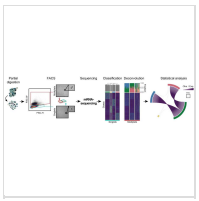

1

Nov 24, 2021

Cell Interaction by Multiplet sequencing (CIM-seq) V.1

Nathanael Andrews[1], Jason T. Serviss[1], Natalie Geyer (Karolinska Institute Stockholm)[1], Agneta B. Andersson[1], Ewa Dzwonkowska[1], Iva Šutevski[1], Rosan Heijboer[1], Ninib Baryawno (Karolinska Institute Stockholm)[1], Marco Gerling[1], Martin Enge[1]

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Single cell sequencing methods facilitate the study of tissues at high resolution, revealing rare cell types with varying transcriptomes or genomes, but so far have been lacking the capacity to investigate cell-cell interactions. Here, we introduce CIM-seq, an unsupervised and high-throughput method to analyze direct physical cell-cell interactions between every cell type in a given tissue. CIM-seq is based on RNA sequencing of incompletely dissociated cells, followed by computational deconvolution of these into their constituent cell types using machine learning. CIM-seq is broadly applicable to studies that aim to simultaneously investigate the constituent cell types and the global interaction profile in a specific tissue.

DOI

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https://www.biorxiv.org/content/10.1101/2020.03.06.980243v1.full

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protocol ,

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Nov 24, 2021

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MATERIALS

Lambda Exonuclease - 5,000 units **New England Biolabs Catalog #M0262L**

HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**

psfTn5 **addgene Catalog #79107**

10% SDS solution **Teknova Catalog #S0287**

SMARTScribe Reverse Transcriptase **Takarabio Catalog #634888**

Magnesium chloride solution for molecular biology (1.00 M) **Sigma – Aldrich Catalog #M1028**

Triton X-100 **Sigma Catalog #93426**

KAPA HiFi PCR kit with dNTPs **Fisher Scientific Catalog #NC0142652**

Recombinant RNAse Inhibitor **Takarabio Catalog #2313A**

Betaine 5M **Sigma Aldrich Catalog #B0300**

ERCC RNA Spike-In Mix **Thermo Fisher Catalog #4456740**

USB Dithiothreitol (DTT), 0.1M Solution **Thermo Fisher Catalog #707265ML**

UltraPure™ DEPC-Treated Water **Thermo Fisher Catalog #750023**

dNTP Mix (10 mM each) **Thermo Fisher Catalog #R0192**

Sera-Mag Speed Beads **Ge Healthcare Catalog #65152105050250**

Hard-Shell® 384-Well PCR Plates thin wall skirted **BioRad Sciences Catalog #HSP3801**

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Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33231**

PEG 8000 - (Polyethylene glycol) **Sigma Aldrich Catalog #P2139**

TAPS **Sigma Aldrich Catalog #T5130**

Oligonucleotides (all ordered from IDT using Standard desalting, except barcodes ordered in solution/plates)

Oligo-dT:
AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTT(N1:34333300)(N2:25252525)
IS_PCR: 5'-AAGCAGTGGTATCAACGCAGAGT-3'
TSO: 5'-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'
ME-A: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'
ME-B: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'
ME-Rev: 5'-/5Phos/CTGTCTCTTATACACATCT-3'

Illumina-compatible barcodes used (Sxxx/Nxxx series, n=784) are available as a supplementary table in the manuscript.

Before preparing cell lysis plates it is recommended to thoroughly clean all equipment with 70% EtOH and RNAse away to prevent contamination and avoid RNA degradation.

Prepare Lysis buffer

1 Prepare **Lysis Buffer:**

NOTE: Reagents are prepared on ice, working quickly. ERCC is stored in single-use aliquots at **-80 °C** , thawed on ice and added last.

