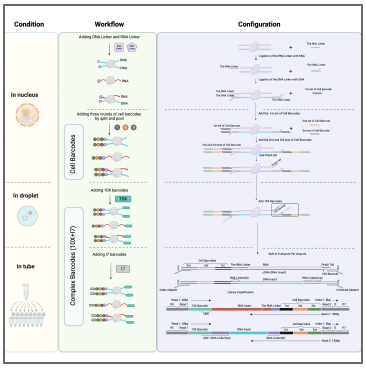

VERSION 1

MAR 11, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.4r3l22kwxl1y/v1

Protocol Citation: Wenxin Zhao, Zhifei Luo, Sheng Zhong 2024. MUSIC Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.4r3l22kwxl1y/v1Version created by Wenxin Zhao

Protocol status: Working
We use this protocol and it's working

MUSIC Protocol V.1

Wenxin Zhao[1], Zhifei Luo[1], Sheng Zhong[1]

[1]Shu Chien-Gene Lay Department of Bioengineering, University of California San Diego

Human BioMolecular Atlas Program (HuBMAP) Method Development Community

MUSIC

1 more workspace

Wenxin Zhao
UCSD

ABSTRACT

Here we introduce the Multi-Nucleic Acid Interaction Mapping in Single Cell (MUSIC) technique for concurrent profiling of multiplex chromatin interactions, gene expression, and RNA-chromatin associations within individual nuclei. MUSIC presents a potent tool for exploring chromatin architecture and transcription at cellular resolution in complex tissues.

Created: Feb 20, 2024

Last Modified: Mar 11, 2024

PROTOCOL integer ID: 95487

Funders Acknowledgement:
NIH grant
Grant ID: DP1DK126138
NIH grant
Grant ID: R01GM138852
NIH grant
Grant ID: UH3CA256960
NIH grant
Grant ID: U01CA200147
NIH grant
Grant ID: R01HD107206
Kruger Research Grant

Crosslinking and nuclei isolation for cell lines

1 Wash cell culture with ice-cold PBS.

2 Incubate cells with 1 mL of Accutase (EMD, SF006) for 3 min at 37°C to dissociate the cells.

3 Resuspend cells with 10 mL of ice-cold PBS to generate single cell suspension.

4 Collect cell pellets by centrifugation at 330 x g for 3 min.

5 Incubate cells with 10 mL of 2 mM disuccinimidyl glutarate (DSG) in 1X PBS in a 15 mL LoBind tube at room temperature for 45 min with gentle rotation.

6 Collect cells by centrifugation at 1,000 x g for 4 min, and wash cells once with 1X PBS.

7 Resuspend cells in 10 mL of 1X PBS containing 3% formaldehyde, and incubate cells for 10 min with a gentle rotation.

8 Quench the reaction by adding 3 mL of 2.5 M glycine per 10 mL of 3% formaldehyde and incubating cells for 5 min with rotation.

9 Wash cells twice with ice-cold 1X PBS containing 0.5% BSA (wt/vol), and centrifuge cells at 1,000 x g for 4 min.

10 Resuspend cells in ice-cold 1X PBS with 0.5% BSA (wt/vol), and aliquot 5M cells into each 1.5 mL tube.

11 Collect cells by centrifugation at 1,000 x g for 5 min and snap-freeze cells in liquid nitrogen, and stored at -80°C.

12 Thaw frozen cells on ice, and incubate cells in 1.4 mL of cell lysis buffer A for every 5M cells for 10 min on ice.

	A	B	C	D
	Component	Working Conc.	Stock Conc.	Volume
	HEPES, pH 7.4	50 mM	1 M	2.5 mL
	NaCl	140 mM	5 M	1.4 mL
	EDTA, pH 8.0	1 mM	0.5 M	100 uL
	EGTA, pH 8.0	1 mM	0.5 M	100 uL
	Glycerol	10%	100%	5 mL
	Triton X-100	0.25%	100%	625 uL
	NP-40	0.5%	100%	250 uL
	Water			10.475 mL
	Total Volume			50 mL

Cell lysis buffer A

13 Collect cell pellets by centrifugation at 900 x g for 4 min at 4°C, and incubate cells in 1.4 mL of cell lysis buffer B on ice for 10 min.

	A	B	C	D
	Component	Working Conc.	Stock Conc.	Volume
	Tris-HCl, pH 8	10 mM	1 M	0.5 mL
	NaCl	200 mM	5 M	2 mL
	EDTA, pH 8.0	1.5 mM	0.5 M	150 uL
	EGTA, pH 8.0	1.5 mM	0.5 M	150 uL
	Water			47.2 mL
	Total Volume			50 mL

Cell lysis buffer B

14 Centrifuge isolated nuclei at 900 x g for 5 min at 4°C.

Crosslinking and nuclei isolation for brain tissues

15 Thaw 50 mg of post-mortem human brain frontal cortex sample on ice in a 1.5ml LoBind tube, and chop the tissue into smaller pieces by pestle.

