CLAIMS

What is claimed is:

1. A method for profiling spatiotemporal gene expression in a cell, the method comprising:

a) incubating a cell in the presence of a pool of nucleoside analogs for an amount of time t_1 to metabolically label nucleic acids synthesized by the cell, wherein each nucleoside analog in the pool of nucleoside analogs comprises a reactive chemical moiety;

b) contacting the metabolically labeled nucleic acids with a population of first oligonucleotide probes, wherein each first oligonucleotide probe in the population of first oligonucleotide probes comprises a reactive chemical moiety that reacts with the reactive chemical moiety of the nucleoside analogs;

c) contacting the metabolically labeled nucleic acids with one or more pairs of oligonucleotide probes comprising a second oligonucleotide probe and a third oligonucleotide probe, wherein:

i) the second oligonucleotide probe comprises a barcode sequence and a portion that is complementary to a metabolically labeled nucleic acid of interest; and

ii) the third oligonucleotide probe comprises a portion that is complementary to the metabolically labeled nucleic acid of interest, a first barcode sequence, a portion that is complementary to a first oligonucleotide probe in the population of first oligonucleotide probes, and a second barcode sequence, wherein the first barcode sequence of the third oligonucleotide probe is complementary to the barcode sequence of the second oligonucleotide probe, and wherein the portion of the third oligonucleotide probe that is complementary to a first oligonucleotide probe in the population of first oligonucleotide probes is split between the 5′ end and the 3′ end of the third oligonucleotide probe;

d) ligating the 5′ end and the 3′ end of the third oligonucleotide probe together to produce a circular oligonucleotide;

e) performing rolling circle amplification to amplify the circular oligonucleotide using the second oligonucleotide probe as a primer to produce one or more concatenated amplicons;

f) embedding the one or more concatenated amplicons in a polymer matrix;

g) contacting the one or more concatenated amplicons embedded in the polymer matrix with a fourth oligonucleotide probe comprising a sequence that is complementary to the second barcode sequence of the third oligonucleotide probe; and

h) imaging the fourth oligonucleotide probe to determine the location of the one or more concatenated amplicons embedded in the polymer matrix.

2. The method of claim 1 further comprising repeating steps (a)-(h) at least one time for a different amount of time t_2 to profile the spatiotemporal expression of the labeled nucleic acids of interest, optionally wherein steps (a)-(h) are repeated for a third amount of time t_3, optionally repeated for a fourth amount of time t_4, optionally repeated for a fifth amount of time t_5, optionally repeated for a sixth amount of time t_6, optionally repeated for a seventh amount of time t_7, optionally repeated for an eighth amount of time t_8, optionally repeated for a ninth amount of time t_9, and optionally repeated for a tenth amount of time t_{10}.

3. The method of claim 1 or 2, wherein spatiotemporal gene expression is profiled in multiple cells simultaneously, optionally wherein spatiotemporal gene expression is profiled in more than 10 cells, more than 20 cells, more than 50 cells, more than 100 cells, more than 200 cells, more than 300 cells, more than 400 cells, more than 500 cells, or more than 1000 cells simultaneously.

4. The method of claim 3, wherein the cells comprise a plurality of cell types, optionally wherein the cell types are selected from the group consisting of stem cells, progenitor cells, neuronal cells, astrocytes, dendritic cells, endothelial cells, microglia, oligodendrocytes, muscle cells, myocardial cells, mesenchymal cells, epithelial cells, immune cells, hepatic cells, smooth and skeletal muscle cells, hematopoietic cells, lymphocytes, monocytes, neutrophils, macrophages, natural killer cells, mast cells, adipocytes, and neurons; and/or

wherein the cell is a permeabilized cell.

5. The method of any one of claims 1-4, wherein the cell is present within an intact tissue, optionally wherein the intact tissue is epithelial tissue, connective tissue, muscular tissue, or nervous tissue, and optionally wherein the intact tissue is *in vivo* during the incubating of step (a)

and prior to the contacting of step (b) and/or wherein the intact tissue is harvested following the incubating of step (a) and prior to the contacting of step (b); and/or wherein the intact tissue is in a non-human experimental animal, optionally wherein the non-human experimental animal is a mouse, rat, dog, pig, or non-human primate.

6. The method of any one of claims 1-5, wherein the metabolically labeled nucleic acids are 5-ethynyl uridine-labeled nucleic acids.

