b) subjecting the compartments to conditions which allow in vitro transcription and/or translation of RNAs and proteins;

c) subjecting the compartments to conditions which allow the modification of DNA and/or RNA targets by RNA-guided nucleic acid modifying enzymes which have functional activity towards said DNA or RNA targets in the presence of a gRNA, thereby producing a population of DNA/RNA molecules that comprises one or more of the following:

i. polynucleotide constructs and/or RNA transcripts or fragments thereof that have been modified by the nucleic acid modifying enzyme(s);

ii. polynucleotide constructs and/or RNA transcripts which have not been modified by the nucleic acid modifying enzyme(s);

d) harvesting the population of DNA/RNA molecules produced in step (c) and subjecting the same to single molecule long-read sequencing;

e) detecting and counting the DNA/RNA molecules referred to in step c)i and/or c)ii based on the sequencing results.

[0008] In one example, the present disclosure refers to a polynucleotide construct comprising: a first polynucleotide sequence encoding a nucleic acid modifying enzyme or a variant thereof, operably linked to a first promoter; and a second polynucleotide sequence comprising a DNA target.

[0009] In another aspect, the present disclosure refers to a polynucleotide construct comprising: a first polynucleotide sequence encoding a nucleic acid modifying enzyme or a variant thereof, operably linked to a first promoter; and a second polynucleotide sequence comprising a DNA template encoding an RNA target; and wherein said RNA target is co-expressed contiguously with the nucleic acid modifying enzyme as a single RNA transcript, driven by the first promoter.

[0010] In yet another example the present disclosure refers to a construct library comprising a plurality of the polynucleotide constructs as disclosed herein, wherein the library is characterized by one or more of the following: a) the plurality of the polynucleotide constructs encode different variants of a nucleic acid modifying enzyme; b) the plurality of polynucleotide constructs encode different DNA or RNA targets. In yet another example the present disclosure refers to a construct library comprising a plurality of the polynucleotide constructs as disclosed herein, wherein the library is characterized by one or more of the following: a) the plurality of the polynucleotide constructs encode different variants of a nucleic acid modifying enzyme; b) the plurality of polynucleotide constructs encode different RNA targets.

[0011] In yet a further example, the present disclosure refers to a construct library comprising a plurality of the polynucleotide constructs as disclosed herein, wherein the library is characterized by one or more of the following: a) the plurality of the polynucleotide constructs encode different variants of a nucleic acid modifying enzyme; b) the plurality of polynucleotide constructs encode different DNA or RNA targets; c) the plurality of polynucleotide constructs encode different gRNAs. In yet a further aspect, the present disclosure refers to a construct library comprising a plurality of the polynucleotide constructs as disclosed herein, wherein the library is characterized by one or more of the following: a) the plurality of the polynucleotide constructs encode different variants of a nucleic acid modifying enzyme; b) the plurality of polynucleotide constructs encode different RNA targets; c) the plurality of polynucleotide constructs encode different gRNAs.

[0012] In another example, the present disclosure refers to a polynucleotide construct comprising: a first polynucleotide sequence encoding a guide RNA (gRNA) operably linked to a first promoter; and a second polynucleotide sequence comprising a DNA target.

[0013] In another aspect, the present disclosure refers to a polynucleotide construct comprising: a first polynucleotide sequence encoding a guide RNA (gRNA) operably linked to a first promoter; and a second polynucleotide sequence comprising a DNA template encoding an RNA target; wherein the expression of said RNA target is co-expressed contiguously with the gRNA as a single RNA transcript, driven by the first promoter.

[0014] In yet another example, the present disclosure refers to a construct library comprising a plurality of the polynucleotide constructs as disclosed herein, wherein the library is characterized by one or more of the following: a) the plurality of polynucleotide constructs encode different DNA or RNA targets; b) the plurality of polynucleotide constructs encode different gRNAs. In yet another aspect, the present disclosure refers to a construct library comprising a plurality of the polynucleotide constructs as disclosed herein, wherein the library is characterized by one or more of the following: a) the plurality of polynucleotide constructs encode different RNA targets; b) the plurality of polynucleotide constructs encode different gRNAs.

