delivering a preselected binding agent into one or a plurality of cells. In such embodiments, it has now been demonstrated that application of suitable fixation methods and solutions can result in the retention of the capability of a binding agent to selectively bind its matched target agent in the fixed tissue. Non-limiting examples of components of fixative solutions that have now been shown to be useful in embodiments of methods of the invention are: alcohols such as methanol and ethanol.

A non-limiting example of components included in a fixative solution that may be used in certain embodiments of the invention comprises a methanol and acetic acid fixative solution. Percentage ratios (v/v) of methanol to acetic acid that may be included in a fixative solution are: 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%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100% methanol to 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, 60%, 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52%, 51%, 50%, 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, and 0% acetic acid, respectively, including all percentages in the range provided.

Another non-limiting example of a fixative solution that may be used in certain embodiments of the invention comprises an ethanol and acetic acid fixative solution. Percentage ratios (v/v) of ethanol to acetic acid that may be included in a fixative solution are: 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%,

5 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%,
64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%,
79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,
94%, 95%, 96%, 97%, 98%, 99%, and 100% ethanol to 80%, 79%, 78%, 77%, 76%, 75%,
74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, 60%,
10 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52%, 51%, 50%, 49%, 48%, 47%, 46%, 45%,
44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%,
29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%,
14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, and 0% acetic acid,
respectively, including all percentages in the range provided.

15 Certain embodiments of methods of the invention may include use a cell fixative
comprising at least one of: MeOH, EtOH, acetone, an amine, a cleavable molecule, a
cleavable polypeptide, a cleavable cross-linker molecule, and an aldehyde. Additional
non-limiting examples of components that may be used in an embodiment of a fixative
method of the invention are: DSP, SPDP, EGS, Hepes-glutamic acid buffer-mediated
20 organic solvent protection effect (HOPE) and osmium. In some aspects of the invention
fixation methods may include use of one or more: heterobifunctional crosslinker,
homobifunctional crosslinker, and zero-length crosslinker.

In some embodiments of the invention, a fixative solution, when contacted with a
host cell is at a temperature of: -40°C, -30°C, -29°C, -28°C, -27°C, -26°C, -25°C, -24°C, -
25 23°C, -22°C, -21°C, -20°C, -19°C, -18°C, -17°C, -16°C, -15°C, -14°C, -13°C, -12°C, -11°C,
-10°C, -9°C, -8°C, -7°C, -6°C, -5°C, -4°C, -3°C, -2°C, -1°C, 0°C, 1°C, 2°C, 3°C, 4°C, 5°C,
6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C,
21°C, 22°C, 23°C, 24°C, 25°C, 30°C, 40°C, or 50°C, including all temperatures in the
range provided. In some embodiments of the invention, a fixative solution may be between
30 -40°C and -25°C; -30°C and -10°C; -25°C and -15°C; -25°C and 1°C; and -25°C and 25°C.

In certain embodiments of the invention a host cell is contacted with a fixative
solution and incubated in that solution for a period of time. An incubation time period
used in a method of the invention may be less than one minute, 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, 35, 40, 45, 50,
35 55, 60, or more minutes, including all times within the range of times provided. In some
aspects of the invention an incubation time of a host cell in a fixative solution is in a range
from: 1-120 minutes, 1 to 60 minutes, 1 to 50 minutes, 1 to 40 minutes, 1 to 30 minutes, 1
to 25 minutes, 1 to 20 minutes, 1 to 15 minutes, 1 to 10 minutes, 1 to 5 minutes, 10 to 20

5 minutes, 10 to 30 minutes, or 15 to 25 minutes.

A host cell of the invention may be washed one or more times subsequent to the fixation incubation. Non-limiting examples of solutions with which a fixed host cell may be contacted and/or washed in an embodiment of the invention are solutions comprising one or more of: PBS, PBS and Triton X-100, a cell permeabilizing agent, binding buffer,
10 one or more target agents, one or more detectably labeled target agents, and one or more detectable labels. Times of periods of washing and/or contact may range from be less than one minute, 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, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, or more minutes, including all times within the range of times provided. In
15 some aspects the incubation time of a host cell in a fixative solution is in a range from: 1-120 minutes, 1 to 60 minutes, 1 to 50 minutes, 1 to 40 minutes, 1 to 30 minutes, 1 to 25 minutes, 1 to 20 minutes, 1 to 15 minutes, 1 to 10 minutes, 1 to 5 minutes, 10 to 20 minutes, 10 to 30 minutes, or 15 to 25 minutes.

Embodiments of methods of the invention include different solutions and times
20 and the disclosure provided herein may be used in combination with art-known procedures to practice aspects of methods of the invention. Addition fixatives that may be used in embodiments of methods of the invention include, but are not limited to: (1) zinc-based fixatives such as, but limited to that presented in Lykidis, D. et al., Nucleic Acids Research, 2007, vol 35, No. 12 e85, in which tissue is stabilized with some advantages
25 over traditional precipitant or cross-linking fixatives with regard to nucleic acid and protein preservation, and (2) aldehyde formulations such as Periodate-Lysine-Paraformaldehyde as described in, for example: Pieri, L. et al., Eur J Histochem. 2002;46(4):365-75, in which sodium periodate and lysine accompany paraformaldehyde in the fixative solution. Each of the abovementioned references are incorporated herein by
30 reference in their entirety.

Sequences and Functional Variants

As described else herein, a preselected binding agent comprises a component that recognizes and selectively binds a matched component in a specific target agent. In some
35 embodiments of the invention, one or both of a preselected binding agent and its matched target agent, in addition to their specific matched sequences, may also include additional sequences, such as one or more amino acid and nucleic acid sequences. It will be

5 understood that in some methods of the invention there may be sequence variation in one or more specific matched pair sequences, and/or in additional sequences that are included in a predetermined binding agent and/or its matched target agent. As used herein, a sequence that includes variation from its “parent” sequence, and retains at least a portion of the function of the parent sequence is referred to as a functional variant.

10 The term “functional variant” as used herein in the context of a preselected binding agent or target agent for use in a methods of the invention, describes a molecule with one or more of the following characteristics: (1) the variant differs in sequence from the molecule of which it is a variant; (2) the variant is a fragment of the molecule of which it is a variant and is identical in sequence to the fragment of which it is a variant, and/or (3)
15 the variant is a fragment and differs in sequence from the fragment of the molecule of which it is a variant. As used herein, the term “parent” in reference to a sequence means a sequence from which a variant originates. For example, though not intended to be limiting, a parent sequence may be a fusion protein comprising a PL62 TALEs sequence (also referred to herein as a PL62 TAL effector sequence) and a functional variant of that
20 parent fusion protein sequence may have 1, 2, 3, 4, 5 or more modifications in its sequence, while retaining at least a portion of the function of the parent in a method of the invention. In another non-limiting example, a parent sequence may be a fusion protein comprising a SRC-140-CODA-3 sequence (SEQ ID NO: 16) and a functional variant of that parent fusion protein sequence may have 1, 2, 3, 4, 5 or more modifications in its
25 sequence, while retaining at least a portion of the function of the parent in a method of the invention.

As used herein the term “modified” or “modification” in reference to a polypeptide sequence or a nucleic acid sequence refers to a change of 1, 2, 3, 4, or more amino acids or nucleic acids, respectively in the sequence as compared to the parent preselected binding
30 agent. As used herein, a sequence modification may be one or more of a substitution, deletion, insertion or a combination thereof. As an example, though not intended to be limiting, the amino acid sequence of a variant fusion protein comprising PL62 TALEs may be identical to its parent fusion protein sequence except that it has one, two, three, four, five, or more amino acid substitutions, deletions, insertions, or combinations thereof,
35 while retaining at least a portion of the function of the parent molecule of which they are a variant.