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Reagent:	Reagent concentration:	µl per reaction:
H20	-	1.31
Inhibitor	1 U/µl	0.05
ERCC (1:600 000)	-	0.05
Triton-X100 (10% solution)	0.2%	0.04
10mM dNTP	2.5mM/each	0.5
100uM dT	2.5µM	0.05
Total	-	2

Add **2 µL lysis buffer mix** to each well. Cover with appropriate lids. Spin down.

Snap freeze on **dry ice**. Store until use at **-80 °C**

Sort cells

2 Sort single cells and multiplets (aggregates of multiple cells) into **2 µL lysis buffer mix.**
Multiplets can be discerned from singlets by gating on the basis of FSC-W (Forward scatter - Width) and FSC-H (Forward scatter - Height) (see **Figure 1**).

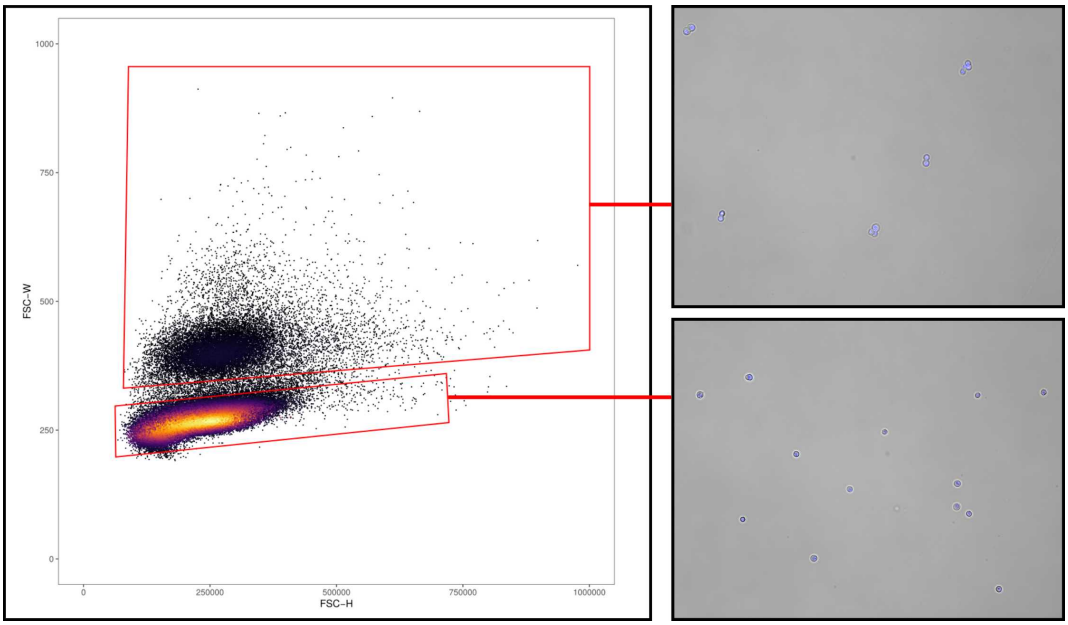

Figure 1. Gating scheme and result of FSC-W and FSC-H based sort of multiplets (top) and singlets (bottom) using HCT116 cells .

Following sort, immediately seal with appropriate seals (approved for -80C > 100C) and centrifuge

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at **2000 x g, 4°C, 00:05:00** .

Snap freeze on **dry ice**. Store until use at **-80 °C** .

Reverse transcription and cDNA amplification

3 Primer annealing

Thaw plate. Spin down. Incubate in thermocycler at **72 °C** for **00:03:00** . Place on ice immediately.

4 Prepare RT master-mix

Made fresh.

Reagent:	Reaction concentration:	Reagent volume:
SmartScribe	15u/µl	0.475
RNase Inhibitor	1.66u/µl	0.125
5x First Strand buffer	1X	1
DTT (100mM)	8.33mM	0.25
Betaine (5M) [fridge]	1.66M	1
MgCl2 (1M) [bench]	10mM	0.03
TSO (100uM)	1.66µM	0.05
H20	-	0.07
Total	-	3

Dispense **3 µL** per well.

