scCircle-seq unveils the diversity and complexity of circular DNAs in single cells

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Abstract

Extrachromosomal circular DNAs (ecDNAs) have emerged as important intra-cellular mobile genetic elements that affect gene copy number and exert in trans regulatory roles within the cell nucleus. Here, we describe scCircle-seq, a method for genomically profiling ecDNAs and unraveling their diversity and complexity in single cells. We implemented and validated scCircle-seq in normal and cancer cell lines, demonstrating that most ecDNAs largely vary between cells and are stochastically inherited during cell division, although their genomic landscape is cell type-specific and can be used to accurately cluster cells of the same origin. ecDNAs are preferentially produced from chromatin regions enriched in H3K9me3 histone mark and are induced during replication stress conditions. Concomitant sequencing of ecDNAs and RNA from the same cell uncovered the absence of correlation between ecDNA copy number and gene expression levels, except for few oncogenes, including MYC, contained within a large ecDNA in colorectal cancer cells. Finally, we applied scCircle-seq to one prostate cancer and two breast cancer specimens, revealing different ecDNA genomic landscapes and, in one triple negative breast cancer sample, the presence of two distinct tumor subclones harboring distinct ecDNA signatures. scCircle-seq is a scalable tool that can be used to dissect the complexity of ecDNAs across different cell and tissue types, and further expands the potential of ecDNAs for cancer diagnostics.

Introduction

Reagents

REAGENTS

- DPBS, with MgCl2 and CaCl2 (Sigma, Cat. No. D8662)
- TrypLE™ Express Enzyme (1X), phenol red (Thermo Fisher Scientific, Cat. No. 12605036)
- PBS (10X), pH 7.4, (Thermo Fisher Scientific, Cat. No. AM9625)
- EDTA (0.5 M), pH 8.0 (Thermo Fisher Scientific, Cat. No. AM9260G)
- TAPS (GOLDBIO, Cat. No. T-780)
- Poly (ethylene glycol) (Sigma, Cat. No. 89510)
- Nuclease-free water (Thermo Fisher Scientific, Cat. No. AM9932)
- UltraPure™ 1M Tris-HCl, pH 8.0 (Thermo Fisher Scientific, Cat. No. 15568025)
- MgCl2 (1 M) (Thermo Fisher Scientific, Cat. No. AM9530G)
- Sodium chloride solution (Sigma, Cat. No. S6546)
- Pierce™ DTT (Dithiothreitol) (Thermo Fisher Scientific, Cat. No. 20290)
- IGEPAL CA-630 (Sigma, Cat. No. I8896)
- Triton X-100 (Sigma, Cat. No. 93418)
- Hoechst 33342 Solution (20 mM, 12.3 mg/ml) (Thermo Fisher Scientific, Cat. No. 62249)
- UltraPure™ BSA (50 mg/mL) (Thermo Fisher Scientific, Cat. No. AM2616)
- Dynabeads™ MyOne™ Silane (Thermo Fisher Scientific, Cat. No. 37002D)
- NEBNext® FFPE DNA Repair Mix (NEB, Cat. No. M6630L)
- QIAGEN Protease (QIAGEN, Cat. No. 19157)
- Protease Inhibitor Cocktail Set VII (Sigma, Cat. No. 539138-1SET)
- Plasmid-Safe™ ATP-Dependent DNase (Lucigen, Cat. No. E3101K)
- ATP Solution (100 mM) (Thermo Fisher Scientific, Cat. No. R0441)
- phi29 DNA Polymerase (NEB, Cat. No. M0269L)
- Deoxynucleotide (dNTP) Solution Mix (NEB, Cat. No. N0447L)
- Exo-Resistant Random Primer (Thermo Fisher Scientific, Cat. No. SO181)
- Pyrophosphatase, inorganic (0.1 U/µL) (Thermo Fisher Scientific, Cat. No. EF0221)
- DNA Clean & Concentrator-5 (Zymo, Cat. No. D4014)
- Tn5 transposase assembled with Nextera adaptors
- Nextera dual index PCR primer (custom-made by IDT based on the sequences in the Nextera XT DNA Library Preparation Kit, Illumina)
- NEBNext Ultrall Q5 PCR Mastermix (NEB, cat. no. M0544L)
- Agencourt RNAClean XP (Beckman Coulter, Cat. No. A63987)
- Agencourt AMPure XP (Beckman Coulter, Cat. No. A63881)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- Bioanalyzer High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4627)

