Sequencing thousands of single-cell genomes with combinatorial indexing

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Abstract

Single-cell genome sequencing has proven valuable for the detection of somatic variation, particularly in the context of tumor evolution. Current technologies suffer from high library construction costs which restrict the number of cells that can be assessed and thus impose limitations on the ability to measure heterogeneity within a tissue. Here, we present Single cell Combinatorial Indexed Sequencing \(SCI-seq) as a means of simultaneously generating thousands of low-pass single cell libraries for somatic copy number variant detection.

Introduction

Here we present the protocol for Single cell Combinatorial Indexing and Sequencing \(SCI-seq) as a means of producing thousands of low-pass single cell genome sequencing libraries for the purpose of copy number variant and aneuploidy detection. The protocol requires nucleosome depletion in order to provide uniform sequence reads throughout the genome. We provide two alternative methods: Lithium Assisted Nucleosome Depletion \(LAND) and crosslinking with SDS \(xSDS). LAND typically produces greater read counts per cell at the cost of decreased coverage uniformity, whereas xSDS produced more uniform coverage at the cost of reduced read counts.

Reagents

• Phosphate Buffer Saline \(PBS, Thermo Fisher, Cat. 10010023) • 0.25% Trypsin \(Thermo Fisher, Cat. 15050057) • Tris \(Fisher, Cat. T1503) • HCl\(Fisher, Cat. A144) • NaCl \(Fisher, Cat. M-11624) • MgCl2 \(Sigma, Cat. M8226) • Igepal CA-630 \(Sigma, I8896) • Protease Inhibitors \(Roche, Cat. 11873580001) • Lithium 3,5-diiodosalicylic acid \(Sigma, Cat. D3635) - LAND Only • Formaldehyde \(Sigma, Cat. F8775) – xSDS Only • Glycine \(Sigma, Cat. G8898) – xSDS Only • HEPES \(Fisher, Cat. BP310) – xSDS Only • NEBuffer 2.1 \(NEB, Cat. B7202) – xSDS Only • SDS \(Sigma, Cat. L3771) – xSDS Only • Triton-X100 \(Sigma, Cat. 9002-93-1) – xSDS Only • DAPI \(Thermo Fisher, Cat. D1306) • TD buffer and NPM from Nextera kit \(Illumina, Cat. FC-121-1031) • 96 Indexed Transposomes \(either assembled using published methods or obtained from Illumina, oligos shown in Table 1) • Indexed i5 and i7 PCR primers \(Table 2) • SYBR Green \(FMC BioProducts, Cat. 50513) • Qiaquick PCR purification kit \(Qiagen, Cat. 28104) • dsDNA High Sensitivity qubit \(Thermo Fisher, Cat. Q32851) • High Sensitivity Bioanalyzer kit \(Agilent, Cat. 5067-4626) • NextSeq sequencing kit \(High or Mid 150-cycle) • Sequencing primers \(Table 3)

Equipment

• Dounce Homogenizer • 35μM Cell Strainer \(BD Biosciences, Cat. 352235) • Sony SH800 cell sorter \(Sony Biotechnology, Cat. SH800) or other FACS instrument capable of DAPI-based single nuclei sorting • CFX Connect RT Thermal Cycler \(Bio-Rad, Cat. 1855200) or other real time thermocycler • Qubit 2.0 Flourometer \(Thermo Fisher, Cat. Q32866) • 2100 Bioanalyzer \(Agilent, Cat. G2939A) • NextSeq 500 \(Illumina, Cat. SY-415-1001)