In embodiments of the invention, a sequence of a binding agent maintains its capability to selectively bind its specific matched target agent, and regions of the binding

5 agent that don't alter the binding properties may include one or more substitutions, deletions, insertions, combinations thereof, or other modifications. As used herein, the term "preselected binding agent" will be understood to encompass functional variants. Methods described herein and other art-known are suitable to assess function of a modified preselected binding agent characteristics such as, but not limited to: expression, localization in a host cell, tissue localization, level of expression, stability, internal
10 localization in a host cell, etc. A functional variant of a preselected binding agent used in a methods of the invention can maintain at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more of a level of function of a characteristic of the parent preselected binding agent, and will
15 maintain the capability to selectively bind to its matched target agent.

Methods of sequence alignments, modifications, etc. are routinely practiced in the art. Sequence modifications that can be included in a fusion protein that result in altered expression, localization, etc. are known in the art and can be used in embodiments of methods of the invention, while maintaining the capability of the preselected binding agent
20 to selectively bind its matched target agent.

A variant of a polypeptide that is part of a preselected binding agent or a variant of a fusion protein used in certain embodiments of the invention may include one or more deletions, point mutations, truncations, amino acid substitutions and/or additions of amino acids or non-amino acid moieties. Modifications of a polypeptide of the invention may be
25 made in certain aspects of the invention by modification of the nucleic acid sequence that encodes the fusion protein.

It will also be understood that conservative amino acid substitutions may be made in a component of a preselected binding agent and/or a fusion protein used in certain methods of the invention. As used herein, a "conservative amino acid substitution" refers
30 to an amino acid substitution that does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Conservative substitutions of amino acids may, in some embodiments of the invention, include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. Polypeptide variants can be
35 prepared according to methods for altering polypeptide sequence and known to one of ordinary skill in the art.

Sequence identity can be determined using standard techniques known in the art. To determine the percent identity (similarity) of two amino acid sequences the sequences

5 are aligned for optimal comparison purposes (e.g., gaps may be introduced in the sequence of one protein for optimal alignment with the other protein). The amino acid residues at corresponding amino acid positions are then compared. When a position in one sequence is occupied by the same amino acid residue as the corresponding position in the other sequence, then the molecules have identity/similarity at that position. The percent identity
10 or percent similarity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity or % similarity = number of identical positions/total number of positions x 100). Such an alignment can be performed using any one of a number of well-known computer algorithms designed and used in the art for such a purpose. Similarly, percent identity/similarity of polynucleotide sequences encoding a
15 polypeptide of the invention can be determined using art-known alignment and comparison methods for nucleic acid molecules.

A site or region for introducing an amino acid sequence modification may be preselected, and the mutation per se need not be preselected. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the
20 target codon or region and the expressed polypeptide screened for the level of desired function or activity. Techniques for making substitution mutations at preselected sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Variant sequences may in some embodiments of the invention be prepared by site specific mutagenesis of nucleic acids in the DNA encoding a polypeptide
25 that is a portion of a binding agent used in a method of the invention, using cassette or PCR mutagenesis or other techniques known in the art, to produce DNA encoding the polypeptide. In certain embodiments of the invention, activity of variant or fragment of a polynucleotide or polypeptide can be tested by cloning the gene encoding the altered polypeptide into a bacterial or mammalian expression vector, introducing the vector into
30 an appropriate host cell, expressing the altered polypeptide, and testing for a functional capability of the polypeptide as disclosed herein. Additional methods for generating recombinant polypeptides are known in the art may include use of prokaryotic and eukaryotic expression systems including but not limited to bacterial and mammalian expression systems.

35 It is understood in the art that the codon systems in different organisms can be slightly different, and that therefore where the expression of a given protein from a given organism is desired, the nucleic acid sequence can be modified for expression within that organism. Thus, in some embodiments, a target agent and/or fusion protein of the

5 invention is encoded by a mammalian-codon-optimized nucleic acid sequence, which may in some embodiments be a human-codon optimized nucleic acid sequence. An aspect of the invention provides a nucleic acid sequence that encodes a fusion protein comprising a preselected binding agent or variant thereof of the invention, and that is optimized for expression with a mammalian cell. In certain aspects of the invention, a nucleic acid
10 sequence is optimized for expression in a human cell.

Host Cells and Subjects

A host cell used in methods and with sequences of the invention may be an excitable cell or a non-excitable cell. A host cell in which a preselected binding agent or functional variant thereof of the invention may be expressed and may be used in methods
15 of the invention may be a prokaryotic or a eukaryotic cell. Useful host cells include, but are not limited to, vertebrate host cells, which in some embodiments of the invention may be mammalian host cells. A non-limiting example of host cells in which a preselected binding agent can be delivered and subsequently imaged using a method of the invention
20 are excitable cells, which include host cells able to produce and respond to electrical signals. Examples of excitable cell types include, but are not limited to neurons, muscles, cardiac cells, and secretory cells (such as pancreatic cells, adrenal medulla cells, pituitary cells, etc.). A host cell into which a preselected binding agent is delivered in an embodiment of a method of the invention is one or more of: a single cell, an isolated cell,
25 a cell that is one of a plurality of cells, a cell that is one in a network of two or more interconnected cells, a cell that is one of two or more cells that are in physical contact with each other, etc.

Non-limiting examples of cells that may be used as host cells in embodiments of methods of the invention include: nervous system cells, neurons, cardiac cells, circulatory
30 system cells, visual system cells, auditory system cells, secretory cells, endocrine cells, and muscle cells. In some embodiments, a cell used in conjunction with the invention may be a healthy normal cell, which is not known to have a disease, disorder or abnormal condition. In some embodiments, a host cell used in conjunction with methods and compositions of the invention is an abnormal cell, for example, a cell obtained from a
35 subject diagnosed as having a disorder, disease, or condition, including, but not limited to a degenerative cell, a neurological disease-bearing cell, a cell model of a disease or condition, an injured cell, etc. In some embodiments of the invention, a cell may be a

5 control cell. In some aspects of the invention a host cell can be a model cell for a disease or condition.

In embodiments of the invention a cell may be a free cell in culture, a free cell obtained from a subject, a cell obtained in a solid biopsy from a subject, organ, or solid culture, etc. In certain embodiments of the invention, means for separating a cell or
10 plurality of cells for fixation, staining, and detection methods of the invention may include art-known means such as, but not limited to: dissecting one or a plurality of cells from the cell's source and sectioning a tissue that includes the cell, for example using cryostat sectioning, vibratome sectioning, microtome sectioning, etc. In some embodiments of the invention, a method of the invention may be performed, in part, on a cell section, which
15 means on a portion of a cell that has been removed from the cell. Routine means of cell sectioning may be used to obtain one or more sections from a tissue and/or cell on which later binding and imaging steps may be performed. Cell sectioning may include one or more of frozen sectioning, paraffin sectioning, sectioning of host cells in tissues infiltrated with one or more agents such as epoxy, methacrylate resins, paraffin wax-based
20 histological waxes, etc. In some embodiments of the invention sections of host cells and tissues may be obtained using cryostat sectioning, vibratome sectioning, microtome sectioning, or other suitable art-known tissue and/or cell sectioning means.

In certain embodiments of the invention, a preselected binding agent is delivered to one or more cells in cell culture, a cell suspension, a cell in a solution, a cell obtained from
25 a subject, and a cell in a subject (*in vivo* cells). Methods of the invention may be carried out in in cultured cells, cultured tissues (e.g., brain slice preparations, etc.), and in living subjects, etc. As used herein, the term "subject" may refer to a: human, non-human primate, cow, horse, pig, sheep, goat, dog, cat, rat, mouse, fly or other host organism. In some aspects of the invention a host subject is an invertebrate subject and in other aspects
30 of the invention a host subject is a vertebrate. In certain embodiments of the invention, a host subject is a mammal. In some aspects of the invention one or more preselected binding agents is delivered into at least one host cell in a host subject and the host cell(s) are subsequently removed from the subject and fixed and further processed according to methods of the invention outside of the subject. A non-limiting example of a case in
35 which such an approach may be used is a situation in which the host subject is a human. Alternatively, a host cell may be removed from a subject prior to delivery of one or more preselected binding agents.

5 In some aspects of the invention one or more preselected binding agents is delivered into at least one host cell in a host subject and the host cell(s) are fixed prior to removal from the subject. Further processing of the fixed cells according to methods of the invention may occur in the subject or the fixed host cells may be removed from the host subject and further processed according to methods of the invention. Non-limiting
10 examples of host subjects in which such an approach may be used are: invertebrates, a rodent, a bird, or other non-human subjects.