7. The method of any one of claims 1-6, wherein spatiotemporal gene expression is profiled for up to 100, up to 200, up to 500, up to 1000, up to 2000, up to 3000, or more than 3000 metabolically labeled nucleic acids of interest simultaneously, preferably wherein spatiotemporal gene expression is profiled for up to 1000 metabolically labeled nucleic acids of interest simultaneously.

8. The method of any one of claims 1-7, wherein the metabolically labeled nucleic acid of interest is a nascent RNA, messenger RNA (mRNA), transfer RNA (tRNA), or ribosomal RNA (rRNA), preferably wherein the metabolically labeled nucleic acid of interest is nascent RNA or mRNA.

9. The method of any one of claims 1-8, wherein the reactive chemical moiety of each nucleoside analog in the pool of nucleoside analogs is a reactive bioorthogonal functional group, optionally wherein the reactive chemical moiety of each nucleoside analog in the pool of nucleoside analogs comprises an azide, an alkyne, a nitrone, an alkene, a tetrazine, or a tetrazole, and preferably wherein the reactive chemical moiety of each nucleoside analog in the pool of nucleoside analogs comprises an alkyne; and/or

wherein each nucleoside analog in the pool of nucleoside analogs is 5-ethynyl uridine, 3′-azido-3′-deoxythymidine, C8-alkyne-deoxyuridine, (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine, or 5-ethynyl-2′-deoxycytidine.

10. The method of any one of claims 1-9, wherein the reactive chemical moiety of each first oligonucleotide probe in the population of first oligonucleotide probes is a reactive bioorthogonal

functional group, optionally wherein the reactive chemical moiety of each first oligonucleotide probe in the population of first oligonucleotide probes comprises an azide, an alkyne, a nitrone, an alkene, a tetrazine, or a tetrazole, and preferably wherein the reactive chemical moiety of each first oligonucleotide probe in the population of first oligonucleotide probes comprises an azide; and/or

wherein reaction of the reactive chemical moiety of the nucleoside analogs and the reactive chemical moiety of each first oligonucleotide probe in the population of first oligonucleotide probes comprises a click chemistry reaction, optionally wherein the click chemistry reaction is a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction.

11. The method of any one of claims 1-10, wherein each first oligonucleotide probe in the population of first oligonucleotide probes further comprises a polymerization blocker, optionally wherein the polymerization blocker is at the 3′ end of each first oligonucleotide probe in the population of first oligonucleotide probes; and/or optionally wherein the polymerization blocker comprises an inverted nucleic acid residue, preferably wherein the polymerization blocker is an inverted thymine residue; and/or

wherein each first oligonucleotide probe in the population of first oligonucleotide probes comprises a poly-A linker sequence, optionally wherein the poly-A linker sequence is about 2-100, about 10-90, about 20-80, about 30-70, or about 40-60 nucleotides long, and preferably wherein the poly-A linker sequence is about 50 nucleotides long; and/or

wherein each first oligonucleotide probe in the population of first oligonucleotide probes comprises the structure:

5′-[reactive chemical moiety]-[poly-A linker sequence]-[portion complementary to third oligonucleotide probe]-[polymerization blocker]-3′,

wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long.

12. The method of any one of claims 1-11, wherein the portion of the first oligonucleotide probe that is complementary to the third oligonucleotide probe is about 3-20, about 4-19, about 5-18, about 6-17, about 7-16, about 8-15, about 9-14, about 10-13, or about 11-12 nucleotides in length; and/or

wherein the portion of the first oligonucleotide probe that is complementary to the third oligonucleotide probe is about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20 nucleotides in length.

13. The method of any one of claims 1-12, wherein the portion of the second oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10-30, about 11-29, about 12-28, about 13-27, about 14-26, about 15-25, about 16-24, about 17-23, about 18-22, or about 19-21 nucleotides in length; and/or

wherein the portion of the second oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more than 30 nucleotides in length; and/or

wherein the barcode sequence of the second oligonucleotide probe is about 1-10 nucleotides long, optionally wherein the barcode sequence of the second oligonucleotide probe is about 5-10 nucleotides long, and preferably wherein the barcode sequence of the second oligonucleotide probe is about 5 nucleotides long; and/or

wherein the second oligonucleotide probe comprises the structure:

5′-[portion complementary to metabolically labeled nucleic acid of interest]-[barcode sequence]-3′,

wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long.