Procedure

1) Preparation of Nucleosome Depleted Nuclei \(1.i: LAND or 1.ii: xSDS) 1.i) LAND Nuclei Preparation & Nucleosome Depletion 1.i.a) Prepare nuclei in accordance with sample type below: Suspension Cell Culture 1.i.a.1) Triturate gently to break up cell clumps. 1.i.a.2) Pellet cells by spinning at 500xg for 5 minutes. 1.i.a.3) Wash with 500 µL ice cold PBS. Adherent Cell Culture 1.i.a.1) Aspirate media and wash cells with 10 mL of PBS at 37°. 1.i.a.2) Add enough 0.25% Trypsin at 37° to cover monolayer. 1.i.a.3) Incubate at 37° for 5 minutes or until 90% of cells are no longer adhering to surface. 1.i.a.4) Add 37° media at 1:1 ratio to quench Trypsin. 1.i.a.5) Pellet cells by spinning at 500xg for 5 minutes. 1.i.a.6) Wash with 500 µL ice cold PBS. Tissue Sample 1.i.a.1) Place tissue sample in 2 mL dounce homoginzer on ice. 1.i.a.2) Add 2 mL of NIB buffer \(10mM TrisHCl pH7.4, 10MM NaCl, 3mM MgCl2, 0.1% igepal, 1x protease inhibitors)to sample and allow to incubate on ice for 5 minutes. 1.i.a.3) Dounce 5 times with loose pestle followed by 15 strokes with tight pestle. 1.i.a.4) Put sample through 35µM cell strainer. \(it may be necessary to use more than one strainer) 1.i.b) Pellet cells or nuclei by spinning at 500xg for 5 minutes. 1.i.c) Resuspend in 200 µL 12.5 mM LIS in NIB buffer \(2.5 µL 1M LIS + 197.5 µL NIB buffer). 1.i.d) Incubate on ice for 5 minutes. 1.i.e) Add 800 µL NIB buffer and 5 µL DAPI\(5 mg/mL). 1.i.f) Gently pass through 35µM cell strainer. 1.ii) xSDS Nuclei Preparation & Nucleosome Depletion 1.ii.a) Prepare nuclei in accordance with sample type below: Suspension Cell Culture 1.ii.a.1) Triturate gently to break up cell clumps. 1.ii.a.2) To 10 mL of cells in media add 406 µL of 37% formaldehyde and incubate at room temp for 10 minutes with gentle shaking. 1.ii.a.3) Add 800 µL of 2.5 M Glycine and incubate on ice for 5 minutes. 1.ii.a.4) Centrifuge at 550xg for 8 minutes at 4°. 1.ii.a.5) Wash with 10 mL of ice cold PBS. 1.ii.a.6) Resuspend cells in 5 mL of ice cold NIB \(10mM TrisHCl pH7.4, 10MM NaCl, 3mM MgCl2, 0.1% igepal, 1x protease inhibitors). 1.ii.a.7) Incubate on ice for 20 minutes with gentle mixing. Adherent Cell Culture 1.ii.a.1) Aspirate media and wash cells with 10 mL of PBS at 37°. 1.ii.a.2) Add enough 0.25% Trypsin at 37° to cover monolayer. 1.ii.a.3) Incubate at 37° for 5 minutes or until 90% of cells are no longer adhering to surface. 1.ii.a.4) Add 37° media at 1:1 ratio to quench Trypsin. 1.ii.a.5) Bring volume to 10ml with media. 1.ii.a.6) Resuspend in 10 mL media, add 406 µL of 37% formaldehyde, and incubate at room temp for 10 minutes with gentle shaking. 1.ii.a.7) Add 800 µL of 2.5 M Glycine and incubate on ice for 5 minutes. 1.ii.a.8) Centrifuge at 550xg for 8 minutes at 4°. 1.ii.a.9) Wash with 10 mL of ice cold PBS. 1.ii.a.10) Resuspend cells in 5 mL of ice cold NIB. 1.ii.a.11) Incubate on ice for 20 minutes with gentle mixing. Tissue Sample 1.ii.a.1) Place tissue sample in 2 mL dounce homoginzer on ice. 1.ii.a.2) Add 2 mL of HEPES NIB \(20mM HEPES, 10MM NaCl, 3mM MgCl2, 0.1% igepal, 1x protease inhibitors) buffer to sample and allow to incubate on ice for 5 minutes. 1.ii.a.3) Dounce 5 times with loose pestle followed by 15 strokes with tight pestle. 1.ii.a.4) Put sample through 35µM cell strainer. \(it may be necessary to use more than one strainer) 1.ii.a.5) Bring volume up to 10ml with HEPES-NIB 1.ii.a.6) To the 10 ml, add 406 µL of 37% formaldehyde. 1.ii.a.7) Add 800 µL of 2.5 M Glycine and incubate on ice 5 minutes. 1.ii.b) Pellet cells or nuclei by spinning at 500xg for 5 minutes and wash with 900 µL of 1x NEBuffer 2.1 1.ii.c) Spin 500 x g for 5 minutes. 1.ii.d) Resuspend in 800 µL 1x NEBuffer 2.1 with 12 µL of 20% SDS and incubate at 42°C with vigorous shaking for 30 minutes. 1.ii.e) Add 200 µL of 10% Triton-X and incubate at 42°C with vigorous shaking for 30 minutes. 1.ii.f) Add 5 µL of 5mg/mL DAPI and pass through a t 35 µm