CONSUMABLES
- Sterile Disposable Scalpel (No. 10) (VWR, Cat. No. 233-5363)
- Eppendorf® DNA LoBind tubes 5 mL (Sigma, Cat. No. EP0030108310)
- Eppendorf® DNA LoBind tubes 0.5 mL (Sigma, Cat. No. EP0030108035)
- Eppendorf® DNA LoBind tubes 1.5 mL (Sigma, Cat. No. EP0030108051)
- 15 mL conical tubes (Fisher Scientific, Cat. No. 430790)
- Falcon® 5 mL Round Bottom Polystyrene Tube, Sterile (Fisher Scientific, Cat. No. 10100151)
- Sapphire Filter tips, low retention (Greiner Bio-One, Cat. No. 771265, 773265, 738265, 750265)
- 50 μm CellTrics® cell strainer (Sysmex, Cat. No. 04-004-2327)
- 20 μm CellTrics® cell strainer (Sysmex, Cat. No. 04-004-2325)
- 10 μm CellTrics® cell strainer (Sysmex, Cat. No. 04-004-2324)
- Qubit™ Assay Tubes (Thermo Fisher Scientific, Cat. No. Q32856)
- Bioanalyzer High-sensitivity DNA Kit (Chips) (Agilent, Cat. No. 5067-4626)

Equipment

- Cell counter (Invitrogen™ Countess™ II FL Automated Cell Counter or equivalent)
- Centrifuge (Eppendorf™ Microcentrifuge 5424 and 5810 or equivalent)
- Sample mixer (Thermo Scientific™ Tube Revolver Rotator or equivalent)
- Non-contact liquid handler (Dispendix GmbH I.DOT)
- Incubator (Binder GmbH KB 53 or Boekel Scientific 241000 or equivalent)
- Thermoshaker (Eppendorf™ Thermomixer® F or equivalent)
- PCR cycler for tubes or strips (Analytik Jena™ Biometra TRIO or equivalent)
- Magnetic stand (Invitrogen™ DynaMag™-2/-5 Magnet or equivalent)
- Fluorometers (Qubit® 2.0 Fluorometer or equivalent)
- Fragment analyzer (Agilent 2100 Bioanalyzer or equivalent)
Procedure

Cell preparation

1. Wash the cells with 1×DPBS containing MgCl2 and CaCl2 pre-warmed to 37 °C and aspirate the DPBS from the flask
2. Add TrypLE™ Express Enzyme and incubate at 37 °C to dissociate cells
3. Add equal volume of culture medium to neutralize the enzyme and resuspend the cells
4. Count cell number and take 1×106 cells

NOTE: For suspension cells, step 1-3 can be skipped

1. Pellet cells at 100-400g for 5 min (differs per cell type)
2. Wash cells with 1×PBS/5mM EDTA for once
3. Pellet cells at 100-400 g for 5 min
4. Resuspend the cells with proper amount of 1xDPBS and dilute them into a proper concentration for mouth-pipetting

Nuclei extraction and DNA nick repair

Prepare the following working solutions

- **Nucleus Isolation Buffer** (1 mL)
  
  Tris-HCl, pH 8.0 (1M)  10 μL  
  NaCl (5M)  2 μL  
  MgCl2 (1M)  3 μL  
  IGEPAL CA-630 (10%)  30 μL  
  BSA (10%)  100 μL  
  DTT (1M)  2 μL  
  Twenn-20 (10%)  10 μL  
  Nuclease-free water  845 μL

**NOTE:** Prepared fresh buffer before nucleus isolation every time.

- **Nick Repair Mix** (40 μL for 100 samples)
  
  NEBNext FFPE DNA Repair Mix  4.9 μL
NEBNext FFPE DNA Repair Buffer (10 x) 15 µL

Linear spike-in DNA (1 pg/µL) 10 µL

Circular spike-in DNA (1 pg/µL) 10 µL

**NOTE:** Prepared fresh nick repair mix right before reaction every time.

- 2 x Triton Lysis (1 mL)
  - Tris-HCl, pH 8.0 (1M) 40 µL
  - NaCl (5M) 8 µL
  - TritonX-100 (10%) 20 µL
  - DTT (1M) 30 µL
  - EDTA (0.5M) 4 µL
  - Nuclease-free water 898 µL