 Some embodiments of the invention include of one or a plurality of host cells in methods and compositions for genetically targeted detection. Such methods may include use of whole, living cells into which are delivered one or more preselected binding agents
15 that include independently selected programmable binding sequences, wherein each preselected binding agent is capable of selectively binding a corresponding matched specific target agent. The whole host cells are then fixed under conditions wherein the one or more preselected binding agents in the fixed host cell is capable of selectively binding the matched specific target agent to which it corresponds. In some embodiments of the
20 invention the whole, now fixed, host cells are contacted with the matched specific target agents corresponding to the one or more preselected binding agents, and binding of the one or more preselected binding agents and their corresponding matched specific target agents is determined in the whole fixed host cell or plurality of whole fixed host cells. In
25 alternative embodiments of the invention the whole, now fixed, host cells are sectioned and the fixed host cell sections are contacted with the matched specific target agents corresponding to the one or more preselected binding agents, and binding of the one or more preselected binding agents and corresponding matched specific target agents is determined in the fixed cell section. As used herein, the term "portion" of a cell may be a section of a cell, and the phrase: "at least a portion of the host cell" means (1) the entire
30 host cell, or (2) some, but less than all of the host cell, non-limiting examples of which are: a section or multiple sections obtained from the host cell.

Controls and Use

 In certain embodiments of compositions and methods of the invention, one or more
35 preselected binding agents are delivered into a host cell, or a localized region of a host cell, for example the soma, dendrite, axon, etc. Methods of the invention can be used to contact the host cell with one or more matched binding agents and to detect the bound pair in the host cell after fixation. Certain embodiments of methods of the invention can be

5 used to assess changes in a host cell, host, tissue, and/or a host subject by comparing results obtained in sample host cells with results in a control host cell. Some embodiments of the invention include delivery of a preselected binding agent into a host cell and contacting the host cell with one or more candidate compounds or treatments prior to fixing the host cell. In some embodiments, a host cell is contacted with a candidate
10 compound or treatment before delivery of one or more preselected binding agents to the host cell. In certain embodiments, the contact with the candidate compound or treatment occurs after delivery of one or more preselected binding agents to the host cell, tissue, and/or subject. Following suitable fixation, the host cells are contacted with matched specific binding agents, binding is detected, and one or more characteristics of the
15 delivered preselected binding agent determined by assessing the binding. Results in host cells contacted with a candidate compound or treatment may be compared to results from host cells prepared, fixed, and detected, under similar conditions except without contact with the candidate compound or treatment. Differences in the binding can be used to assess the impact of the candidate compound or treatment on one or more characteristics
20 of the preselected binding agent.

It will be understood that results obtained from the host cell, tissue, or subject that was not contacted with the candidate compound or treatment may be referred to as “control results” and the non-contacted host cells as “control host cells”. As used herein a control may be as described above and also may be a predetermined value, which can take
25 a variety of forms. It can be a single cut-off value, such as a median or mean. It can be established based upon comparative groups. Other examples of comparative groups may include host cells or tissues that have a disorder or condition and groups without the disorder or condition. Another comparative group may be host cells from a group with a family history of a disease or condition and host cells from a group without such a family
30 history. A predetermined value can be arranged, for example, where a tested population is divided equally (or unequally) into groups based on results of testing. Those skilled in the art are able to select appropriate control groups and values for use in comparative methods of the invention.

A host cell, tissue, and/or a subject that include a host cell can be monitored for the
35 presence or absence of a change that occurs in test conditions versus a control condition. For example, in a host cell, a characteristic such as level of expression or cell localization may differ in a host cell contacted with a candidate compound or treatment compared to the level of expression or localization in a control host cell. In some embodiments of

5 methods of the invention a characteristic that can be assessed by detecting binding of a preselected target agent and its matched binding agent may be one or more of: a downstream effect on one or more additional cells, which occurs due to the modulation of an activity in the host cell to which the preselected binding agent was delivered.

Candidate-compound candidate-treatment identification methods of the invention
10 may be carried out in a host cell in a subject or in cultured or *in vitro* host cells. Candidate-compound identification methods of the invention that are performed in a host subject, may include delivery of a preselected binding agent into a cell in the subject, contacting the subject with the candidate compound or treatment (before and/or after delivering the preselected binding agent), fixing the host cells, and contacting the fixed
15 host cell with a matched binding agent, and detecting the binding as an assessment of one or more characteristics of the preselected binding agent. A result of contacting a host cell, tissue, and/or subject with a candidate compound or candidate treatment can be measured and compared to a control value as a determination of the presence or absence of an effect of the candidate compound or candidate treatment, respectively, on a characteristic in the
20 host cell.

Further information that can be obtained using methods of the invention to detect bound pairs of binding agents and their specific target agents in one or a plurality of fixed host cells includes but is not limited to: the level of expression of the preselected binding agent and the effect on expression and/or localization of the binding agent resulting when
25 prior to fixation, the host cell is contacted with a candidate modulatory compound, toxin, or other test agent. Methods of the invention can be used to assess one or more aspects of up to dozens or hundreds of different preselected binding agents a host cell or plurality of host cells. Non-limiting examples of procedures in which methods of the invention may be used are: screening for effects of candidate agents on host cells; assessing the internal
30 environment of host cells and its response to external changes; identifying specific individual host cells in a plurality of host cells in which two or more of the host cells comprise independently selected different preselected binding agents; and determining localization and expression of one or more specific binding agents in neuronal host cells as a means of mapping projections and connections of such cells at the level of single cell
35 resolution.

Compositions

5 An agent, host cell, compound, or molecule used in a method of the invention may be part of a composition. As used herein, a composition means a solution in which the agent, compound, or molecule is present. Non-limiting examples of compositions are: culture media, a diluent, a solution, or other environment in which the agent, host cell, compound or molecule is located. A non-limiting example of a composition is a solution
10 that comprises a vector for delivery into a host cell in a method of the invention. Another non-limiting example of a composition is a solution in which target agents are present when contacted with a fixed host cell. In addition to one or more of an agent, host cell, vector, molecule, or compound, molecule, a composition used in certain methods of the invention may include one or more of: a liquid, an aqueous solution, culture media, a
15 solute, a preservative, a fixative, or other suitable component.

 In some aspects of the invention, a composition may include all or part of a fixed cell that includes at least one preselected programmable binding agent bound to its matched specific target agent, thereby forming a bound complex. The preselected binding agent includes an independently selected programmable binding sequence, and the
20 preselected binding agent is selected, at least in part, based on its capability to selectively bind its matched specific target agent. In such compositions of the invention, the fixed cell or the part of the fixed cell include two or more different independently preselected programmable binding agents bound to their matched specific target agents each forming a bound complex.

25 A composition used in a method of the invention can but need not be a pharmaceutical composition. The term “pharmaceutical composition” as used herein, means a composition that comprises at least one pharmaceutically acceptable carrier that is useful in preparing a pharmaceutical composition that is generally safe, non-toxic, and neither biologically nor otherwise undesirable. A pharmaceutical composition may be
30 used in certain embodiments of methods of the invention, if, as a non-limiting example, one or more preselected binding agents are to be delivered into a host cell in a host subject. In certain embodiments of the invention, at least one pharmaceutical composition is utilized in the delivery of one or more preselected binding agents to one or more host cells in a subject, host cells comprising the delivered preselected binding agent are
35 removed from the subject and are fixed subsequent to their removal. In such cases the use of pharmaceutical compositions may be advantageous for the health and safety of the host subject.

5 In certain aspects of the invention a pharmaceutical composition comprises one or more preselected binding agents, encoding molecules, a vector that are delivered to a host cell, a host tissue, host subject, and/or a host cell in a subject, in combination with one or more additional preselected binding agents and/or in combination with one or more additional molecules, therapeutic agents, candidate agents, candidate treatments, and
10 therapeutic regimens that are also administered to the host cell, host tissue, host subject, and/or host cell in a subject. A pharmaceutical composition used in the foregoing methods may, in addition to one or more preselected binding agents, contain an effective amount of a candidate compound or therapeutic compound to alter a characteristic of a preselected binding agent in a host cell in the host subject. In some embodiments of the invention, a
15 pharmaceutical composition of the invention may include a pharmaceutically acceptable carrier.

 Pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers and other materials that are well-known in the art. Exemplary pharmaceutically acceptable carriers are described in U.S. Pat. No. 5,211,657 and others
20 are known by those skilled in the art. In certain embodiments of the invention, such preparations may contain salt, buffering agents, preservatives, compatible carriers, aqueous solutions, water, etc.

 Delivery of a preselected binding agent into a host cell may be achieved by various means described herein and other art-known means. Such administration may be done
25 once, or a plurality of times prior to fixation of the host cell. If administered multiple times to a host subject, one or more preselected binding agents, polypeptides, encoding nucleic acids, and/or vectors may be administered via different routes. For example, though not intended to be limiting: a first (or the first few) administrations may be made directly into a tissue in the host subject while later administrations may be systemic.

30 The amount of a preselected binding agent delivered into a host cell, or the amount of a target agent that contacts a fixed host cell are amounts sufficient to permit sufficient binding of the preselected target agent to its matched target agent in a fixed cell and to permit detection of a detectable label attached to the target agent for determination of one or more characteristics of the preselected binding agent in the host cell. Suitable amounts
35 can be readily determined by a practitioner using teaching provided herein in conjunction with art-known methods, and without a need for undue experimentation.

Examples

5 Example 1

Studies were performed to assess embodiments of visualization methods of the invention in cells. In some of the studies cells were transfected with and expressed PL61, which specifically interacts with Hairpin 13 but not Hairpin 14. In other studies, cells were transfected with and expressed PL62, which specifically interacts with Hairpin 14 but not Hairpin 13. The comparison of the results demonstrated the ability of
 10 embodiments of methods of the invention to permit specific visualization of polypeptides in cells.

Materials and Methods15 *Cells*

Human embryonic kidney (HEK) cells

Hairpins

Hairpins (sequences shown below) were diluted to 1 μ M in TALE binding buffer and
 20 heated to 95°C for 90 seconds, and then held at room temperature for 30 minutes.

TALE binding buffer

The buffer was prepared with 25 mM Tris, 250 mM NaCl, 5% glycerol and 5 mM TCEP, pH 8.5.

25

Sequences

PL61: CAG-NLS-TALE(13)-mRuby2, which expresses a TAL effector that targets the sequence set forth as: tgctacgactaggtatacct (SEQ ID NO: 1). This sequence is from the mouse genome and is not present in human genome. A fusion protein expressed from
 30 PL61: CAG-NLS-TALE(13)-mRuby2 is also referred to herein as PL61 TALEs.

PL62: CAG-NLS-TALE(14)-mRuby2, which expresses a TAL effector that targets the sequence set forth as: tcggacggcccttcgccatc (SEQ ID NO: 2). This sequence from the mouse genome and is not present in human genome. A fusion protein expressed from
 35 PL62: CAG-NLS-TALE(14)-mRuby2 is also referred to herein as PL62 TALEs.

Hairpin 13: /5Cy5/agatatgctacgactaggtatacctccgttaaaaaaacggaggtatacctagtcgtagcatatct (SEQ ID NO: 3). SEQ ID NO: 3 comprises the target sequence set forth as SEQ ID NO: 1

- 5 and a detectable label. SEQ ID NO: 3 may be referred to herein as a specific target agent that is matched to the preselected binding agent, the TALE(13) effector. SEQ ID NO: 20 is the DNA sequence set forth as SEQ ID NO: 3 without the 5Cy5 label.

Hairpin 14:

- 10 5Cy5/agatatcgacggcccttcgccatcccggttaaaaaaacgggatggcgaagggccgtccgatatct (SEQ ID NO: 4). SEQ ID NO: 4 comprises the target sequence set forth as SEQ ID NO: 2 and a detectable label. SEQ ID NO: 4 may be referred to herein as a specific target agent that is matched to the preselected binding agent, the TALE(14) effector. SEQ ID NO: 21 is the DNA sequence set forth as SEQ ID NO: 4 without the 5Cy5 label.

15

Experimental procedures were as follows:

- (1) HEK cells were cultured on glass-bottom culture wells (Grace BioLabs) that had been pretreated with matrigel as per routine practice.
- 20 (2) The cells were then transfected with plasmids PL61 or PL62 (described below), using Trans-IT X2 transfection reagent according to manufacturer's instructions (Mirus Bio, Madison, WI).
- (3) Cells were given 24 hours to express the construct, and expression was validated by
- 25 the presence of fluorescence in the cells.

(4) Cells were fixed using one of the following Fixation Protocols.

Fixation Protocol 1

Cells were contacted with 10% formalin at room temperature for 10 minutes.

30

Fixation Protocol 2

Cells were contacted with a solution comprising 4% formaldehyde/2.5% glutaraldehyde in PBS for 10 minutes at room temperature.

35 Fixation Protocol 3

Cells were contacted with a solution precooled to -20°C and comprising 95% methanol and 5% acetic acid. After application of this solution to the cells, the cells were placed at -20°C for 10 minutes.

5

Fixation Protocol 4

Cells were contacted with a solution pre-cooled to -20°C and comprising 95% ethanol and 5% acetic acid that. Following application of this solution to the cells, the cells were placed at -20°C for 10 minutes.

10

Fixation Protocol 5

Cells were contacted with 100% acetone that was pre-cooled to -20°C. Following application of this solution to the cells, the cells were placed at -20°C for 10 minutes.

15 (5) After the fixation procedure, the fixed cells were processed as follows:

- a) all fixed cells were washed twice in PBS;
- b) cells that had been fixed with aldehyde solutions were washed for 15 minutes in a solution of 0.5% Triton X-100 in PBS to permeabilize the cells;
- c) all cells were washed twice in TALE binding buffer (see below);
- 20 d) all cells were contacted with a solution of 1µM hairpins in TALE binding buffer for one hour; and
- e) all cells were washed in TALE binding buffer to remove excess hairpins.

25

(6) All processed cells were imaged using standard fluorescence-imaging methods.

Results

Fig. 1A-H and Fig. 2A-H show results from studies using the methanol fixation method (#3 above) and the ethanol fixation method (#4 above). These studies demonstrated successful visualization in the cells using embodiments of methods of the invention.

30

Example 2

Experiments were performed to assess different fixation protocols. In one study, ethanol fixation methods were assessed. See Example 1 for sequences and methods.

35

Ethanol fixation study:

In HEK cells transfected with PL61, fixed with the ethanol protocol (method #4, Example 1), and stained with Hairpin 13, Fig. 1A shows the location of the TALEs,

5 evidenced by the detected fluorescence of mRuby. Co-localization of the PL61 TALEs and the hairpin 13 was evidenced in Fig. 1B, which shows detected fluorescence of Cy5 (attached to hairpin 13), in the same location as the mRuby signal of Fig. 1A.

In HEK cells transfected with PL62, fixed with the ethanol protocol (method #4, Example 1), and stained with Hairpin 13, Fig. 1C shows the location of the TALEs, evidenced by the detected fluorescence of mRuby. Fig. 1D shows a lack of detected fluorescence indicating that there was no co-localization of the PL62 TALEs and hairpin 13.

In experiments that included HEK cells transfected with and expressed PL62, fixed as using Fixation Protocol 4, Example 1, and stained with Hairpin 14. Fig. 1E shows the location of the PL62 TALEs, evidenced by the detected fluorescence of mRuby. Fig. 1F shows a lack of detected fluorescence, which indicated that there was no co-localization of the PL62 TALEs and hairpin 14.

In studies that included HEK cells transfected and expressing with PL62, fixed with the ethanol protocol (Fixation Protocol 4, Example 1), and stained with Hairpin 14, Fig. 1G shows the location of the PL62 TALEs, evidenced by the detected fluorescence of mRuby. Co-localization of the PL62 TALEs and the hairpin 14 was evidenced in Fig. 1H, which shows detected fluorescence of Cy5 (attached to hairpin 14), in the same location as the mRuby signal of Fig. 1G.

