ECCITE-seq

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Method Article

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Abstract

Here, we describe Expanded CRISPR-compatible Cellular Indexing of Transcriptomes and Epitopes by sequencing \(ECCITE-seq) for the high-throughput characterization of at least five modalities of information from each single cell: transcriptome, immune receptor clonotypes, surface markers, sample identity and single guide RNA \(sgRNAs). ECCITE-seq adapts CITE-seq and Cell Hashing to a 5' tag-based scRNA-seq assay, integrating clonotype and cell surface marker information to RNA-based cellular phenotypes in immune cells. Additionally, ECCITE-seq allows the direct detection of sgRNAs, through a minor modification to the scRNA-seq workflow, facilitating high throughput and sensitive single cell perturbation screens compatible with existing guide libraries and commonly used vectors.

Reagents

Reagents: •Flow-cytometry grade monoclonal antibodies, unlabeled and purified •FC blocking reagent \(e.g. BioLegend FcX) •8-strip PCR tubes, emulsion safe \(e.g. TempAssure PCR 8-strips, USA Scientific) •Bioanalyzer chips and reagents \(DNA High Sensitivity kit, Agilent) •SPRIselect reagent \(GE Healthcare, B23317) •E-gel 2%-4% \(Invitrogen, USA) •Low-bind 1.5 mL tubes \(Common lab suppliers) •PCR Thermocycler \(e.g. Bio-Rad, T100) •Magnetic tube rack \(e.g. Invitrogen, USA) •Qubit \(Invitrogen, USA) •Hemocytometer \(e.g. Fuchs Rosenthal) •DMSO \(Common lab suppliers). •PBS \(Common lab suppliers) •Tween20 \(Common lab suppliers) •TE pH 8.0 \(Common lab suppliers) •BSA \(DNAse, RNAse and protease free, e.g. VWR #0332-25G) •Dead Cell Removal Kit \(e.g. Miltenyi) Oligonucleotide sequences: Protein tags: These contain standard small TruSeq RNA read 2 sequences and can be amplified using Illumina's Truseq Small RNA primer sets \(RPIx – primers, see example RPI1 below). See example below with a 12nt barcode: example: /5AmMC12/CCTTGGCACCCGAGAATTCCAxxxxxxxxCCCATATAAGAAA Hashtags: These contain standard TruSeq DNA read 2 sequences and can be amplified using truncated versions of Illumina's TruSeq DNA primer sets \(see example D701_s below). See example below with a 12nt barcode: example: /5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTxxxxxxxxCCCATATAAGAAA Guide tags arise after reverse transcription of sgRNAs primed by the oligo: sg_RT_v4: AGCAAGTGAGAAGCATCGTGTCAAAGCACCGACTCGGTGCCAC Oligos required for tag library amplification and TCR γ/δ enrichment: •10x Genomics SI-PCR primer 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC**T**C •Protein-tag additive primer 5'CCTTGGCACCCGAGAATT**C**C •Hashtag additive primer 5'GTGACTGGAGTTCAGACGTGTGC**T**C •Guide-tag additive primer 5'AGCAAGTGAGAAGCATCGTG**T**C •Illumina Small RNA RPI1 primer \(for protein-tag amplification; i7 index, Oligonucleotide sequences © 2015 Illumina, Inc) 5'CAAGCAGAAGACGGCATACGAGxxxxxxxxGTGACTGGAGTTCCTTGGCACCCGAGAATTC**C**A •Illumina TruSeq D701_s primer \(for hashtag amplification; i7 index, shorter than the original Illumina sequence) 5'CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTGACTGGAGTTCAGACGTGT**G**C •Next_nst_x: \(custom primer for guide-tag amplification; i7 index, scaffold annealing region) 5'CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATTTCTAGCTCTAAA**A**C •R1_hTRDC primer: \(for Human γ/δ mix 1) 5'AGCTTGACAGCATTGTACTTCC •R1_hTRGC primer: \(for Human γ/δ mix 1) 5'TGTGTCGTTAGTCTTCATGGTGTTCC •R2_hTRDC primer: \(for Human γ/δ mix 2) 5'TCCTTCACCAGACAAGCGAC •R2_hTRGC primer: \(for Human γ/δ mix 2) 5'GATCCCAGAATCGTGTTGCTC * Phosphorothioate bond B C or G or T; not A nucleotide

