Transcrptome analysis via photo-isolation chemistry

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Method Article

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Abstract

To gain insights into tissue-specific gene expression in multicellular systems, gene expression profiles are required to be precisely linked with spatial information. Here, we establish a hight-depth spatial transcriptomics method, photo-isolation chemistrty (PIC), which is able to isolate gene expression profiles only from UV-irradiatied region out of whole tissue section. This method performs reverse transcription on tissue sections using photocaged oligo DNAs. After the UV irradiation, the cDNAs in the irradiated regions are allowed to be amplified and sequenced, thereby providing gene expression profiles linked with spatial information.

Introduction

Reagents

Basic Reagents

- Nuclease-free water (Nacali Tesque, cat. no. 06442-95)

- Phosphate Buffered Saline (PBS) Tablets, pH7.4 (TaKaRa, cat. no.T9181)

- O.C.T compound (Sakura Finetek Japan, cat. No. 4583)

- Dry ice

- iso-Pentane (Nacali Tesque, cat. no. 26404-75)

- Triton X-100 (Nacali Tesque, cat. no. 35501-02)

- Polyoxyethylene Sorbitan Monolaurate (Tween-20) (Nacali Tesque, cat. no. 28353-85)

- 5 mol/l-Hydrochloric Acid (5 N HCl) (Nacali Tesque, cat. no. 7647-01-0)

- 1 mol/l-Tris (hydroxymethy) aminomethane-Hydrochloride Buffer Solution (pH8.0) (Nacali Tesque, cat. no. 06938-15)

- 16% Formaldehyde (w/v), Methanol-free (Thermo; cat. no. 28906)

- 0.1%-tTBS (10x TBST) (pH 7.4) (Nacali Tesque, cat. no. 12750-81)

- Blocking One-P (Nacali Tesque, cat. no. 05999084)

- 5 mol/l-Sodium Chloride Solution (5 M NaCl) (Nacali Tesque, cat. no. 06900-14)

- Potassium Acetate (Nacali Tesque, cat. no. 28404-15)

- Magnesium Acetate Tetrahydrate (MgOAc) (Nacali Tesque, cat. no. 20821-85)

- 0.5 mol/l-EDTA Solution (pH 8.0) (Nacali Tesque, cat. no. 06894-85)

- 50% w/v Polyethylene Glycol 8,000 (Nacali Tesque, cat. no. 26065-54)

- Proteinase K solution (20 mg/ml) (KANTO CHEMICAL CO., INC. cat. no. 34060-96)

- Qiagen MinElute PCR Purification kit (QIAGEN, cat. no. 28006)

- SuperScript II Reverse Transcriptase (Thermo, cat. no. 18064071)

- 5xFirst Strand Buffer (Thermo, cat. no. 18064071)

- 0.1 M DTT (Thermo, cat. no. 18064071)

- RNaseOUT (Thermo, cat. no. 10777019)

- Deoxynucleotide (dNTP) Solution Mix (NEB, cat. no. N0447L)

- Second Strans Buffer (Thermo, cat. no. 10812014)

- DNA Polymerase I (E.coli) (Thermo, cat. no. 18010025)

- E.coli DNA ligase (Thermo, cat. no. 18052019)

- RNaseH (E.coli) (Thermo, cat. no. 18021071)

- MEGAscript T7 Transcription Kit (Thermo, cat. no. AMB13345)

- ExoSAP-IT Express PCR Product Cleanup (Thermo,cat. no. 75001.1.ML)

- AMPure XP beads (Beckman Coulter, cat. no. A63881)

- RNAClean XP beads (Beckman Coulter; cat. no. A63987)

- Ethanol (96-100% (vol/vol)) (Nacali Tesque, cat. no.14713-95)

- Nuclear Violet LCS1 (AAT Biooquest, Inc, cat. no. 17543)

- SlowFade Diamond (Thermo, cat. no. S36963)

- Pusion High-Fidelity PCR Master Mix (NEB, cat. no. M0531L)

Reagent setup

- Phosphate-buffer saline (PBS) : Dissolve 1 tablet of Phosphate Buffered Saline (PBS) Tablet, pH7.4 in 1000 ml of dH_2O, and autoclave

- 4% PFA in PBS : Mix 1 ml 16% Formaldehyde (w/v), Methanol-free and PBS

- 10% (vol/vol) Triton X-100 : Dilute 5 ml of Triton X-100 with Nuclease-free water to a final volume of 50 ml