14. The method of any one of claims 1-13, wherein the first barcode sequence of the third oligonucleotide probe is about 1-10 nucleotides long, optionally wherein the first barcode sequence of the third oligonucleotide probe is about 5-10 nucleotides long, and preferably wherein the first barcode sequence of the third oligonucleotide probe is about 5 nucleotides long; and/or

wherein the second barcode sequence of the third oligonucleotide probe is about 1-10 nucleotides long, optionally wherein the second barcode sequence of the third oligonucleotide

probe is about 5-10 nucleotides long, and preferably wherein the second barcode sequence of the third oligonucleotide probe is about 5 nucleotides long; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the first oligonucleotide probe is about 3-20, about 4-19, about 5-18, about 6-17, about 7-16, about 8-15, about 9-14, about 10-13, or about 11-12 nucleotides in length; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the first oligonucleotide probe is about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20 nucleotides in length; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10-30, about 11-29, about 12-28, about 13-27, about 14-26, about 15-25, about 16-24, about 17-23, about 18-22, or about 19-21 nucleotides in length; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more than 30 nucleotides in length; and/or wherein the third oligonucleotide probe comprises the structure:

5′-[first portion complementary to a first oligonucleotide probe]-[first barcode sequence]-[portion complementary to metabolically labeled nucleic acid of interest]-[second barcode sequence]-[second portion complementary to a first oligonucleotide probe]-3′,

wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long; and/or

wherein the second barcode sequence of the third oligonucleotide probe is a gene-specific sequence used to identify the metabolically labeled nucleic acid of interest.

15. The method of any one of claims 1-14, wherein the fourth oligonucleotide probe comprises a fluorophore.

16. The method of any one of claims 1-15, wherein the step of performing rolling circle amplification further comprises providing amine-modified nucleotides, wherein the amine-

modified nucleotides are incorporated into the one or more concatenated amplicons, optionally wherein the step of embedding the one or more concatenated amplicons in a polymer matrix comprises reacting the amine-modified nucleotides of the one or more concatenated amplicons with methacrylic acid *N*-hydroxysuccinimide and co-polymerizing the one or more concatenated amplicons and the polymer matrix.

17. The method of any one of claims 1-16, wherein the polymer matrix is a hydrogel, optionally wherein the hydrogel is a polyvinyl alcohol hydrogel, a polyethylene glycol hydrogel, a sodium polyacrylate hydrogel, an acrylate polymer hydrogel, or a polyacrylamide hydrogel.

18. The method of any one of claims 1-17, wherein the step of contacting the one or more concatenated amplicons embedded in the polymer matrix with the fourth oligonucleotide probe is performed to identify the metabolically labeled nucleic acid of interest, optionally wherein identifying the metabolically labeled nucleic acid of interest comprises performing SEDAL sequencing, and optionally wherein SEDAL sequencing is performed two, three, four, five, or more than five times.

19. The method of any one of claims 1-18, wherein the step of imaging comprises fluorescent imaging, and/or wherein the step of imaging comprises confocal microscopy or epifluorescence microscopy.

20. The method of any one of claims 1-19 further comprising profiling additional molecules within the cell, optionally wherein the additional molecules are RNAs, DNAs, proteins, carbohydrates, small molecules, metabolites, and/or lipids; and/or

further comprising determining the cell type of the profiled cell by comparing the spatiotemporal gene expression profile of the cell to reference data comprising spatiotemporal gene expression profiles of various cell types; and/or

further comprising overexpressing or knocking out one or more genes in the cell to determine whether the one or more genes are involved in the spatiotemporal expression of the metabolically labeled nucleic acid of interest.

21. A plurality of oligonucleotide probes comprising a first oligonucleotide probe, a second oligonucleotide probe, and a third oligonucleotide probe, wherein

i) the first oligonucleotide probe comprises a reactive chemical moiety;

ii) the second oligonucleotide probe comprises a barcode sequence and a portion that is complementary to a metabolically labeled nucleic acid of interest; and

iii) the third oligonucleotide probe comprises a portion that is complementary to the metabolically labeled nucleic acid of interest, a first barcode sequence, a portion that is complementary to the first oligonucleotide probe, and a second barcode sequence, wherein the portion of the third oligonucleotide probe that is complementary to the first oligonucleotide probe is split between the 5′ end and the 3′ end of the third oligonucleotide probe,

wherein the first barcode sequence of the third oligonucleotide probe is complementary to the barcode sequence of the second oligonucleotide probe.