**NOTE:** Store at -20 °C for up to 1 year.

- 2 x Trans8k (1 mL)
  - TPAS buffer (1M, pH 8.5) 20 µL
  - MgCl2 (1M) 10 µL
  - PEG 8k (50%) 320 µL
  - Nuclease-free water 650 µL

**NOTE:** Store at -20 °C for up to 1 year.

**Nuclei extraction and DNA nick repair**

1. Mouth-pipette the single cell into PCR tube with 6.75 µL *Nucleus Isolation Buffer* and 0.25 µL Dynabeads™ MyOne™ Silane beads
2. Incubate the PCR tubes on ice for 30 minutes
3. Vortex the PCR tubes for 1 minute and then centrifuge at 4 °C for 5 minutes
4. Transfer 5.6 µL of supernatant into another PCR without disturbing the bead pellets

**NOTE:** The supernatant can be subjected to Smart-seq2 protocol or be stored in -20 °C for up to a week
1. Dispense 0.4 μL Nick Repair Mix to each sample without touching the liquid surface.
2. Spin down the liquid and mix by gently vortexing them

NOTE: It is important to vortex gently in case genomic DNA is heavily fragmentized.

1. Incubate the samples at 20 °C for 1 hour with the lid set at 50 °C in the PCR cycler.

Single cell lysis

1. Prepare the Lysis Buffer (for 110 samples):
   QIAGEN Protease (60 mg/ml) 4.4 μL
   2 x Triton lysis 155 μL

NOTE: Prepare the fresh lysis buffer every time before using.

1. Dispense 1.54 μL Lysis Buffer to each sample without touching the liquid surface
2. Spin down the liquid and mix by gently vortexing them

NOTE: It is important to vortex gently in case genomic DNA is heavily fragmentized.

1. Incubate the samples in the PCR cycler at 50 °C for 1 hour with a cap temperature of 90 °C
2. Prepare the Inhibition Mix (for 110 samples):
   Protease Inhibitor Cocktail Set VII 5.5 μL
   Nuclease-free water 49.5 μL

1. Dispense 0.5 μL Inhibition Mix to each sample without touching the liquid surface
2. Spin down the liquid and mix by gently vortexing them

NOTE: It is important to vortex gently in case genomic DNA is heavily fragmentized.

1. Incubate the samples at RT for 1 hour

Linear DNA digestion

1. Prepare the Digestion Mix (for 110 samples):
   ATP Solution (100 mM) 11 μL
   Plasmid-Safe Reaction Buffer (10 x) 55 μL
   Plasmid-Safe ATP-Dependent DNase (10 U/μL) 55 μL
**DTT**

(25 mM) 11 μL

Nuclease-free water 0 μL

**NOTE:** The quantity of the Plasmid-Safe ATP-Dependent DNase can be from 1 U to 5 U for each sample depending on the ploidy of the cell types.

1. Dispense 1.2 μL **Digestion Mix** to each sample without touching the liquid surface
2. Spin down the liquid and mix by gently vortexing them

**NOTE:** It is important to vortex gently in case genomic DNA is heavily fragmentized.

1. Incubate the samples in the PCR cyclers with lid set at 90 °C following the program below
   i. 37°C 20 h
   ii. 70 °C 10 min
   iii. 4°C forever

**Amplification of circular DNA**

1. Prepare the **Amplification Mix** (for 105 samples):
   - phi29 DNA Polymerase (10 U/μL) 84 μL
   - phi29 buffer (10 x) 105 μL
   - dNTP Solution Mix (10 mM) 105 μL
   - Exo-Resistant Random Primer (500 mM) 105 μL
   - Pyrophosphatase, inorganic (0.1 U/μL) 10.5 μL
   - Nuclease-free water 115.5 μL

1. Dispense 5 μL **Amplification Mix** to each sample without touching the liquid surface
2. Spin down the liquid and mix by gently vortexing them

**NOTE:** It is important to vortex gently in case genomic DNA is heavily fragmentized.

1. Incubate the samples in the PCR cyclers with lid set at 90°C following the program below
   i. 30°C 3-8 h
ii. 65 °C 10 min

iii. 4°C forever

NOTE: For fresh cells, 3 h amplification is enough but for deep fixed cells, amplification time should be at least 6 h.