25 Example 3

Methanol fixation study:

Experiments were performed to assess different fixation protocols. In one study, methanol fixation methods were assessed. See Example 1 for sequences and methods.

In HEK cells transfected with PL61, fixed with the methanol protocol (method #3 in Example 1), and stained with Hairpin 13, Fig. 2A shows the location of the TALEs, evidenced by the detected fluorescence of mRuby. Co-localization of the PL61 TALEs and the hairpin 13 was evidenced in Fig. 2B, which shows detected fluorescence of Cy5 (attached to hairpin 13), in the same location as the mRuby signal of Fig. 2A.

In HEK cells transfected with PL62, fixed with the methanol protocol (method #3., Example 1), and stained with Hairpin 13, Fig. 2C shows the location of the TALEs, evidenced by the detected fluorescence of mRuby. Fig. 2D shows a lack of corresponding detected fluorescence, which demonstrated that there was no co-localization of the PL62 TALEs and hairpin 13.

5 In experiments that included HEK cells transfected with and expressed PL62, fixed as using Fixation Protocol 3 (Example 1), and stained with Hairpin 14. Fig. 2E shows the location of the PL62 TALEs, evidenced by the detected fluorescence of mRuby. Fig. 2F shows a lack of detected fluorescence, which indicated that there was no co-localization of the PL62 TALEs and hairpin 14.

10 In studies that included HEK cells transfected and expressing with PL62, fixed with the methanol protocol (Fixation Protocol 3, Example 1), and stained with Hairpin 14, Fig. 2G shows the location of the PL62 TALEs, evidenced by the detected fluorescence of mRuby. Co-localization of the PL62 TALEs and the hairpin 14 was evidenced in Fig. 2H, which shows detected fluorescence of Cy5 (attached to hairpin 14), in the same location as
15 the mRuby signal of Fig. 2G.

The studies and results demonstrate the effective use of embodiments of methods of the invention to specifically label and visualize target proteins expressed in cells using nucleic acid molecules that bind to the targeted proteins.

20 Example 4

Additional Tale experiments were performed using methods described in Example 1, except using a Tale with the sequence set forth as SEQ ID NO: 5 also referred to herein as SRC-60B. Imaging results of experiments demonstrated that SRC-60B (SEQ ID NO: 5) bound the following sequence:

25 /5Atto488N/AGGAATTCATACTGCTCGACGTAAGTACCCGTTAAAAAAACGG
GTCAGTTACGTCGAGCAGATATGAATTCCT (SEQ ID NO: 6).

Example 5

30 Studies were performed to assess embodiments of visualization methods that include use of zinc fingers as programmable DNA binding proteins in cells. The results demonstrated the ability of embodiments of methods of the invention to permit specific visualization of polypeptides in cells. These studies included staining zinc fingers, (programmable DNA binding proteins) with conjugate DNA strands following methanol or PFA fixation.

35

Materials

Cells used: Human embryonic kidney (HEK) cells

5 *Probes*

Two probe sequences (sequences shown below) were mixed at a 1:1 ratio in ZBB, heated to 95°C and then brought to room temperature.

ZF binding buffer

- 10 The zinc binding buffer (ZBB) included PBS with 5% BSA, 20 μ M ZnCl₂, 1 mM MgCl₂ and 100 μ g/ml salmon sperm DNA.

ZF imaging buffer

- The zinc binding buffer (ZIB) included PBS with 20 μ M ZnCl₂, 1 mM MgCl₂ and 100
15 μ g/ml salmon sperm DNA

Sequences

The DNA sequences of three zinc fingers used in experiments were:

- SRC-140-4 set forth herein as SEQ ID NO: 7.
20 SRC-140-5 set forth herein as SEQ ID NO: 8.
SRC-140-CODA-3 set forth herein as SEQ ID NO: 9.

- A double-stranded probe was used in experiments with SRC-140-4. The probe was prepared by annealing the following two sequences: acatcGAGGACGGCagcgt (SEQ ID
25 NO: 10) and
acgctGCCGTCCTCgatgTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/ (SEQ ID NO: 11).

- A double-stranded probe was used in experiments with SRC-140-5. The probe was
30 prepared by annealing the following two sequences: ttgaaGAAGATGGTgagcgt (SEQ ID NO: 12) and
agcgcACCATCTTctcaaTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/ (SEQ ID NO: 13).

- 35 For experiments with SRC-140-CODA-3, the following two probe sequences were used:
gccgaGAAGACGAatacatTATAAGCGGGAGATTCGTCCTCATA/3AlexF546N/ (SEQ ID NO: 14) and atgtaTTCGTCTTctggc (SEQ ID NO: 15).

- 5 The DNA sequence of the complete plasmid encoding 140-CODA-3 is set forth herein as SEQ ID NO: 16.

Methods

- 10 (1) HEK cells were cultured on glass-bottom culture wells (Grace BioLabs) that had been pretreated with matrigel as per routine practice.

- (2) The cells were then transfected with plasmids set forth herein as : SRC-140-4, set forth herein as SEQ ID NO: 7 and SRC-140-5, set forth herein as SEQ ID NO: 8, using Trans-IT X2 transfection reagent according to manufacturer's instructions (Mirus Bio, Madison, WI). In some studies cultured HEK cells were transfected with plasmids set forth herein as SRC-140-CODA-3, set forth herein as SEQ ID NO: 9, using Trans-IT X2 transfection reagent according to manufacturer's instructions (Mirus Bio, Madison, WI).

- 20 (3) Cells were given 24 hours to express the construct, and expression was validated by the presence of fluorescence in the cells.

- (4) Cells were fixed using one of the following Fixation Protocols.

- 25 *Fixation Protocol 1 – Standard 10% fixation protocol, with Alternative fixation protocols with 100% methanol and 70% ethanol.*

1. Fix cells 20 minutes in 10% normal-buffered formalin, or 4% paraformaldehyde.
2. Permeabilize cells 5 minutes in 0.1% triton-X in 1x PBS.
 - a. Alternative: permeabilize cells 5 minutes in 100% methanol or permeabilize cells 5 minutes in 70% ethanol.
- 30 3. Wash cells one time (1x) for 5 minutes in 1x PBS.
 - a. Alternative: For cells permeabilized with methanol or ethanol in Step (4)2.a, wash cells two times (2x), 5 minutes each time in PBS.
4. Block 30 minutes in zinc binding buffer (ZBB).
5. Incubate blocked cells 30 minutes with hairpins at room temperature (RT).
- 35 6. Wash cells 3 times, 10 minutes each time in zinc imaging buffer (ZIB).
7. Replace ZIB solution with DAPI in ZIB and image cells after 5 minutes

5 *Fixation Protocol 2: Standard methanol fixation protocol*

1. Fix cells 20 minutes in ice-cold 95% methanol/5% acetic acid.
2. Wash cells 2 times, 5 minute each time, in 1x PBS.
3. Block 30 minutes in zinc binding buffer (ZBB).
4. Incubate blocked cells 30 minutes with hairpins.
- 10 5. Wash cells 3 times, 10 minutes each time, in zinc imaging buffer (ZIB).
6. Replace ZIB with DAPI in ZIB and image cells after 5 minutes.

(5) All processed cells were imaged using standard fluorescence-imaging methods.

15 Results

Experimental results from experiments using SRC-140-CODA-3 are shown in Fig. 3A-H, in which the Zinc fingers were observed to bind to their cognate DNA sequences following fixation with PFA (see Step 4, Fixation Protocol 1) and with methanol (see Step 4, Fixation Protocol 2). Staining after fixation in methanol was roughly 5x stronger than staining after fixation in PFA. As in the case of the TALEs (see previous Examples), the fingers only bound to their cognate binding sequences. Similar results were seen in studies performed using SRC-140-4 and its probes, and in studies performed using SRC-140-5 and its probes.