22. The plurality of oligonucleotide probes of claim 21, wherein the reactive chemical moiety of the first oligonucleotide probe is a reactive bioorthogonal functional group, optionally wherein the reactive chemical moiety of the first oligonucleotide probe comprises an azide, an alkyne, a nitrone, an alkene, a tetrazine, or a tetrazole, and preferably wherein the reactive chemical moiety of the first oligonucleotide probe comprises an azide.

23. The plurality of oligonucleotide probes of claim 21 or 22, wherein the first oligonucleotide probe further comprises a polymerization blocker, optionally wherein the polymerization blocker is at the 3′ end of the first oligonucleotide probe, and/or optionally wherein the polymerization blocker comprises an inverted nucleic acid residue, preferably wherein the polymerization blocker is an inverted thymine residue.

24. The plurality of oligonucleotide probes of any one of claims 21-23, wherein the first oligonucleotide probe further comprises a poly-A linker sequence, optionally wherein the poly-A linker sequence is about 2-100, about 10-90, about 20-80, about 30-70, or about 40-60 nucleotides long, and preferably wherein the poly-A linker sequence is about 50 nucleotides long; and/or

wherein the first oligonucleotide probe comprises the structure:

5′-[reactive chemical moiety]-[poly-A linker sequence]-[portion complementary to third oligonucleotide probe]-[polymerization blocker]-3′,
wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long.

25. The plurality of oligonucleotide probes of any one of claims 21-24, wherein the portion of the first oligonucleotide probe that is complementary to the third oligonucleotide probe is about 3-20, about 4-19, about 5-18, about 6-17, about 7-16, about 8-15, about 9-14, about 10-13, or about 11-12 nucleotides in length; and/or

wherein the portion of the first oligonucleotide probe that is complementary to the third oligonucleotide probe is about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20 nucleotides in length.

26. The plurality of oligonucleotide probes of any one of claims 21-25, wherein the portion of the second oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10-30, about 11-29, about 12-28, about 13-27, about 14-26, about 15-25, about 16-24, about 17-23, about 18-22, or about 19-21 nucleotides in length; and/or

wherein the portion of the second oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more than 30 nucleotides in length; and/or

wherein the barcode sequence of the second oligonucleotide probe is about 1-10 nucleotides long, optionally wherein the barcode sequence of the second oligonucleotide probe is about 5-10 nucleotides long, and preferably wherein the barcode sequence of the second oligonucleotide probe is about 5 nucleotides long; and/or

wherein the second oligonucleotide probe comprises the structure:

5′-[portion complementary to nucleic acid of interest]-[barcode sequence]-3′,
wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long.

27. The plurality of oligonucleotide probes of any one of claims 21-26, wherein the first barcode sequence of the third oligonucleotide probe is about 1-10 nucleotides long, optionally wherein the first barcode sequence of the third oligonucleotide probe is about 5-10 nucleotides long, and preferably wherein the first barcode sequence of the third oligonucleotide probe is about 5 nucleotides long; and/or

wherein the second barcode sequence of the third oligonucleotide probe is about 1-10 nucleotides long, optionally wherein the second barcode sequence of the third oligonucleotide probe is about 5-10 nucleotides long, and preferably wherein the second barcode sequence of the third oligonucleotide probe is about 5 nucleotides long; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the first oligonucleotide probe is about 3-20, about 4-19, about 5-18, about 6-17, about 7-16, about 8-15, about 9-14, about 10-13, or about 11-12 nucleotides in length; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the first oligonucleotide probe is about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20 nucleotides in length; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10-30, about 11-29, about 12-28, about 13-27, about 14-26, about 15-25, about 16-24, about 17-23, about 18-22, or about 19-21 nucleotides in length; and/or

wherein the portion of the third oligonucleotide probe that is complementary to the metabolically labeled nucleic acid of interest is about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more than 30 nucleotides in length; and/or

wherein the third oligonucleotide probe comprises the structure:

5′-[first portion complementary to first oligonucleotide probe]-[first barcode sequence]-[portion complementary to nucleic acid of interest]-[second barcode sequence]-[second portion complementary to first oligonucleotide probe]-3′,

wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long; and/or

wherein the second barcode sequence of the third oligonucleotide probe is a gene-specific sequence used to identify a nucleic acid of interest.