16 Incubate samples with 10 mL of 2 mM disuccinimidyl glutarate (DSG) in 1X PBS in a 15 mL LoBind tube at room temperature for 45 min with gentle rotation.

17 Wash once with 10 mL of 1XPBS by centrifugation at 1,000 x g for 4 min.

18 Resuspend the sample in 15 mL of 1X PBS containing 3% formaldehyde, and incubate for 10 min with a gentle rotation.

19 Quench the crosslinking reaction by the addition of 5 mL of 1.25 M glycine followed by an incubation of 5 min with a rotation.

20 Centrifuge the sample at 1,000 x g for 4 min and washed twice with ice-cold 1X PBS containing 0.3% BSA (wt/vol).

21 Chromium Nuclei Isolation kit (10X genomics, 1000494) was used to isolate nuclei from crosslinked cortex samples according to the section for “single cell gene expression & chromium fixed RNA profiling” (page 25 to page 30 from sample user guide).

22 All the following steps (steps 23-71) are the same for either cell line or human cortex samples.

Nuclei permeabilization

23 Thoroughly resuspend and permeabilize the nuclei in 200 µL of 1X rCutSmart buffer (NEB, B7204S) containing 0.25% SDS at 62°C for 10 min using Eppendorf Thermomixer C (Eppendorf).

24 Add 60 µL of 1X rCutSmart buffer containing 10% Triton X-100 (wt/vol) into the SDS solution above, and incubate the reaction at 37°C for 15 min while shaking at 800 rpm.

25 Wash treated nuclei once with 1X rCutSmart buffer by centrifugation at 900 x g for 2 min at 4°C.

5' Phosphorylation

26 Resuspend nuclei in 250 µL of 5’ Phosphorylation Master Mix followed by an incubation at 37°C while rotating at 800 rpm for 1 hour.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	10X T4PNK buffer	1X	25
	T4PNK (10 U/uL)	0.5 U/uL	12.5
	ATP (10 mM)	1 mM	25
	RNasin (40 U/uL)	1 U/uL	6.25
	Water		181.25
	Total Volume		250

5’ Phosphorylation Master Mix

27 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

	A	B	C	D
	Component	Working Conc.	Stock Conc.	Volume
	PBS, pH 7.5	1X	10X	5 mL
	EDTA, pH 8.0	1 mM	0.5 M	100 uL
	EGTA, pH 8.0	1 mM	0.5 M	100 uL

	A	B	C	D
	Triton X-100	0.1%	100%	50 uL
	Water			44.75 mL
	Total Volume			50 mL

PBS Wash Buffer 1

	A	B	C	D
	Component	Working Conc.	Stock Conc.	Volume
	PBS, pH 7.5	1X	10X	5 mL
	Triton X-100	0.1%	100%	50 uL
	BSA	0.3%	10%	1.5 mL
	Water			43.45 mL
	Total Volume			50 mL

PBS Wash Buffer 2

RNA linker ligation

28 Incubate the isolated nuclei with 250 µL of the RNA Ligation Mix at 25°C for 2 hours then 16°C overnight with an intermittent mixing at 800 rpm (30 seconds on and 270 off).

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	10X T4 RNA ligation buffer	1X	25
	T4 RNA ligase 1 (10 U/uL)	0.4 U/uL	10
	PEG (50%)	15 %	75
	ATP (10 mM)	1 mM	25
	RNasin (40 U/uL)	1 U/uL	6.25
	100 uM RNA linker	4 uM	10
	DMSO		18.75
	Water		80
	Total Volume		250

RNA Ligation Mix

29 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

Chromatin digestion

30 Resuspend the nuclei in a Digestion Master Mix followed by an incubation at 37°C for 3 h while rotating at 800 rpm.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	10X rCutSmart buffer	1X	30
	HpyCH4V (5 U/uL)	0.5 U/uL	30
	RNasin (40 U/uL)	1 U/uL	7.5
	Water		232.5
	Total Volume		300

Chromatin Digestion Master Mix

31 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

dA-tailing

32 Incubate the cells at 37 ℃ for 1.5 h with rotation at 800 rpm in 250 µL of the dA-tailing mix.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	10X NEBNext dA-Tailing reaction buffer	1X	25
	Klenow fragment (5 U/uL)	0.2 U/uL	10
	RNasin (40 U/uL)	1 U/uL	6.25
	Water		208.75
	Total Volume		250

dA-Tailing mix

33 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

DNA linker ligation

34 Incubate the cells at 20 °C overnight with rotation at 800 rpm (30s on and 270s off) in 250 µL of the DNA linker ligation mix.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	NEBNext Quick Ligation Reaction Buffer (5X)	0.8X	40
	Instant Sticky-end Ligase Master Mix (2X)	0.2X	25
	1, 2-Propanediol (100%)	6%	15
	DNA linker (45 uM)	4.5 uM	25
	RNasin (40 U/uL)	1 U/uL	6.25
	Water		138.75
	Total Volume		250

DNA linker ligation mix

35 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

5' Phosphorylation

36 Resuspend nuclei in 250 µL of 5’ phosphorylation master mix followed by an incubation at 37°C while rotating at 800 rpm for 1 hour.