25 Example 6

The studies performed in Example 5 are repeated as described except using ethanol fixation is used instead of methanol fixation. In results of these studies the Zinc fingers are observed to bind to their cognate DNA sequences following fixation with ethanol. As in the case of the TALEs, the fingers only bind to their cognate binding sequences.

30

Example 7

In vivo studies were performed to test the binding of ZIFs to their hairpins in mouse brain. In these experiments, a mouse brain was injected intracranially with an AAV PHP.eB coding for a zinc finger protein (see Chan, K. et al., (2017) Nature Neuroscience Vol. 20, 1172-1179 and Deverman, B., et al., (2016) Nature Biotechnology Vol. 34:204-209, the content of each of which is incorporated by reference herein in its

5 entirety). The vector expressed the SRC-140-4 protein, which is encoded by nucleic acid sequence set forth as SEQ ID NO: 7), in the mouse brain.

a) In some studies, after 3 weeks, the mouse was transcardially perfused with methanol, the brain removed and immersed in methanol. After 2 days in methanol, the brain was equilibrated in PBS with 30% sucrose until it sank, and 50µm sections were cut
10 on the cryostat. The sections were equilibrated in ZBB for 12 hours, and stained with the double-stranded probes in ZBB similar to as described for cell culture in other Examples herein. Results indicated the co-localization of the GFP signal and the zinc finger binding signal.

b) In some studies, at two weeks post-injection, the mouse was perfused with ice-
15 cold 95% methanol, 5% acetic acid and the brain sliced into 50µm coronal sections on a vibratome. Following blocking in zinc binding buffer, sections were stained and Fig. 4A-B shows staining with the fluorescent cognate oligonucleotide hairpins (Fig. 4A) and with DAPI (Fig. 4B).

20 Equivalents

Although several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or
25 modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used.
30 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and
35 claimed. The present invention is directed to each individual feature, system, article, material, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, and/or methods, if such features, systems,

5 articles, materials, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

Where a range of values is provided, it is understood that each intervening value is encompassed. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

10 All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least
15 one.” The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified,
20 unless clearly indicated to the contrary.

All references, patents and patent applications and publications that are cited or referred to in this application are incorporated by reference in their entirety herein.

What is claimed is:

25

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Claims

1. A method of genetically targeted detection, comprising:
 - a) delivering to at least one host cell one or more preselected binding agents comprising an independently selected programmable binding sequence, wherein each preselected binding agent is capable of selectively binding a corresponding matched specific target agent;
 - b) fixing the at least one host cell under conditions wherein the one or more preselected binding agents in the fixed host cell is capable of selectively binding the matched specific target agent to which it corresponds;
 - c) contacting the at least one fixed host cell with a composition comprising the matched specific target agents corresponding to the one or more preselected binding agents; and
 - d) determining the binding of the one or more preselected binding agents and corresponding matched specific target agents in the at least one fixed host cell.
2. The method of claim 1, wherein the preselected binding agent comprises one or more of a polypeptide molecule and a nucleic acid molecule.
3. The method of claim 1 or 2, wherein the preselected binding agent comprises a polypeptide/nucleic acid complex.
4. The method of any one of claims 1-3, wherein each of the independently selected programmable binding sequences correspond to a nucleic acid sequence in a matched specific target agent.
5. The method of claim 1, wherein 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different independently selected programmable binding sequences are delivered into the host cell.
6. The method of claim 5, wherein each of the programmable binding sequence corresponds to and is capable of selectively binding a matched specific target agent.
7. The method of any one of claims 1-6, wherein the preselected binding agent comprises at least one of: a nucleic acid-binding molecule and a nucleic acid analog.

5 8. The method of claim 7, wherein the nucleic acid-binding molecule is one or more
of: a nucleic acid-binding polypeptide, a DNA-binding molecule, and a DNA-binding
polypeptide.

9. The method of any one of claims 1-8, wherein the preselected binding agent
10 comprises a transcription activator-like effector (TALE) sequence.

10. The method of any one of claims 1-8, wherein the preselected binding agent
comprises a megaTAL effector sequence.

15 11. The method of any one of claims 1-8, wherein the preselected binding agent
comprises a CRISPR/Cas9 sequence or Cas9 analog sequence effector sequence.

12. The method of claim 11, wherein the Cas9 analog sequence comprises a Cas13a
(c2c2), Cpf1, or MIMIVIRE sequence.

20 13. The method of any one of claims 1-8, wherein the preselected binding agent
comprises a zinc finger (ZF) effector sequence.

14. The method of any one of claims 1-13, wherein the specific target agent comprises
25 at least one double-stranded nucleic acid molecule.

15. The method of any one of claims 1-13, wherein the specific target agent comprises
at least one single-stranded nucleic acid molecule.

30 16. The method of any one of claims 1-15, wherein the specific target agent comprises
one or more independently selected detectable labels.

17. The method of any one of claims 1-15, wherein the preselected programmable
binding agent comprises a detectable label.

35 18. The method of claim 16 or 17, wherein the detectable label comprises a fluorescent
label.

5 19. The method of any one of claims 1-18, wherein fixing the at least one host cell comprises contacting the host cell with at least one cell fixative.

20. The method of claim 19, wherein the cell fixative comprises at least one of: MeOH, EtOH, acetone, an amine, a cleavable molecule, a cleavable polypeptide, a
10 cleavable cross-linker molecule, and an aldehyde.

21. The method of any one of claims 1-20, wherein fixing comprises contacting the at least one host cell with an alcohol solution.

15 22. The method of claim 21, wherein the alcohol solution comprises at least one of MeOH and EtOH.

23. The method of any one of claims 1-22, wherein the at least one host cell is a plurality of host cells comprising one or more cell types.

20 24. The method of any one of claims 1-23, wherein a means for determining a characteristic of the one or more preselected binding agents in the fixed host cell, comprises detecting the one or more specific target agents bound to the matched preselected binding agent in the contacted fixed host cell.

25 25. The method of claim 24, wherein the characteristic is one or more of: localization, presence, absence, and amount.

26. The method of any one of claims 1-25, wherein the at least one host cell is
30 obtained from a subject.

27. The method of any one of claims 1-25, wherein the at least one host cell is obtained from culture.

35 28. The method of any one of claims 1-27, wherein the at least one host cell comprises a neuron.

5 29. The method of any one of claims 1-28, wherein the one or more preselected binding agent is a plurality of independently preselected binding agents.

30. The method of any one of claims 1-29, wherein the preselected binding agent is delivered to the at least one host cell using a physical delivery means.

10

31. The method of claim 30, wherein the physical delivery means comprises at least one of microinjection, electroporation, ballistic delivery, and laser delivery.

32. The method of any one of claims 1-29, wherein the preselected binding agent is
15 delivered to the at least one host cell by means of a vector.

33. The method of claim 32, wherein the vector is a viral vector or a non-viral vector.

34. The method of claim 33, wherein the viral vector is a lentivirus vector (LV), an
20 adenovirus vectors (AdV), an adeno-associated virus vectors (AAV), or a herpes simplex-1 virus vectors (HSV-1s).

35. The method of claim 33, wherein the non-viral vector is a lipid nanoparticle (LNP), a liposome, a polymer, or a cell-derived membrane vesicle (CMV).

25

36. The method of any one of claims 1-29, wherein the preselected binding agent is delivered to the at least one host cell by a means comprising a cell-penetrating molecule.

37. The method of claim 36, wherein the cell-penetrating molecule comprises a cell-
30 penetrating polypeptide.

38. The method of any one of claims 1-37, wherein determining the binding of the one or more preselected programmable binding agents and corresponding matched specific target agents comprises:

- 35
- i. contacting the corresponding matched specific target agents with two or more independently selected detectable labels;
 - ii. imaging the detectable labels;
 - iii. quenching the detectable labels; and

5 iv. repeating steps (i.)-(iii.) one or more times.

39. The method of any one of claims 1-37, wherein determining the binding of the one or more preselected binding agents and corresponding matched specific target agents comprises simultaneously contacting one or more of the corresponding matched specific target agents with two or more independently selected detectable labels and sequentially
10 detecting the detectable labels.