Sample dilution and clean-up

For the tube:

1. Clean up the samples in the first column of the plate with 1x Agencourt AMPure XP according to its protocol and elute each sample with 30 μL elution buffer
2. Measure the concentration of each sample.

For the plate:

1. Take 1 μL of the original solution and dilute 20 x with Nuclease-free water in a new plate.
2. Clean up the samples in the first column of the plate with 1x Agencourt AMPure XP according to its protocol and elute each sample with 30 μL elution buffer
3. Measure the concentration of each sample and calculate the average amount of DNA for each plate.

NOTE: Usually the total mass of DNA would be around 40-400 ng per sample and if the total mass is below 10 ng, it might result from the unspecific amplification of environmental DNA.

Library preparation

1. Prepare the following Mix:

   - **Tagmentation Mix** (for 50 samples):

     Trans8k (2 x) 250 μL

     Tn5 transposase assembled with Nextera adaptors (0.2 μM) 10 μL

   NOTE: For 4 ng input DNA (size ranging from 2 kb to 6 kb), 1 μL of 0.1 μM Tn5 is enough to generate a nice library. And the concentration of Tn5 can be changed accordingly with the amount of DNA input.

   - **Tnp Removal Mix** (for 50 reactions):

     NaCl (5M) 6 μL

     EDTA (0.5M) 9 μL

     QIAGEN Protease (60 mg/ml) 4 μL
Nuclease-free water 79 μL

1. Dispense 4 ng amplified DNA of each sample into a new PCR tube and add nuclease-free water to make the total volume to 4.8 μL.

1. Dispense 5.2 μL **Tagmentation Mix** to each sample and mix by vortexing them

**NOTE:** The samples should be thoroughly vortexed for the Tagmentation Mix is viscous and hard to mix.

Place the samples in the PCR cyclers with lid set at 90 °C following the program below

i. 4 °C forever

ii. 55 °C 10 min

iii. 4 °C forever

**NOTE:** Place the sample tubes into the PCR cycler as fast as possible and rest the samples on ice before putting them into PCR cycler. After it, skip the i step (4 °C forever) which is for temporarily repressing the mix from early tagmentation reaction.

1. Take out the samples tube immediately after the reaction and rest them on ice.

2. Dispense 2 μL **Tnp Removal Mix** to each sample and mix by vortexing.

**NOTE:** Dispense the Tnp Removal Mix to each sample as fast as possible.

Place the samples in the PCR cyclers with lid set at 90 °C following the program below

i. 4 °C forever

ii. 50 °C 30 min

iii. 70 °C 15 min

iv. 4 °C forever

**NOTE:** Skip the i step after all the samples are placed in the PCR cycler.

1. Prepare the Library Preparation Mix (for 50 samples):

Q5 Master Mix (2 x) 725 μL

MgCl2 (0.1M) 25 μL

1. Dispense 14.5 μL **Library Preparation Mix** to each tube and 2 μL Nextera dual index PCR primer (10 μM) to each sample and mix by vortexing.
Place the samples in the PCR cyclers with lid set at 105 °C following the program below

i. 4 °C forever

ii. 72 °C 5 min

iii. 98 °C 30 sec

iv. 98 °C 10 sec

v. 62 °C 1 min

vi. 72 °C 2 min

Go to step iv 10 times

vii. 72 °C 5 min

viii. 4 °C forever

**NOTE:** Skip the i step after all the samples are placed in the PCR cycler. If you input less than 2 ng of DNA, increase the PCR cycle number to 12.

Library clean-up

1. Mix 96 samples with different indexes into 1 tube
2. Do double-size selection to the mixed library with 0.3 x Agencourt AMPure XP and 0.8 x Agencourt AMPure XP and elute the purified library with 30 μL elution buffer
3. Check the size distribution of the final libraries with Bioanalyzer

**NOTE:** An ideal size of the final library would be a platform-like distribution ranging from 400 bp to 900 bp.

Troubleshooting

Time Taken

In total, this protocol takes about 2 days from the samples to libraries.

References