37 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

Cell counting

38 Resuspend the nuclei in 900 µL of PBS Wash Buffer 2 with 0.2 U/µL of RNase Inhibitor, and filter the nuclei through a 10 µM cell strainer (pluriStrainer, 43-10010-50).

39 Stain 6 µL of the nuclei suspension with 6 µL of Ethidium homodimer-1, and count the number of nuclei by Countess II Automated Cell Counter (ThermoFisher).

Ligation of cell barcodes

40 Aliquot up to 10w of cells for split-pool, and collect the rest of the cells to assess the DNA length after chromatin fragmentation.

41 Anneal cell barcodes.

41.1 Make 100 µl of 45 µM DNA oligo duplex plates, in each well of the PCR plate, the following reaction will occur (total 100 µL):

	A	B	C	D
	Component	Stock Conc.	Working Conc.	Volume (uL)
	Top strand oligo	100 uM	45 uM	45
	Bottom strand oligo	100 uM	45 uM	45
	NaCl	5 M	50 mM	1
	ddH2O			9
	Total Volume			100

Oligo Annealing

Annealing using hybridization program:
Heat to 95°C and maintain the temperature for 2 min.
Cool to 22°C at a rate of -1°C/min.

Cool to 4 °C for temporary storage.

41.2 Aliquot 2.4 µL of annealed oligo duplex into several new 96-well plates. Seal the plate and centrifuge at 800 g for 2 min.

42 Fill the nuclei suspension to 1144 µL with PBS Wash Buffer 2 and 24 µL of RNAse inhibitor, and pipette 11.2 µL of cell solution into each well of the 96-well plate that contains cell barcodes.

43 Pipette 6.4 µL of ligation master mix into each well of the 96-well plate above.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	NEBNext Quick Ligation Reaction Buffer (5X)	0.8X	960
	Instant Sticky-end Ligase Master Mix (2X)	0.2X	600
	1, 2-Propanediol (100%)	6%	360
	Total Volume		1920

Ligation master mix

44 Ligate Barcode Set 1 with the linkers in each well in the Ligation Master Mix at 20°C overnight with an intermittent mixing at 1600 rpm (30 seconds on and 270 off).

45 Quench the ligation by an incubation for 10 min at 20°C at 1600 rpm (30s on and 270s off) in 60 µL of quenching buffer for each well of the 96-well plate.

46 The nuclei solutions from the 96 wells were pooled together into a 15 mL LoBind tube. 95 µL of quenching buffer was added to each well to rinse and collect any remaining nuclei and pooled into the same 15 mL tube.

47 Centrifuge the pooled nuclei at 900 x g for 4 min, and transfer the nuclei into a 1.5 mL tube with 0.5 mL of remaining supernatant. Rinse the 15 mL tube with 500 µL of PBS Wash Buffer 2, and collect the residual nuclei into the same 1.5 mL tube.

48 Wash the nuclei three times with 900 µL of PBS Wash Buffer 2 by the centrifugation at 900 x g for 2 min.

49 Repeat Steps 42-48 for the 2nd and 3rd rounds of split-pool.

3' Dephosphorylation

50 Incubate the cells at 37 ℃ for 1h at 800 rpm in the 3’ Dephosphorylation Buffer.

	A	B	C	D
	Component	Stock Conc.	Working Conc.	Volume (uL)
	Tris-HCl, pH 6.5	1 M	350 mM	350
	MgCl2	1 M	10 mM	50
	DTT	100 mM	10 mM	100
	Water			500
	Total Volume			1000

5X PNK Buffer

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	5X PNK Buffer	1X	50
	T4PNK (10 U/uL)	0.5 U/uL	12.5
	RNasin (40 U/uL)	1 U/uL	6.25
	Water		181.25
	Total Volume		250

3’ Dephosphorylation Buffer

51 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

PolyA tailing

52 Incubate the nuclei at 37 ℃ for 10 min at 800 rpm in the PolyA Tailing Buffer.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	10X E.coli Poly(A) Polymerase Reaction Buffer	1X	25
	E.coli Poly(A) Polymerase (5 U/uL)	0.08 U/uL	4
	ATP (10 mM)	1 mM	25
	RNasin (40 U/uL)	1 U/uL	6.25
	Water		189.75
	Total Volume		250

PolyA Tailing Buffer

53 Wash the nuclei once with 900 µL of PBS Wash Buffer 1 and three times with 900 µL of PBS Wash Buffer 2 at 900 x g for 2 min at 4°C.