40. The method of claim 39, wherein the detectable labels are detected with a means comprising a fluorescent in situ hybridization (FISH) method.

15 41. The method of any one of claims 1-40, wherein the cell is a vertebrate cell, optionally a mammalian cell, optionally a human cell.

42. The method of any one of claims 1-41, further comprising sectioning the host cell
20 after fixation of the host cell.

43. A composition comprising: at least a portion of a fixed cell comprising at least one preselected programmable binding agent bound to its matched specific target agent, forming a bound complex, wherein the preselected binding agent comprises an
25 independently selected programmable binding sequence, and wherein the preselected binding agent is selected, in part, based on its capability to selectively bind its matched specific target agent.

44. The composition of claim 43, wherein the at least a portion of the fixed cell
30 comprises two or more different independently preselected programmable binding agents bound to their matched specific target agents each forming a bound complex.

45. The composition of claim 43 or 44, wherein the bound complex comprises a detectable label.

35 46. The composition of any one of claims 43-45, wherein the preselected binding agent comprises one or more of a polypeptide molecule and a nucleic acid molecule.

5 47. The composition of any one of claims 43-45, wherein the preselected binding agent comprises a polypeptide/nucleic acid complex.

48. The composition of any one of claims 43-47, wherein each of the independently selected programmable binding sequences correspond to a nucleic acid sequence in a
10 matched specific target agent.

49. The composition of any one of claims 43-48, wherein the preselected binding agent comprises at least one of: a nucleic acid-binding molecule and a nucleic acid analog.

15 50. The composition of claim 49, wherein the nucleic acid-binding molecule is one or more of: a nucleic acid-binding polypeptide, a DNA-binding molecule, and a DNA-binding polypeptide.

51. The composition of any one of claims 43-50, wherein the preselected binding agent
20 comprises a transcription activator-like effector (TALE) sequence.

52. The composition of any one of claims 43-50, wherein the preselected binding agent comprises a megaTAL effector sequence.

25 53. The composition of any one of claims 43-50, wherein the preselected binding agent comprises a CRISPR/Cas9 sequence or Cas9 analog sequence effector sequence.

54. The composition of claim 53, wherein the Cas9 analog sequence comprises a Cas13a (c2c2), Cpf1, or MIMIVIRE sequence.

30

55. The composition of any one of claims 43-50, wherein the preselected binding agent comprises a zinc finger (ZF) effector sequence.

56. The composition of any one of claims 43-55, wherein the specific target agent
35 comprises at least one double-stranded nucleic acid molecule.

57. The composition of any one of claims 43-55, wherein the specific target agent comprises at least one single-stranded nucleic acid molecule.

5

58. The composition of claim 45, wherein the detectable label comprises a fluorescent label.

59. The method of any one of claims 43-58, wherein the fixed cell is a neuron.

10

60. The composition of any one of claims 43-59, wherein the fixed cell is a vertebrate cell, optionally is a mammalian cell, and optionally is a human cell.

15

61. The composition of any one of claims 43-60, wherein the preselected binding agent is part of a fusion protein.

62. The composition of any one of claims 43-61, wherein the at least a portion of the fixed cell includes all of the fixed cell.

20

63. The composition of any one of claims 43-61, wherein the at least a portion of the fixed cell comprises a section obtained from the fixed cell.

Fig. 1A

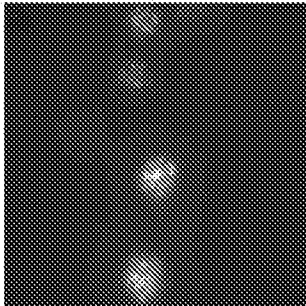


Fig. 1B

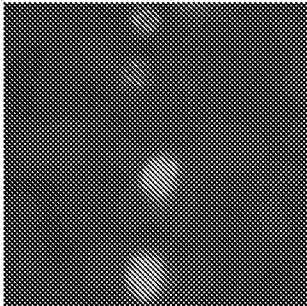


Fig. 1C

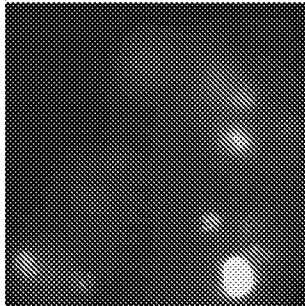


Fig. 1D

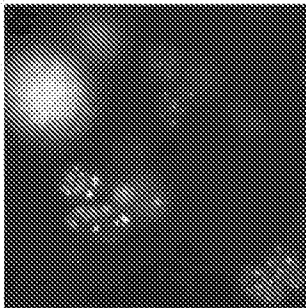
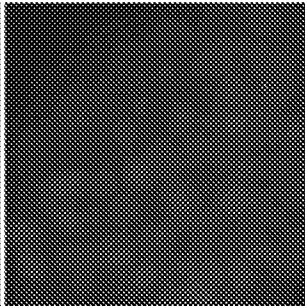


Fig. 1E

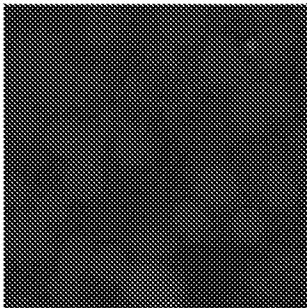


Fig. 1F

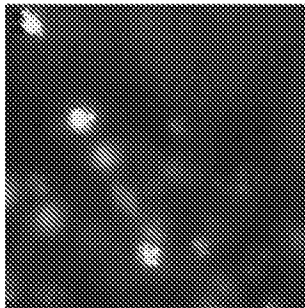


Fig. 1G

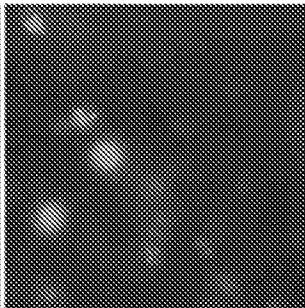


Fig. 1H

Fig. 2A

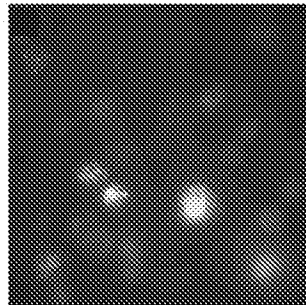


Fig. 2B

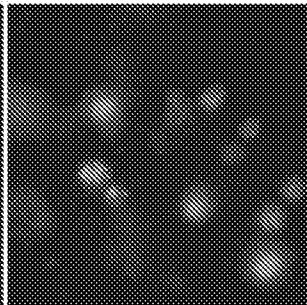


Fig. 2C

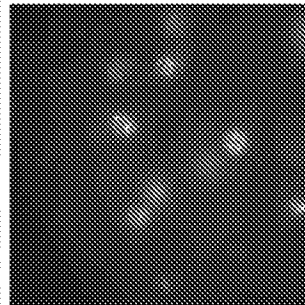


Fig. 2D

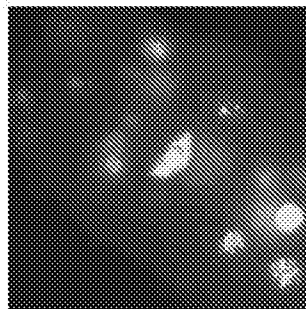
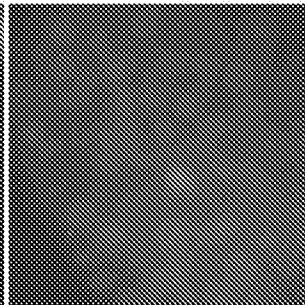