- 5% (vol/vol) Triton X-100 in PBS : Mix 1 ml of 10% (vol/vol) Triton X-100 with 1 ml of PBS

- 10% (vol/vol) Tween-20 : Dilute 5 ml of Tween-20 with Nuclease-free water to a final volume of 50 ml

- 0.1 N HCl in Nuclease-free water : Mix 200 ml of 5 N HCl with 9.8 ml Nuclease-free water

- 1xTBST : Mix 5ml of 10xTBST with 45 ml of Nuclease-free water

- Proteinase K lysis solution : Mix 970 µl of PBS, 10 µl of 10% Tween and 20 µl of Proteinase K (20mg/mL)

- Blocking solution : Mix 5 ml of Blocking One-P with 5 ml of 1xTBST

- 1 M KOAc : Dissolve 19.63 g of Potassium Acetate in dH_2O, make up to a final volume of 200 ml, and autoclave

- 1.5 M MgOAc : Dissolve 64.35 g of Potassium Acetate in dH_2O, make up to a final volume of 200 ml, and autoclave

- Beads binding buffer : Mix 4 ml of 50% w/v Polyethylene Glycol 8,000 , 5 ml of 5M NaCl, and 1 ml of Nuclease-free water

- Fragmentation Buffer : Mix 5 ml of 1M KOAc, 1 ml of 1.5 M MgOAc, 2 ml of 1M Tris-HCl (pH8.0), and 2 ml of Nuclease-free water

- 80% Ethanol : Mix 40 ml of Ethanol (96-100% (vol/vol)) and 10 ml of Nuclease-free water

- 70% Ethanol : Mix 35 ml of Ethanol (96-100% (vol/vol)) and 15 ml of Nuclease-free water

Oligos

t = NPOM-caged dT

- Caged Oligo DNA ; 5'-GCCGGTAATACGACTCACTATAGGGtttGAGttCtACAGTCCGACGATCNNNNNNTCGAAGttTTTTTTTTTTTTTTTTTTTTTTV-3'

- RandomhexRT ; 5'- GCCTTGGCACCCGAGAATTCCANNNNNN -3'

- RNA PCR primer 1 ; AATGATACGGCGACCACCGAGATCTACACAATACATCGTTCAGAGTTCTACAGTCCGA

- RNA PCR primer 2 ; CAAGCAGAAGACGGCATACGAGATAGTGCTTCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

Equipment

- PAP PEN (BMS, cat. no. BC-PAPPEN-S)

- Cryostat (Leica)

- Leica DM5000 B fluorescence microscope with an EL6000 100E Hg lamp (100% power) and a Leica A filter cube (11513873) at a wavelength of 340–380 nm

- Polypropylene staing jar (Thermo, cat. no. 1-7934-01)

- Hybridization oven (TAITEC, cat. no. HB-80)

- Humidified chamber (Cosmo Bio Co., Ltd., cat. no. 10HTLS)

- NGS Magna Stand 96 well (NIPPON Genetics Co., Ltd. , cat. no. FD-SSMAG96)

- Thermal cycler (Applied Biosystems)

- Bioanalyzer 2100 (Agilent Technologies)

- Library Quantification Kit (Clontech, cat. No. 638324)

Procedure

Preparation of Fresh Frozen Sections

1 Crush dry ice into pieces of 2-3 cm in size

2 Put the crushed dry ice lumps into a 100 ml beaker, then fill it with iso-pentane

3 Put the dissected tissues into an embedding container filled with O.C.T compound

4 Flash-freeze the tissues by submerging into the iso-pentane cooled with dry ice.

5 Make the sections at a thickness of 10 μm with a cryostat and mount them on MAS-coated glass slides (Matsunami).

Note: Subsequent processing can be facilitated by creating the hydrophobic barriers around the sections with a pap pen facilitates

Fixation and permeabilization

6 Wash the tissue sections twice with PBS to remove the O.C.T compound

7 Fix the sections with 4% PFA in PBS for 10 min at room temperature

8 Wash the sections twice with PBS

9 Incubate the sections for 3 min with PBS containing 3% Triton X-100 at room temperature

10 Wash the sections twice with PBS

11 Incubate the sections with 0.1N HCl in nuclease-free water for 5 min at room temperature

12 Remove the supernatant

13 Incubate the sections with 1M Tris-HCl (pH8.0) for 5 min at room temperature

14 Wash the sections twice with PBS

15 Immerse the sections in PBS preheated to 65°C in a staining jar and incubate in a hybridization oven for 5min

16 Immerse the sections in a staining jar containing chilled PBS

In situ RT

17 Mix 0.5 μl of 500 ng/μl caged Oligo DNA, 0.5 μl of 10 mM dNTPs, and 5 μl of nuclease-free water in a 0.2 ml tube, and heat to 65°C and then quickly cool to 4°C using Thermal cycler