cell strainer. 2) Sorting and Tagmentation 2.i) Prep tagmentation plate with 10 µL 1x TD buffer \(for 1 plate: 500 µL NIB buffer + 500 µL TD buffer) 2.ii) Sort 2000 single nuclei into each well of the tagmentation plate. NOTE: At this step the number of nuclei per well can be varied slightly as long as the number of nuclei per well is consistent for the whole plate. It is also possible to multiplex different samples into different wells of the plate as the transposase index will be preserved. 2.iii) Gate according to Figure 1. 2.iv) Spin down plate. 2.v) Add 1 µL 2.5 µM of uniquely indexed transposome to each well. 2.vi) Seal plate and incubate at 55° for 15 minutes with gentle shaking. 2.vii) Let plate return to room temperature then place on ice. 2.viii) Pool all wells, add 5 µL DAPI\(5 mg/mL), and pass through 35µM cell strainer. 3) Second Sort and PCR Indexing 3.i) Prepare a master mix for each well with: 0.25 µL 20mg/mL BSA, 0.5 µL 1% SDS, and 7.75 µL H2O 3.ii) Add 8.5 µL of master mix and 2.5 µL of each \(i5 and i7) 10 µM primer to each well of a 96 well plate. 3.iii) Sort 15-22 single nuclei into each well using the most stringent sort settings. 3.iv) Spin down plate. 3.v) LAND ONLY: Incubate for 5 min at 55° to denature transposase. 3.v) xSDS ONLY: Incubate at 68° for 45 minutes to denature transposase and reverse crosslinks. 3.vi) Add 12 µL \(7.5 µL NPM + 4 µL H2O + 0.5 µL 100x sybr green) to each well of strip tube \(for 1 plate: 750 µL NPM + 400 µL H2O + 50 µL sybr) 3.vii) Perform the following PCR cycles: 72°C for 5 minutes, 98°C for 30 seconds, then continual cycles of \(98°C for 10 seconds, 63°C for 30 seconds, 72°C for one minute followed by a plate read and an additional 10 seconds at 72°C). These cycles should be repeated until the majority of wells are exhibiting exponential amplification as determined by SYBR green fluorescence. 4) Library Clean Up and Quantification 4.i) Pool 5 µL from each well of the PCR plate 4.ii) Purify using Qiaquick PCR Purification column and elute in 30 µL of EB. 4.iii) Run 2 µL of cleaned up pooled library on dsDNA HS qubit. 4.iv) Use qubit reading to dilute library to ~4 ng/µL and run 1 µL on a High Sensitivity Bioanalyzer chip. 4.v) Use bioanalyzer concentration results for the range of 200 bp - 1 kb to dilute the pool to 1 nM for sequencing. 5) Sequencing 5.i) Set up NextSeq run as per manufacturers instructions for a 1 nM sample except for the following changes. 5.ii) Library pool should be loaded at a concentration of 0.8 pM and a total volume of 1.5 mL and deposited into catridge position 10. 5.iii) Setup custom primers by diluting 9 µL of 100 µM stock sequencing primer 1 into a total of 1.5 mL of HT1 buffer into cartridge position 7; 9 µL of 100 µM stock sequencing primer 2 into a total of 1.5 mL of HT1 buffer into cartridge position 8; and 18 µL of each custom index sequencing primer at 100 µM stock concentrations to a total of 3 mL of HT1 buffer into cartridge position 9. 5.iv) Nextseq should be operating in standalone mode. Choose the SCIseq custom chemistry recipe \(Amini et. al. 2014). Select dual index. Enter appropriate number of read cycles \(50 recommended). And 18 cycles for each index. Select the custom checkbox for all reads and indices. 6) Process sequence data using the software included with this protocol \(also available at http://sci-seq.sourceforge.net) according to the accompanying instructions.