[0015] In another aspect, the present disclosure refers to one or more compartments, each comprising a polynucleotide construct as disclosed herein, wherein the compartments are segregated from each other.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0017] **Figure 1.** Diagram illustrating a non-limiting list of the key concepts and steps of this invention, wherein the compartmentalization occurs via generation of water-in-oil emulsion droplets.

[0018] **Figure 2.** Diagram illustrating non-limiting examples of polynucleotide constructs as disclosed in the present disclosure. Note that ‘Cas nuclease’ can be replaced with any nucleic acid modifying enzyme, and can also refer to Cas variants such as inactivated Cas nuclease, or Cas protein fused to or associated with function-conferring domains.

[0019] **Figure 3.** A diagrammatic representation of how DNA/RNA molecule reads are counted for the calculation of enzymatic activity, in one example where the enzyme is a Cas

nuclease and the modification is DNA cleavage. In this example, the DNA target site resides 3' of the encoded Cas variant. The nanopore-sequencing reads (aligned against a reference sequence) with aligned 3' ends that map to the reference sequence at sites 3' downstream of the window of expected Cas cleavage sites are considered uncleaved (dark grey bars of "nanopore-seq aligned reads"; Figure 3), while read alignments with 3' ends lying within the

Claims

1. A method comprising the steps of:

a) segregating a plurality of polynucleotide constructs into compartments, wherein each compartment comprises a single polynucleotide construct, wherein each polynucleotide construct comprises

i) a first polynucleotide sequence encoding a nucleic acid modifying enzyme or a variant thereof, operably linked to a first promoter; and

ii) a second polynucleotide sequence comprising a DNA target or a DNA template encoding an RNA target, wherein when the second polynucleotide sequence comprises a DNA template encoding an RNA target, said RNA target is co-expressed contiguously with the nucleic acid modifying enzyme as a single RNA transcript, driven by the first promoter;

and wherein the plurality of the polynucleotide constructs encode different variants of the nucleic acid modifying enzyme, and/or different DNA or RNA targets;

b) subjecting the compartments to conditions which allow *in vitro* expression of RNAs and proteins;

c) subjecting the plurality of the compartments to conditions which allow the modification of DNA/RNA targets by nucleic acid modifying enzymes which have modification activity towards said DNA or RNA targets, thereby producing a population of DNA/RNA molecules that comprises one or more of the following:

i. polynucleotide constructs and/or RNA transcripts or fragments thereof that have been modified by the nucleic acid modifying enzyme(s);

ii. polynucleotide constructs and/or RNA transcripts which have not been modified by the nucleic acid modifying enzyme(s);

d) harvesting the population of DNA/RNA molecules produced in step (c) and subjecting the same to single molecule sequencing;

e) detecting and counting the DNA/RNA molecules referred to in step c)i and c)ii based on the sequencing results.

2. The method according to claim 1, wherein the nucleic acid modifying enzyme is an RNA-guided nucleic acid modifying enzyme, each compartment further comprises a guide RNA or a nucleotide template encoding the same.

3. The method according to claim 1, wherein the nucleic acid modifying enzyme is an RNA-guided nucleic acid modifying enzyme, each polynucleotide further comprises a third polynucleotide sequence encoding a variant guide RNA (gRNA); and wherein the plurality of the polynucleotide constructs encode different variants of the nucleic acid modifying enzyme, and/or different DNA or RNA targets, and/or different gRNAs.