Sonication

54 Filter nuclei in PBS+0.04% BSA via a 10 µM filter.

55 Stain 6 µL of the nuclei suspension with 6 µL of Ethidium homodimer-1, and count the number of nuclei by Countess II Automated Cell Counter (ThermoFisher).

56 Transfer 5k cells to a Covaris microtube-15, and filled the nuclei suspension to 15 µL with 1X PBS with 0.04% BSA (wt/vol). Sonicate the nuclei using Covaris M220 Focused-ultrasonicator with water temperature 6ºC, incident power 50 W, duty factor 5 for 5 min.

10X GEM

57 Load the RT Mix to the 10X Chromium controller according to Steps 1.1 to 1.5 in the protocol of Chromium Next GEM Single Cell 3' Reagent kit. Proceed to GEM generation (~18 min).

	A	B
	Reagent	Volume (uL)
	Reducing Agent B	18.8
	RT Reagent B	2
	RT Enzyme C	8.7
	Sonicated nuclei	20
	Water	20.5
	Total Volume	70

RT Mix

58 Take out all emulsions (~120-150 µl) to a PCR tube. Incubate the GEM in Thermocycler using the following program:

	A	B
	Temp. (°C)	Time
	53	45 min
	85	5 min
	Slowly cool down at 0.1°C/s	15 min
	4	Hold

Thermocycler Program for Reverse Transcription

59 Recover nuclei acids form emulsion with 1:1 (~150 µl) of recovery reagent (Go to 10X protocol). Wait for 2 mins. Discard all organic (pink) phase. Fill the aqueous phase to 200 µL with nuclease free water.

Reverse crosslinking

60 Aliquot 25 µl of the recovered nuclei solution into each of 8 LoBind 1.5 mL tubes with 25 µl of 2X reverse crosslinking mix to each of the 8 tubes.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	NaCl (5 M)	0.4 M	20
	SDS (20%)	o.4%	5
	EDTA (0.5 M)	50 mM	25
	EGTA (0.5 M)	50 mM	25
	Proteinase K (0.8 U/uL)	0.04 U/uL	12.5
	Water		162.5
	Total Volume		250

2X Reverse Crosslinking Master Mix

61 Incubate the tubes at 50 ℃ for 2h at 800 rpm (30s on and 270s off) and 55 ℃ overnight at 800 rpm (30s on and 270s off).

62 Purify the nuclei acid in 8 tubes with NEB RNA clean up kit (NEB, 76307-460) and elute in 21 µl of water.

Pre-PCR extension

63 Transfer 30 µl of the pre-PCR Master Mix into each of 8 PCR tubes. Transfer 20 µl of the eluted DNA into each of the 8 PCR tubes. Incubate eluted DNA at 55 ℃ for 15 min.

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	10X Isothermal Amplification Buffer II	1X	5
	Bst 3.0 DNA polymerase (8 U/uL)	0.32 U/uL	2
	MgSO4 (100 mM)	6 mM	3
	dNTP Mix (10 mM)	1.4 mM * 4	7
	RNasin (40 U/uL)	0.5 U/uL	0.625
	Water		12.375
	Total Volume		30

pre-PCR Master Mix

64 Purify with 1.8X (90 µL) of RNA clean Ampure beads, and elute the sample with 20 µl of water.

Library amplification

65 Conduct PCR for 13 cycles using the Ultra 2 program:

	A	B	C
	Reagent	FInal Conc.	Volume (uL)
	NEBNext Q5 Mater Mix	1X	25
	Illumina Universal Adaptor (10 uM)	0.5 uM	2.5
	Illumina Index Adaptor 1-8 (10 uM)	0.5 uM	2.5
	Sample		20
	Total Volume		50

PCR Master Mix

66 Purify the library with 1.2X (60 µL) of RNA clean Ampure beads. Elute with 12.5 µL of water.

Library size selection

67 Pool the 8 PCR elution, and load the elution into 5 wells of 4% E-gel. Check the size distribution.

68 Excise bands from 300-1200 bp. Extract the DNA with NEB Monarch gel purification kit (NEB, T1020S) using two columns, and elute the DNA with 15 µL of the elution buffer for each column.

Quantity/quality check and sequencing

69 Check the quantity with Qubit.

70 Check the size distribution with Tape station.

71 Sequence the library. Set the Read 1 of the sequencer to 28 bp, the Index 1 to 8 bp, and the Read 2 to 150 bp.