Cover plate with new film and spin down.

5 Incubate in thermocycler

42 °C **01:30:00**

70 °C **00:05:00**

4 °C **hold**

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6 cDNA preamplification

Made fresh.

Reagents	Reaction concentration:	Reagent volume:
H2O	-	1.0688
Kapa HiFi HotStart ReadyMix (2x)	1X	6.25
IS_PCR primer (10uM)	0.1µM	0.125
Lambda Exonuclease	0,045u/µl	0.05625
Total	-	7.5000

Dispense **7.5 µL** per well . Total reaction volume will be 12.5µl.
Spin down. Cover with new lid.

7 Incubate in thermocycler with the following program:

Step	Temperature	Time	Cycles
Lambda exonuclease	37ºC	30 min	1x
Initial denaturation	95ºC	3 min	1x
Denaturation	98ºC	20 sec	18-24x
Annealing	67ºC	15 sec	
Elongation	72ºC	4 min	
Final elongation	72ºC	5 min	1x
	4C	Hold	

8

cDNA cleanup

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

We prepare SPRI-beads in 20% PEG-8000 solution as in:
https://openwetware.org/wiki/SPRI_bead_mix#Ingredients_for_50_mL_2

Using 20% SPRI-bead solution:

1. Add 0.7x the reaction volume of SPRI beads per well. Mix well by pipetting. (i.e **8.75 µL** SPRI-bead solution for **12.5 µL** reaction volume)
2. Incubate **00:05:00** **Room temperature**
3. Place on magnetic stand for **00:03:00**
4. Carefully remove supernatant
5. Add 40 µl 80% EtOH and incubate **00:00:30**
6. Remove EtOH (without disturbing the beads)
7. Wash again with EtOH. Make sure to remove well.
8. Allow beads to air-dry for **00:10:00** - **00:15:00**
9. Remove plate from magnetic stand
10. Elute beads in **15 µL** EB or TE buffer. Mix well by pipetting
11. Incubate **00:05:00** **Room temperature**
12. Place on magnetic plate for **00:03:00**
13. *Optional: Carefully remove supernatant to the elution plate*

9 cDNA quantification

We measure concentration of random wells using Qubit HS dsDNA, adapted to a 96-well plate reader.

1. Add **97 µL** of 1X Qubit HS dsDNA solution to a flat-bottom, black plate
2. Add **3 µL** of cDNA sample
3. Add Standards (NOTE: We make a 8-step ladder from 0ng/µl --> 10ng/µl Qubit Standard DNA in TE buffer)
3. Read in plate reader using 485nM excitation/528nm emission
4. Calculate cDNA concentration

10 (optional) cDNA quality control

Using Agilent HS 5000 DNA chips (or equivalent)

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

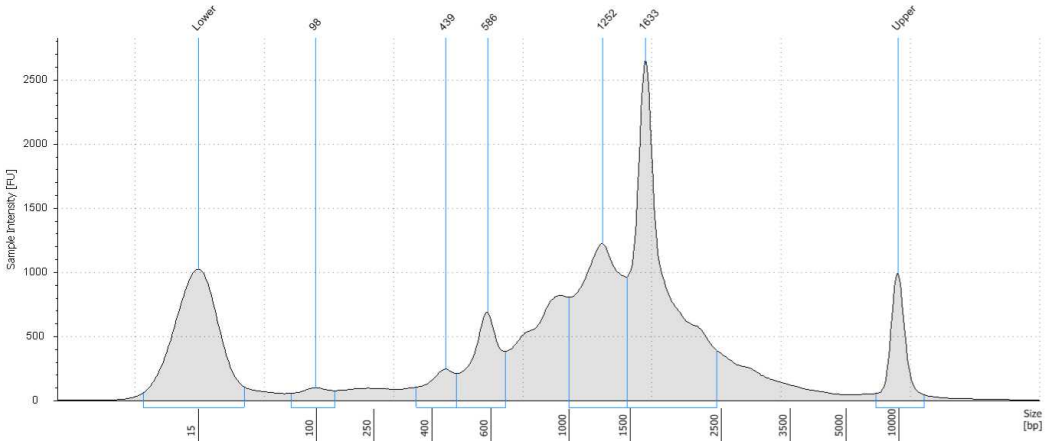

Figure 2. cDNA profile of single cell run on HS D5000 Agilent tapestation

11 Make cDNA dilution plate

Dilute cDNA in water based on average concentration from Qubit measurements.
Target concentration 150pg per µl in **15 µL** .