4. A method comprising the steps of:

a) segregating a plurality of polynucleotide constructs into compartments, wherein each compartment comprises a single polynucleotide construct, wherein each polynucleotide construct comprises:

i) a first polynucleotide sequence encoding a guide RNA (gRNA) operably linked to a first promoter;

ii) a second polynucleotide sequence comprising a DNA target or a DNA template encoding an RNA target, wherein when the second polynucleotide sequence comprises a DNA template encoding an RNA target, said RNA target is co-expressed contiguously with the gRNA as a single RNA transcript, driven by the first promoter;

wherein the plurality of the polynucleotide constructs encode different gRNAs, and/or different DNA or RNA targets; and wherein each compartment further comprises an RNA-guided nucleic acid modifying enzyme or a variant thereof or a nucleotide template encoding the same;

b) subjecting the compartments to conditions which allow in vitro transcription and/or translation of RNAs and proteins;

c) subjecting the compartments to conditions which allow the modification of DNA and/or RNA targets by RNA-guided nucleic acid modifying enzymes which have functional activity towards said DNA or RNA targets in the presence of a gRNA, thereby producing a population of DNA/RNA molecules that comprises one or more of the following:

i. polynucleotide constructs and/or RNA transcripts or fragments thereof that have been modified by the nucleic acid modifying enzyme(s);

ii. polynucleotide constructs and/or RNA transcripts which have not been modified by the nucleic acid modifying enzyme(s);

d) harvesting the population of DNA/RNA molecules produced in step (c) and subjecting the same to single molecule long-read sequencing;

e) detecting and counting the DNA/RNA molecules referred to in step c)i and/or c)ii based on the sequencing results.

5. The method of any of claims 1 to 4, wherein the method further comprises evaluating the modifying activity of one or more nucleic acid modifying enzymes against one or more of the DNA/RNA targets, by calculating the number of polynucleotide constructs and/or RNA transcripts that have been modified by the nucleic acid modifying enzyme($\sum \text{counts}^{\text{modified}}$), and comparing it against the number of polynucleotide constructs and/or RNA transcripts that have not been modified by the nucleic acid modifying enzymes ($\sum \text{counts}^{\text{unmodified}}$), or against the total number of polynucleotide constructs and/or RNA transcripts ($\sum \text{counts}^{\text{modified+unmodified}}$).

6. The method of claim 5, wherein the enzymatic activity is represented by a value calculated using any one of the following formulas:

1) $\textit{enzymatic activity} \approx \sum \text{counts}^{\text{modified}} / \sum \text{counts}^{\text{unmodified}}$;

2) $\textit{enzymatic activity} \approx \sum \text{counts}^{\text{modified}} / \sum \text{counts}^{\text{modified+unmodified}}$.

7. The method of any of claims 1 to 6, wherein step d) further comprises breaking the compartments by physical or chemical methods.

8. The method of any of claims 1 to 7, wherein step d) further comprises purifying the harvested DNA/RNA molecules to remove excess DNA, RNA and/or proteins from the reaction.

9. The method of any of claims 1 to 8, wherein the harvested population of DNA/RNA molecules are not subjected to further modifications before being subjected to the single molecule sequencing reaction, except for modifications required of the single molecule sequencing.

10. The method of any of claims 1 to 9, wherein the detection and counting of the DNA/RNA molecules which have or have not been modified by the nucleic acid modifying

enzyme(s) is based only on data generated during the single molecule sequencing and does not require further modifications or processing of the DNA/RNA molecules.

11. The method of any one of claims 5 to 10, wherein the modification activity is cleavage activity, and the detection and calculation of modified and unmodified polynucleotide constructs or RNA transcripts are achieved by aligning sequencing reads of the DNA/RNA molecules against a reference sequence which contains a window of cleavage sites for the nucleic acid modifying enzyme(s), wherein

i) when the sequencing read of a DNA/RNA molecule is mapped across both 5' and 3' of the window of cleavage sites, the DNA/RNA molecule is an unmodified polynucleotide construct or RNA target;

ii) when the end of the sequencing read of a DNA/RNA molecule is mapped to within the window of cleavage sites, the DNA/RNA molecule is a modified polynucleotide construct or RNA target;

iii) when the end of the sequencing read of a DNA/RNA molecule does not map within the window of cleavage sites, nor across both 5' and 3' of the window of cleavage sites, the DNA/RNA molecule is non-informative and is not used for the measurement of modification activity.