Fig. 2E

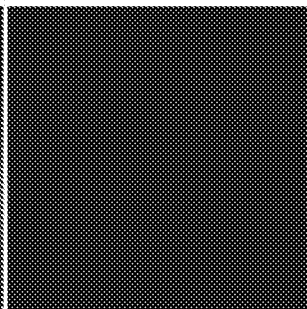


Fig. 2F

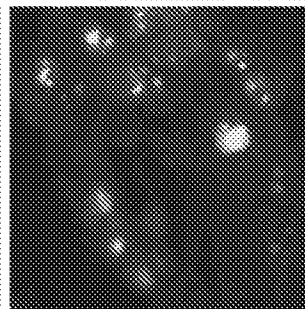


Fig. 2G

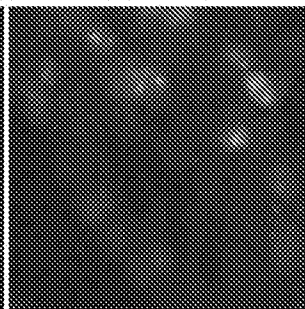


Fig. 2H

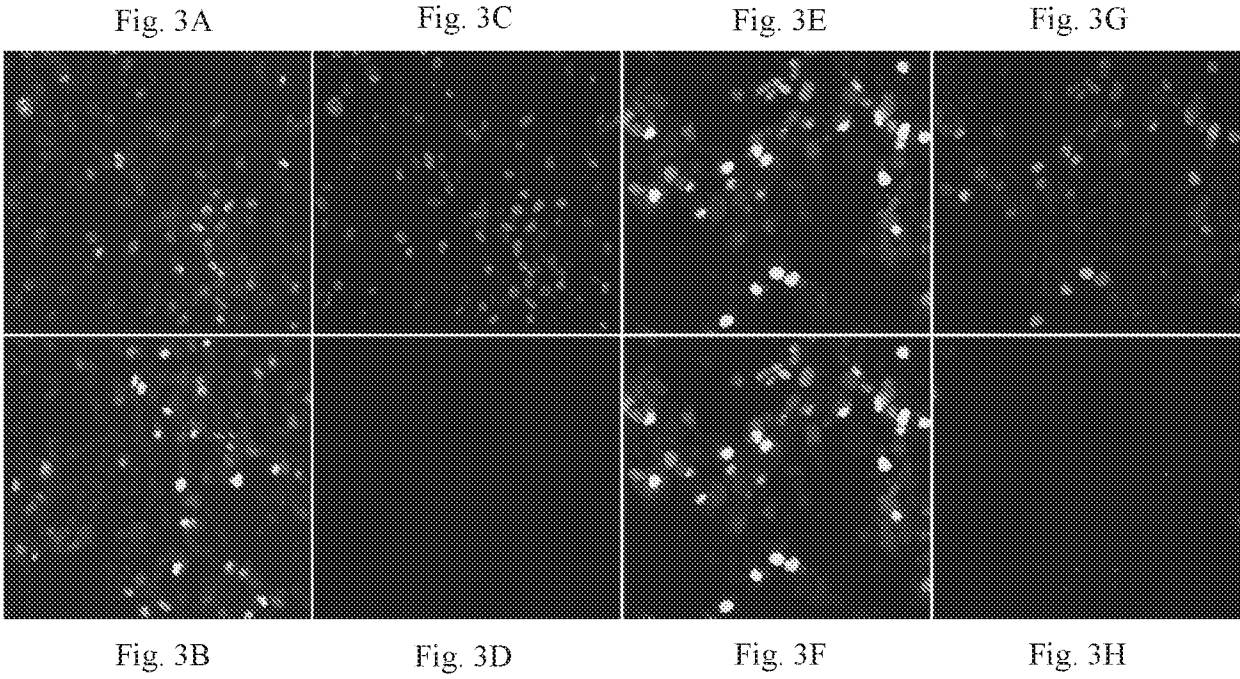


Fig. 4A

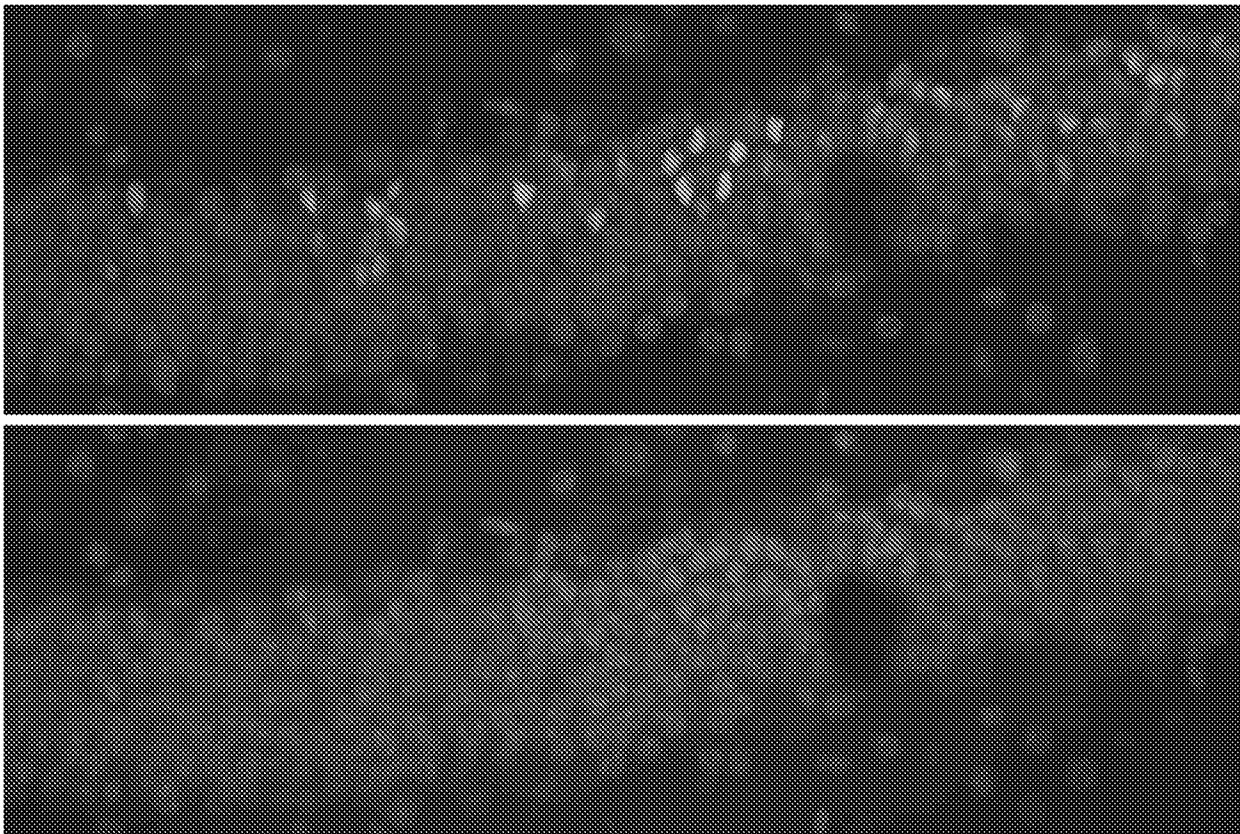


Fig. 4B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/025369

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 9/22; C12Q 1/6813; C12Q 1/6876 (2020.01)

CPC - C12N 9/22; C12N 2310/20; C12Q 1/6876; C12Q 2563/107; C12Q 2563/179 (2020.05)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

see Search History document

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

see Search History document

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

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2018/0340221 A1 (SIGMA-ALDRICH CO. LLC) 29 November 2018 (29.11.2018) entire document	1-3, 5, 6, 43-45, 58
A	US 2018/0320226 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 08 November 2018 (08.11.2018) entire document	1-3, 5, 6, 43-45, 58
A	US 2017/0268035 A1 (HOWARD HUGHES MEDICAL INSTITUTE) 21 September 2017 (21.09.2017) entire document	1-3, 5, 6, 43-45, 58
A	US 2019/0040370 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 07 February 2019 (07.02.2019) entire document	1-3, 5, 6, 43-45, 58
A	WU et al. "Progress and Challenges for Live-cell Imaging of Genomic Loci Using CRISPR-based Platforms," Genomics, Proteomics, & Bioinformatics, 30 January 2019 (30.01.2019), Vol. 17, Iss. 2, Pgs. 119-128. entire document	1-3, 5, 6, 43-45, 58

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

27 May 2020

Date of mailing of the international search report

16 JUN 2020

Name and mailing address of the ISA/US

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Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/025369

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4, 7-42, 46-57, 59-63
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.