cDNA tagmentation

12 Tn5 digestion

Tn5 is produced from psfTn5 (Addgene #79107), purified to ~3mg/ml and assembled with Illumina Tn5 adapters (see oligos) as in Picelli et al, 2014.

Picelli S, Björklund AK, Reinius B, Sagasser S, Winberg G, Sandberg R (2014). Tn5 transposase and tagmentation procedures for massively scaled sequencing projects.. Genome research.
https://doi.org/10.1101/gr.177881.114

13 Prepare Tn5 master mix

NOTE: TAPS-PEG Buffer contains PEG, which is viscous. Buffer should equilibrate to room temperature before use to allow proper mixing.

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

Reagent	Reaction conc.	µl per reaction
Nuclease free H2O	-	1.05
TAPS-PEG (50mM TAPS, 25mM MgCl2, 40% PEG-8000)	10mM TAPS, 5mM MgCl2, 8% PEG-8000	0.5
psfTn5, loaded with 50µM MEDS-A/B		0.25
Total		1.8

Dispense **1.8 µL** per well in a new plate(tagmentation plate)

14

Add **0.7 µL** cDNA (normalized to 150pg/µl)
Mix well by vortexing plate. Cover with new lid and spin down.

15 Incubate in thermocycler at **55 °C** **00:10:00**

Remove immediately and stop reaction by adding **1 µL** per well of 0.1% SDS.

Vortex, spin down and incubate **00:07:00** at **Room temperature**

cDNA library PCR and barcoding

16 **Make PCR master-mix**

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

Reagents	µl per reaction
H2O	13.25
5x buffer	5
dNTPs	0.75
KAPA	0.5
Total	19.50

Dispense **19.5 µL** per well to tagmentation plate (containing **3.5 µL** sample after step 14)

17 Add primers/barcodes

2 µL per well (from 384-well index plates, with 3.75µM/each forward/reverse primers; **see oligos in materials**).

Total reaction volume is **25 µL** (**3.5 µL** sample + **19.5 µL** PCR mix and **2 µL** primers).

18

Vortex. Spin down and cover. Incubate in thermocycler as following:

Step	Temperature	Time	Cycles
Gap fill	72ºC	3 min	1x
First denature	95ºC	30 sec	1x
Denature	95ºC	15 sec	12x
Denature	67ºC	30 sec	
Denature	72ºC	45 sec	
Final extension	72ºC	4 min	1x
	4-10ºC	hold	

19

Pool **2.5 µL** from each well to an 1.5ml Eppendorf tube.

20 Library cleanup

1. Add 0.9x pooled library volume of SPRI-bead solution. Incubate for **00:05:00** at **Room temperature** .
2. Place on magnetic rack for **00:03:00** .

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

3. Remove supernatant without disturbing magnetic beads.
4. Add at least **1 mL** 80% EtOH (fresh). Incubate for **00:00:30** .
5. Remove supernatant.
6. Repeat EtOH wash.
7. Air dry for **00:10:00** - **00:15:00** .
8. Re-suspend beads thoroughly in 100 µl EB or TE buffer.
9. Place eppendorf on magnetic rack for **00:03:00** .
10. Transfer supernatant to new 1.5ml Eppendorf tube.
11. Repeat cleanup (from step 1-7) and elute in 30 µl EB or TE buffer.
12.(Optional) Place eppendorf on magnetic rack for **00:03:00** and transfer supernatant to new tube.