Equipment

• Magnetic tube rack • Bioanalyzer \(Agilent, USA) • PCR Thermocycler • Qubit \(Invitrogen, USA)

Procedure

Preparation of CITE-seq oligos and antibodies: As described in Stoeckius et al 2018 cell hashing manuscript: https://doi.org/10.1186/s13059-018-1603-1 CITE-seq run: 1. Cell staining o Obtain all single cell suspensions from different samples/conditions that will be multiplexed in the run. Keep samples in separate tubes until after cell hashing and shortly before loading cells into the single cell RNA-seq instrument. When aiming to super-load the same sample into one run, divide the sample up into equal proportions before staining with distinct cell hashing antibodies. Keep cell suspensions on ice \(unless otherwise stated) at all times. o Carefully count all cells to ensure accurate quantitation. • Make note of cell viability \(>95%) and also include dead cells in the total cell count\! • If you observe many dead cells, live cell enrichment \(e.g. Dead Cell Removal kit) is recommended\! o Resuspend ~1-2 million cells in 100 µl Staining buffer \(2%BSA, 0.01%Tween in PBS). o Add 10 µl Fc Blocking reagent \(FcX, BioLegend). o Incubate for 10 minutes at 4˚C. o While cells are incubating in Fc Block, prepare antibody-pool using 1-1.5 µg \(or titrated amounts) of each CITE-seq antibody and 1 µg of single cell hashing antibody \(pool). o Add antibody-oligo pool to cells. o Incubate for 30 minutes at 4˚C. o Wash cells 3 times with 1 mL Staining buffer spin 5 minutes 300g at 4˚C. o Resuspend cells in PBS and filter through 40 µm strainers \(e.g. Flowmi cell strainer). o Verify cell concentration by counting on hemocytometer after filtration. o Pool all different samples/conditions at desired proportions and immediately proceed to next step. Run 10x Genomics single cell V\(D)J assay according to manufacturer's instructions with the following modifications: 2. Guide-tag RT supplement At reverse transcription step: spike-in the guide RT primer o Add 5.9 µl of 2 µM gd_RT_v4 primer in the RT reaction \(0.12 µM final concentration). Subtract that volume from the water added to the cells. 3. cDNA PCR additive\(s) At cDNA amplification step: spike-in additive primers to increase yield of applicable tag products o Add 1 µl of 2 µM protein-tag additive, o Add 1 µl of 1 µM hashtag additive o Add 1 µl of 1 µM guide-tag additive 4. Separation of tag and cDNA libraries After cDNA amplification: separate protein tags and guide tags \(~180-200bp) from cDNAs \(>300bp) o Perform 0.6x SPRI selection to separate protein and guide tags from full-length cDNAs. o DO NOT DISCARD SUPERNATANT FROM 0.6x SPRI. THIS CONTAINS THE ADTs and hashtags\! • Add 0.6x SPRI to cDNA reaction as described in 10x Genomics or Drop-seq protocol. • Incubate 5 minutes and place on magnet. • Supernatant contains ADTs and hashtags. • Beads contain full length mRNA-derived cDNAs. o mRNA-derived cDNA >300bp \(beads fraction). • Proceed with standard 10x V\(D)J solution for cDNA sequencing library preparation. o Hashtags, Protein tags and guide tags ~180-200bp \(supernatant fraction) • Purify tags using two 2x SPRI purifications per manufacturer protocol: • Add 1.4x SPRI to supernatant to obtain a final SPRI volume of 2x SPRI • Transfer entire volume into a low-bind 1.5mL tube and incubate 10 minutes at room temperature. • Place tube on magnet and wait ~2 minutes until solution is clear. • Carefully remove and discard the supernatant. • Add 400 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds. • Carefully remove and discard the ethanol wash. • Centrifuge tube briefly and return it to magnet. • Remove and discard any remaining ethanol. • Resuspend in beads in 50 µl water. • Perform another round of 2x SPRI purification by adding 100 µl SPRI reagent directly onto resuspended beads. • Mix by pipetting and incubate 10 minutes at room temperature. • Place tube on magnet and wait ~2 minutes until solution is clear. • Carefully remove and discard the supernatant. • Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds. • Carefully remove and discard the ethanol wash. • Repeat wash • Centrifuge tube briefly and return it to magnet. • Remove and discard any remaining ethanol and allow the beads to air dry for 2 minutes • Resuspend beads in 45 µl water for each element captured \(135 µl for example if you need to make protein-tag, hashtag- and guide-tag libraries). • Pipette mix vigorously and incubate at room temperature for 5 minutes. • Place tube on magnet and transfer clear supernatant into new tube. This will be your input for the following PCR reactions: 5. Hashtag library preparation PCR in 100 µL using 25 pmol of each primer: 45 µl bead elution 50 µl 2x KAPA Hifi PCR Master Mix 2.5 µl 10 µM SI-PCR oligo 2.5 µl 10 µM TruSeq DNA D7xx_s primer \(containing i7 index) Cycling conditions: 95˚C 3 min 95˚C 20 sec | 64˚C 30 sec | ~8-12 cycles 72˚C 20 sec | 72˚C 5 min Expected product: 180bp 6. Protein-tag library preparation PCR in 100 µL using 25 pmol of each primer: 45 µl bead elution 50 µl 2x KAPA Hifi PCR Master mix 2.5 µl 10 µM SI-PCR oligo 2.5 µl 10 µM TruSeq Small RNA RPIx primer \(containing i7 index) Cycling conditions: 95˚C 3 min 95˚C 20 sec | 60˚C 30 sec | ~6-10 cycles 72˚C 20 sec | 72˚C 5 min Expected product: 180bp 7. Guide-tag library preparation 1. Guide-tag library \(this is an enrichment PCR to improve yield and specificity, indexing to follow in subsequent PCR): PCR in 100 µL using 25 pmol of each primer: 45 µl bead elution 50 µl 2x KAPA Hifi Master mix 2.5 µl 10 µM SI-PCR primer 2.5 µl 10 µM gd_add_v4 primer Cycling conditions: 95˚C 3 min 95˚C 20 sec | 64˚C 30 sec | ~8-10 cycles 72˚C 20 sec | 72˚C 5 min Expected product: 220bp • Purify all tag