Figures

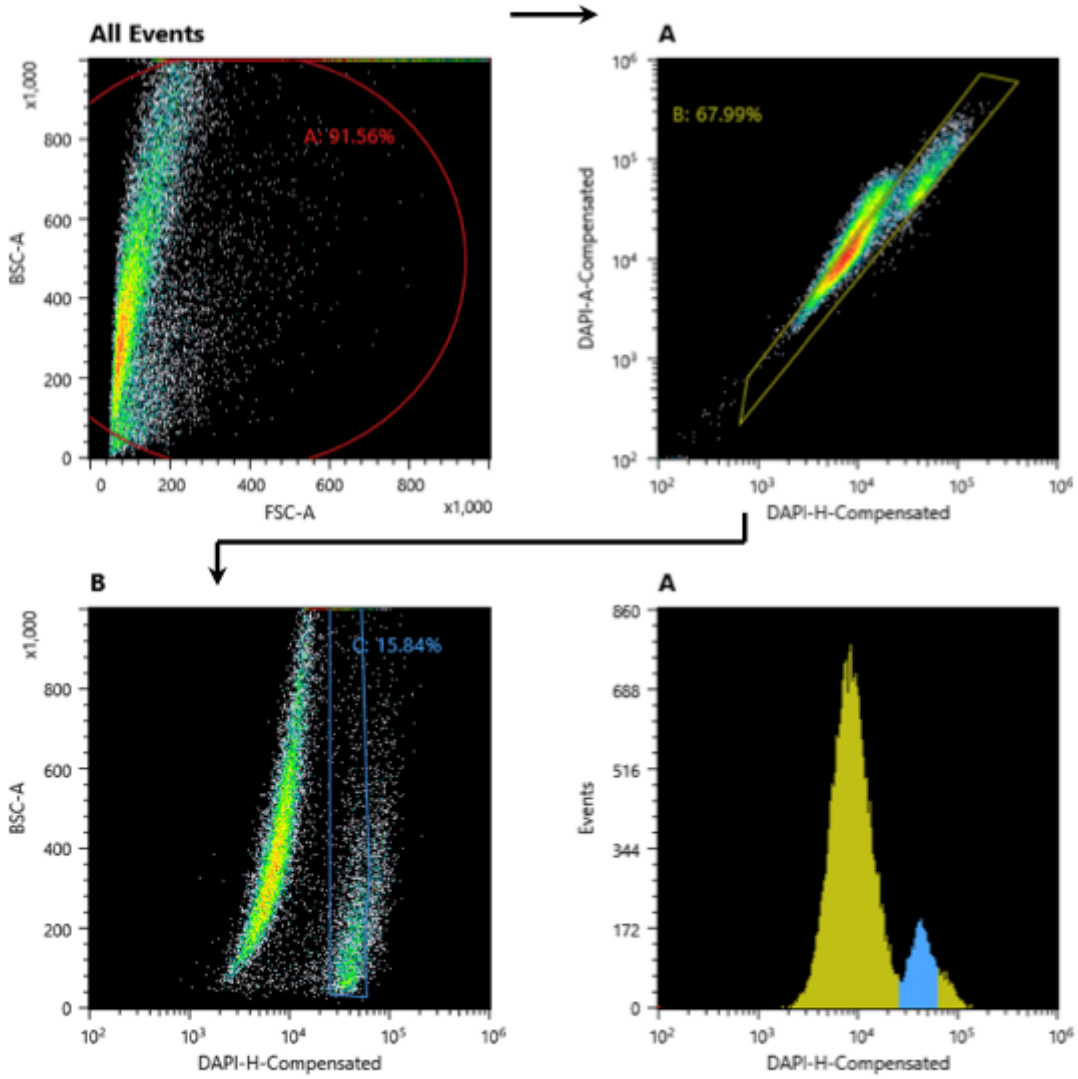

Figure 1

Gating for SCI-seq FANS Sorting

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.xlsx
- supplement0.zip