28. The plurality of oligonucleotide probes of any one of claims 21-27 further comprising a fourth oligonucleotide probe, optionally wherein the fourth oligonucleotide probe comprises a sequence that is complementary to the second barcode sequence of the third oligonucleotide probe, and/or optionally wherein the fourth oligonucleotide probe comprises a fluorophore.

29. An oligonucleotide probe comprising a portion that is complementary to a nucleic acid of interest, a first barcode sequence, a portion that is complementary to an additional oligonucleotide probe, and a second barcode sequence, wherein the portion of the oligonucleotide probe that is complementary to the additional oligonucleotide probe is split between the 5′ end and the 3′ end of the oligonucleotide probe.

30. The oligonucleotide probe of claim 29, wherein the first barcode sequence is complementary to a barcode sequence on the additional oligonucleotide probe; and/or

wherein the first barcode sequence is 5-10 nucleotides long, preferably wherein the first barcode sequence is 5 nucleotides long; and/or

wherein the second barcode sequence is 5-10 nucleotides long, preferably wherein the second barcode sequence is 5 nucleotides long; and/or

wherein the oligonucleotide probe comprises the structure:

5′-[first portion complementary to first oligonucleotide probe]-[first barcode sequence]-[portion complementary to nucleic acid of interest]-[second barcode sequence]-[second portion complementary to first oligonucleotide probe]-3′,

wherein]-[comprises an optional nucleotide linker, optionally wherein each instance of the optional nucleotide linker is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides long.

31. A kit comprising the plurality of oligonucleotide probes of any one of claims 21-28 or the oligonucleotide probe of claim 29 or 30, optionally

wherein the kit comprises multiple pluralities of oligonucleotide probes of any one of claims 21-28, wherein each plurality of oligonucleotide probes is used to profile spatiotemporal gene expression of a different metabolically labeled nucleic acid of interest, or wherein the kit comprises multiple oligonucleotide probes of claim 29 or 30, wherein each oligonucleotide probe comprises a portion that is complementary to a different nucleic acid of interest; and/or

wherein the kit comprises more than 1, more than 2, more than 3, more than 4, more than 5, more than 10, more than 20, more than 30, more than 40, more than 50, more than 100, more than 200, more than 500, or more than 1000 pluralities of oligonucleotide probes, or wherein the kit comprises more than 1, more than 2, more than 3, more than 4, more than 5, more than 10, more than 30, more than 40, more than 50, more than 100, more than 200, more than 500, or more than 1000 oligonucleotide probes; and/or

the kit further comprises cells; one or more enzymes, optionally wherein the one or more enzymes comprise a ligase and/or a polymerase; amine-modified nucleotides; and/or reagents and monomers for preparing a polymer matrix.

32. A system for profiling spatiotemporal gene expression in a cell comprising

a) a cell;

b) a pool of nucleoside analogs, wherein each nucleoside analog in the pool of nucleoside analogs comprises a reactive chemical moiety;

c) a population of first oligonucleotide probes, wherein each oligonucleotide probe in the population of first oligonucleotide probes comprises a reactive chemical moiety that reacts with the reactive chemical moiety of the nucleoside analogs; and

d) one or more pairs of oligonucleotide probes comprising a second oligonucleotide probe and a third oligonucleotide probe, wherein:

i) the second oligonucleotide probe comprises a barcode sequence and a portion that is complementary to a nucleic acid of interest; and

ii) the third oligonucleotide probe comprises a portion that is complementary to the nucleic acid of interest, a first barcode sequence, a portion that is complementary to a first oligonucleotide probe in the population of first oligonucleotide probes, and a second barcode sequence, wherein the first barcode sequence of the third oligonucleotide probe is complementary to the barcode sequence of the second oligonucleotide probe, and

wherein the portion of the third oligonucleotide probe that is complementary to the first oligonucleotide probe is split between the 5′ end and the 3′ end of the third oligonucleotide probe.

33. The system of claim 32 further comprising a microscope, a computer, a CPU, computer memory and/or storage, and/or a camera.