libraries using 1.6x SPRI purification by adding 160 µl SPRI reagent. • Incubate 5 minutes at room temperature. • Place tube on magnet and wait 1 minute until solution is clear. • Carefully remove and discard the supernatant. • Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds \(first Ethanol wash). • Carefully remove and discard the ethanol wash. • Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds \(second Ethanol wash). • Carefully remove and discard the ethanol wash. • Centrifuge tube briefly and return it to magnet. • Remove and discard any remaining ethanol and allow the beads to air dry for 2 minutes. • Resuspend beads in 20 µl water. • Pipette mix vigorously and incubate at room temperature for 5 minutes. • Place tube on magnet and transfer clear supernatant to PCR tube. • Protein-tag and hashtag libraries are ready to be sequenced. • Proceed with indexing PCR for the guide-tag library PCR in 100 µL using 25 pmol of each primer: x µl bead elution \(carry over 5-10 ng of the 1st PCR product) 50 µl 2x KAPA Hifi Master mix 2.5 µl 10 µM SI-PCR primer 2.5 µl 10 µM Next_nst_x primer \(containing i7 index) 100 µl total Cycling conditions: 95˚C 3 min 95˚C 20 sec | 54˚C 30 sec | ~6-10 cycles 72˚C 20 sec | 72˚C 5 min Expected product: 210bp Purify library using 1.6x SPRI as described above. Elute in 20 µl water • Quantify all tag libraries by standard methods \(QuBit, BioAnalyzer, qPCR) 8. Preparation of the TCR γ/δ library The TCR γ/δ library is prepared by following the 10x Genomics protocol for generating TCR α/β libraries in Chapter 5 of the 10x protocol, but substituting primers as detailed below: PCR1: Human γ/δ mix 1 \(R1_hTRDC + R1_hTRGC) instead of T cell 1 PCR2: Human γ/δ mix 2 \(R2_hTRDC + R2_hTRGC) instead of T cell 2 PCR1: 2-5 µl full length cDNA 50 µl 2x KAPA Hifi Master mix 5 µl cDNA additive 2 µl 20 µM SI-PCR primer 2 µl 20 µM human γ/δ mix 1 Water to 100 µl PCR2: 35 µl bead elution 50 µl 2x KAPA Hifi Master mix 5 µl cDNA additive 2 µl 20 µM SI-PCR or P5 primer 2 µl 20 µM human γ/δ mix 2 Water to 100 µl Cleanups and PCR conditions are identical to the 10x protocol, with the exception that γ/δ libraries often require extra cycles of amplification \(~12) due to the comparative rarity of γ/δ T cells compared to α/β T cells in most cell populations. TCR γ/δ enriched libraries are further processed according to the 10x Genomics Single Cell V\(D)J protocol. Sequencing CITE-seq libraries: • Protein-tag, hashtag- and guide-tag libraries can be pooled with cDNA sequencing libraries at different proportions depending on the size of the antibody and guide panels. Typically, to obtain sufficient read coverage for all libraries we sequence protein-tag libraries in 3-10% of a lane, hashtag libraries in 2-5% of a lane and guide-tag libraries in 5-10% of a lane with the remainder dedicated to the cDNA library.