18 Add 2 µl of 5× First Strand Buffer, 1 µl of 0.1 M DTT, 0.5 µl of 40 U/µl RNase Out, and 0.5 µl of 200 U/µl Superscript II Reverse Transcriptase to the primer mix prepared above (17)

19 Drop 10 µl of the RT mix prepared above (18) onto the section and seal with a coverslip

20 Incubate the sections at 42 °C for 1 hr in a PBS-humidified chamber

21 Immerse the sections in PBS preheated to 70 °C in a staining jar and keep at 70 °C in a hybridization oven for 10min

22 Immerse the sections in a staining jar cold containing chilled PBS

23 Wash the sections twice with PBS

Option : Immunostaing

24 Incubate the sections for 15 min with blocking solution at room temperature

25 Incubate the sections with primary antibodies diluted in blocking solution for 2 hr at room temperature or overnight at 4 °C in a hybridization oven

26 Wash the sections three times with TBST

27 Incubate the sections with 2nd antibodies diluted in blocking solution for 1 hr at room temperature or overnight at 4 °C in a hybridization oven

28 Wash the sections three times with TBST

29 Incubate the sections with Nuclear Violet LCS (1:1000) with blocking solution for 15 min at room temperature in a hybridization oven

30 Mount the sections with SlowFade Diamond before coverslipping

UV irradiation

31 Irradiate 365 nm-centered UV light to the ROIs for 15 min

Note: Since the caged compound (NPOM) used in PIC is cleaved most efficiently at 365 nm, the irradiation can be performed with the fluorescence filter cube that are conventionally used to excite nuclear staining dyes with blue fluorescence such as DAPI and Hoechst. We used Leica A filter cube (#11513873; 340–380 nm excitation) for uncaging under a Leica DM5000 B fluorescence microscope illuminated with an EL6000 100 W Hg lamp.

Cell lysis

32 Strip the cover slip, and wash the sections three times with PBS

33 Drop 40 µ l of proteinase K lysis solution to the sections

34 Incubate the sections in a hybridization oven at 42 °C for 30 min

35 Scrape the sections and collect the cell lysates and debris in a 1.5 ml tube

36 Drop 40 µl of proteinase K lysis solution to the section, again

37 Incubate the sections in a hybridization oven at 42 °C for 30 min, again

38 Collect the cell lysates and debris in the same tube as (36) (total volume ≃ 80 µl)

39 Incubate for 30 min at 55 °C

40 Purify cDNA:mRNA hybrids with Qiagen MinElute PCR Purification kit

41 Elute with 15 µl of nuclease-free water

Second-strand DNA synthesis

42 Mix the eluted 15 µl of cDNA:mRNA hybrids with 2 µl of 5xFirst Strand Buffer, 2.31 µl of 5x Second Strand Buffer, 0.23 µl of 10 mM dNTPs, 0.08 µl of E.coli DNA ligase (10 U/µl), 0.3 µl of E.coli DNA polumerase (10 U/µl) and 0.08 µl of E.coli RNaseH (2 U/µl)

43 Incubate for 2 hr at 16 °C

44 Mix 20 µl of second-strand DNA sample and 24 µl of Ampure binding buffer mix (Ampure XP beads : Beads binding buffer = 1 : 5) and vortex thoroughly

45 Incubate for 15 min at room temperature

46 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

47 Remove the supernatant

48 Add 200 µl of 80% Ethanol and incubate for 30 sec at room temperature

49 Remove the supernatant

50 Add 200 µl of 80% Ethanol and incubate for 30 sec at room temperature

51 Remove the supernatant

52 Spin down the sample tube

53 Place the sample tube on the magnetic stand and incubate for 2 min at room temperature

54 Remove the supernatant and air dry

55 Suspend the beads with 6.4 µl of nuclease-free water by pipetting

in virto transcription

56 Mix the 6.4 µl of beads suspension containing double-strand cDNAs with 1.6 µl of ATP, 1.6 µl of GTP, 1.6 µl of CTP, 1.6 µl of UTP, 1.6 µl of 10xT7 reaction buffer, and 1.6 µl of T7 Enzyme from the MEGAscript T7 Transcription Kit