12. A polynucleotide construct comprising:

a first polynucleotide sequence encoding a nucleic acid modifying enzyme or a variant thereof, operably linked to a first promoter; and

a second polynucleotide sequence comprising a DNA template encoding an RNA target; and

wherein said RNA target is co-expressed contiguously with the nucleic acid modifying enzyme as a single RNA transcript, driven by the first promoter.

13. A construct library comprising a plurality of the polynucleotide constructs of claim 12, wherein the library is characterized by one or more of the following:

a. the plurality of the polynucleotide constructs encode different variants of a nucleic acid modifying enzyme ;

b. the plurality of polynucleotide constructs encode different RNA targets.

14. The polynucleotide construct according to claim 12, wherein the polynucleotide construct further comprises a third polynucleotide sequence encoding a guide RNA (gRNA).

15. A construct library comprising a plurality of the polynucleotide constructs of claim 14, wherein the library is characterized by one or more of the following:

a. the plurality of the polynucleotide constructs encode different variants of a nucleic acid modifying enzyme;

b. the plurality of polynucleotide constructs encode different RNA targets;

c. the plurality of polynucleotide constructs encode different gRNAs.

16. A polynucleotide construct comprising:

a first polynucleotide sequence encoding a guide RNA (gRNA) operably linked to a first promoter; and

a second polynucleotide sequence comprising a DNA template encoding an RNA target; wherein the expression of said RNA target is co-expressed contiguously with the gRNA as a single RNA transcript, driven by the first promoter.

17. A construct library comprising a plurality of the polynucleotide constructs of claim 16, wherein the library is characterized by one or more of the following:

a. the plurality of polynucleotide constructs encode different RNA targets;

b. the plurality of polynucleotide constructs encode different gRNAs.

18. The method of any one of claims 1 to 11, or the polynucleotide construct of any of claims 12, 14, and 16, wherein the first and second polynucleotide sequences are fully or partially overlapping.

19. The method of any one of claims 1 to 11, or the polynucleotide construct of any of claims 14, and 16, wherein the DNA or RNA target comprises a protospacer that is at least partially complementary to the guide RNA.

20. The method of any one of claims 1 to 11, or the method or polynucleotide construct of any of claims 18 and 19, wherein the DNA target also comprises a proximal Protospacer Adjacent Motif (PAM) sequence.

21. The method of any one of claims 1 to 11, the polynucleotide construct of any of claims 12, 14 and 16, or the method or polynucleotide construct of any of claims 19 to 21, wherein when the polynucleotide construct comprises a DNA template encoding an RNA target, the RNA target further comprises a proximal Protospacer Flanking Sequence (PFS).

22. The method of any one of claims 1 to 11, or the polynucleotide construct of any of claims 12, 14, or the method or polynucleotide construct of any of claims 19 to 21, wherein the nucleic acid modifying enzyme is a CRISPR-associated protein (Cas).

23. The method of any one of claims 1 to 11, or the method or polynucleotide construct of any of claims 18 to 22, wherein the variant nucleic acid modifying enzyme contains one or more inactivated catalytic sites, and is capable of binding and inhibiting the expression of a DNA target, without modifying the DNA target.

24. The method of any one of claims 1 to 11, or the polynucleotide construct of any of claims 12, 14, or the method or polynucleotide construct of any of claims 18 to 23, wherein the variant nucleic acid modifying enzyme is fused with one or more additional functional domains capable of modifying DNA or RNA.

25. One or more compartments, each comprising a polynucleotide construct as defined in any of claims 12, 14, and 16, wherein the compartments are segregated from each other.

26. The method according to any one of claims 1 to 11, 18 to 24, or the one or more compartments of claim 25, wherein each of the compartments further comprises *in vitro* transcription and translation (IVTT) reagents, said IVTT reagents enable the *in vitro* transcription and/or translation of proteins and/or RNAs.

27. The method of any one of claims 1 to 11, 18 to 24, or the one or more compartments of claim 25 or 26, wherein the compartments are emulsion droplets.

28. The method of any one of claims 1 to 11, 18 to 24, or the one or more compartments of any one of claim 25 to 27, wherein the segregation is achieved using microfluidics, hydrogel-limited diffusion, or partitioned wells.