21 Pooled library QC

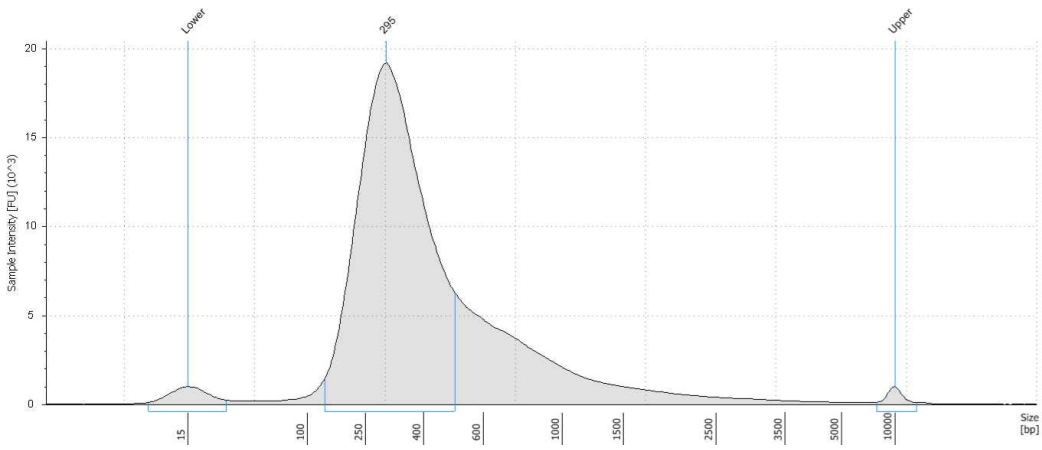

Figure 3. cDNA profile of a library of 784 cells (both single cells and multiplets) on HS D5000 Agilent tapestation.

Data pre-processing

22

A series of **pre-processing steps** must be performed in order to generate a counts file:

1. Trim reads, remove adapter sequences and align RNAseq data to reference genome using STAR:

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013). STAR: ultrafast universal RNA-seq aligner.. Bioinformatics (Oxford, England). https://doi.org/10.1093/bioinformatics/bts635

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

2. Remove duplicate reads using Picard:

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data.. Genome research. https://doi.org/10.1101/gr.107524.110

3. Generate transcript counts file using HTSeq:

Anders S, Pyl PT, Huber W (2015). HTSeq--a Python framework to work with high-throughput sequencing data.. Bioinformatics (Oxford, England). https://doi.org/10.1093/bioinformatics/btu638

Dimensionality reduction and classification

23

Once a counts file has been generated the data can be analyzed. CIM-seq requires four arguments in order to run:

1. The raw counts data with gene IDs as rownames and sample IDs as colnames.

2. The ERCC spike-in counts data with gene IDs as rownames and sample IDs as colnames.

3. The dimensionality reduced representation of the data.

4. A class for each of the individual singlets.

In order to generate the last two of these we recommend using the Seurat package in R, as CIM-seq is implemented in R as well. A number of tutorials for Seurat can be found on the Satijalab website:

https://satijalab.org/seurat/vignettes.html

CIM-seq

24

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6

CIM-seq can be downloaded from:
https://github.com/EngeLab/CIMseq

Or installed directly in R using the devtools package:

devtools::install_github("EngeLab/CIMseq")

The CIM-seq vignette can be found at:

https://github.com/EngeLab/CIMseq/tree/master/vignettes

Citation: Nathanael Andrews, Jason T. Serviss, Natalie Geyer (Karolinska Institute Stockholm), Agneta B. Andersson, Ewa Dzwonkowska, Iva ÃÂ utevski, Rosan Heijboer, Ninib Baryawno (Karolinska Institute Stockholm), Marco Gerling, Martin Enge Cell Interaction by Multiplet sequencing (CIM-seq) https://dx.doi.org/10.17504/protocols.io.bd7zi9p6