57 Incubate for 17 hr at 37 °C

58 Add 1 µl of TURBO DNase from the MEGAscript T7 Transcription Kit and incubate for 15 min at 37 °C

59 Add 3 µl of ExoSAP-IT Express PCR Product Cleanup and incubate for 5 min at 37 °C

60 Add 5.5 µl of fragmentation Buffer and incubate for 3 min at 94 °C and quickly cool to 4°C using Thermal cycler

61 Place the sample tube on ice and add 2.75 µl of 0.5 M EDTA pH8.0

62 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

63 Collect 28 µl of supernatant in a new 0.2 ml tube

aRNA Cleanup

64 Mix 28 µl of aRNA samples with 50.4 µl of RNAClean XP beads and vortex thoroughly

65 Incubate for 10 min at room temperature

66 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

67 Remove the supernatant

68 Add 200 µl of 70% Ethanol and incubate for 30 sec at room temperature

69 Remove the supernatant

70 Add 200 µl of 70% Ethanol and incubate for 30 sec at room temperature

71 Remove the supernatant

72 Spin down the sample tube

73 Place the sample tube on the magnetic stand and incubate for 2 min at room temperature

74 Remove the supernatant and air dry

75 Suspend the beads with 7 µl of nuclease-free water by pipetting

76 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

77 Collect 5 µl of supernatant in a new 0.2 ml tube

Library preparation

78 Mix eluted 5 µl of aRNAs with 1 µl of RandomhexRT (250 ng) and 0.5 µl of 10 mM dNTPs

79 Heat the tubes at 65°C and then quickly cool to 4°C using Thermal cycler

80 Add 2 µl of 5× First Strand Buffer, 1 µl of 0.1 M DTT, 0.5 µl of 40 U/µl RNase Out, and 0.5 µl of 200 U/µl Superscript II Reverse Transcriptase

81 Incubate for 10 min at 25°C, then for 1 hr at 42°C

82 Add 8.4 µl of nuclease-free water, 1.8 µl of RNA PCR primer 1 (10 µM), 1.8 µl of RNA PCR primer 2 (10 µM) and 22.5 µl of Pusion High-Fidelity PCR Master Mix

83 Amplify by PCR (98°C for 30 s, followed by 11 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 30 s, with final extension at 72°C for 10 min)

84 Add 1 µl of RNaseA (10 mg/ml) and incubate for 30 min at 37 °C

85 Add 6 µl of nuclease-free water and 32.5 µl Ampure XP beads, and vortex thoroughly

86 Incubate for 15 min at room temperature

87 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

88 Collect the supernatant in a new 0.2 ml tube

89 Add 12.5 µl Ampure XP beads, and vortex thoroughly

90 Incubate for 10 min at room temperature

91 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

92 Remove the supernatant

93 Add 200 µl of 80% Ethanol and incubate for 30 sec at room temperature

94 Remove the supernatant

95 Add 200 µl of 80% Ethanol and incubate for 30 sec at room temperature

96 Remove the supernatant

97 Spin down the sample tube

98 Place the sample tube on the magnetic stand and incubate for 2 min at room temperature

99 Remove the supernatant and air dry

100 Suspend the beads with 32 µl of nuclease-free water by pipetting

101 Incubate for 2 min at room temperature

102 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

103 Collect 30 µl of supernatant in a new 0.2 ml tube

104 Add 19.5 µl Ampure XP beads, and vortex thoroughly

105 Incubate for 15 min at room temperature

106 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

107 Collect the supernatant in a new 0.2 ml tube

108 Add 7.5 µl Ampure XP beads, and vortex thoroughly

109 Incubate for 10 min at room temperature

110 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

111 Remove the supernatant

112 Add 200 µl of 80% Ethanol and incubate for 30 sec at room temperature

113 Remove the supernatant

114 Add 200 µl of 80% Ethanol and incubate for 30 sec at room temperature

115 Remove the supernatant

116 Spin down the sample tube

117 Place the sample tube on the magnetic stand and incubate for 2 min at room temperature

118 Remove the supernatant and air dry

119 Suspend the beads with 17.5 µl of nuclease-free water by pipetting

120 Place the sample tube on the magnetic stand and incubate for 5 min at room temperature

121 Collect 15 µl of supernatant in a new 0.2 ml tube

122 Quantify cDNA library using high-sensitivity DNA chips on a Bioanalyzer 2100 and Library Quantification Kit

123 Sequence with Illumina sequencer (1–3 million reads per sample)

124 Analyze the sequence data according to CEL-seq2 protocol (Hashimshony, T. et al. Genome Biol 2016)