References

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Figures

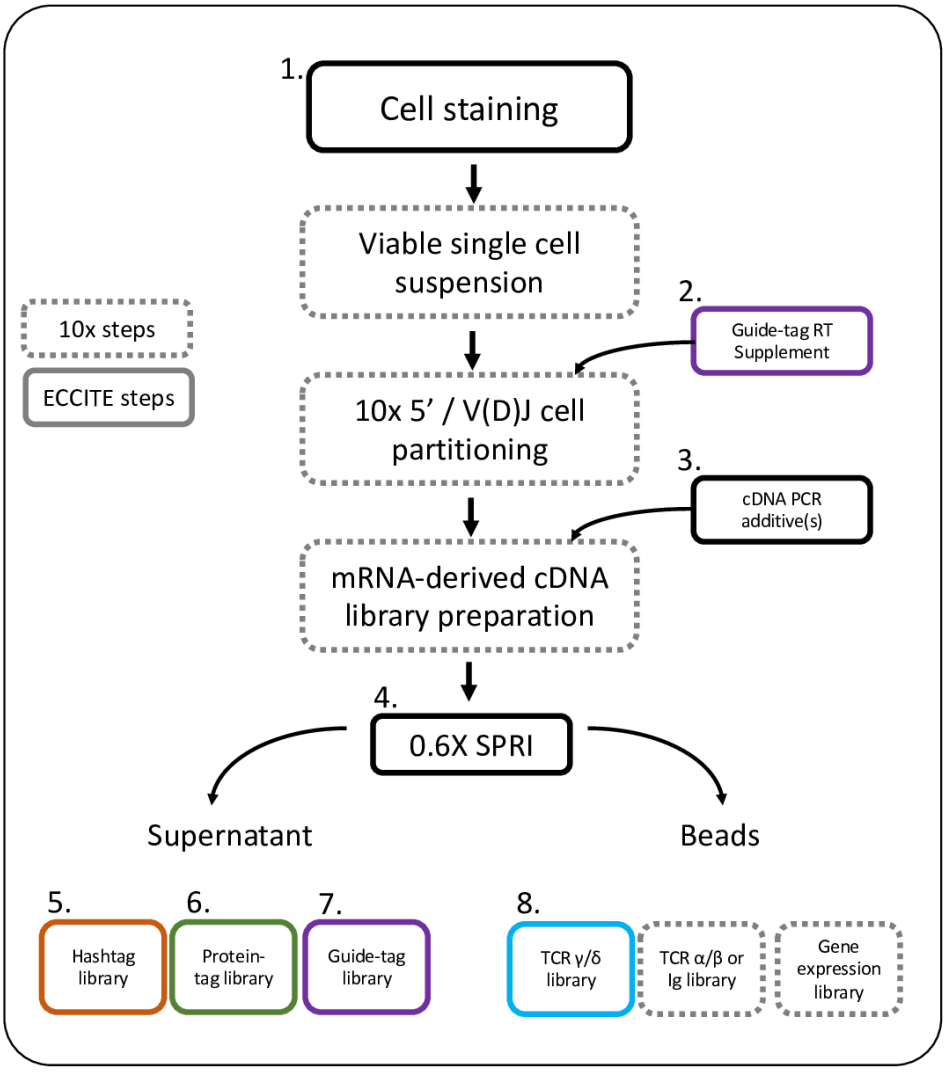

Figure 1

Protocol flowchart ECCITE-seq workflow: sections of the protocol that differ from the 10x Genomics V(D)J kit are highlighted. Refer to the associated section number for step-by-step guidance.