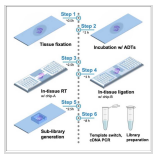

Sep 22, 2022

Spatial Multi-omics Sequencing for Fixed Tissue via DBiT-seq

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ABSTRACT

This protocol describes the use of Deterministic Barcoding in Tissue for spatial omics sequencing (DBiT-seq) platform to construct a multi-omics atlas on fixed frozen tissue samples. This approach uses a microfluidic-based method to introduce combinatorial DNA oligo barcodes directly to the cells in a tissue section fixed on a glass slide. This technique does not directly resolve single cells but can achieve a near-single-cell resolution for spatial transcriptomics and spatial analysis of a targeted panel of proteins.

DOI

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EXTERNAL LINK

https://www.sciencedirect.com/science/article/pii/S2666166721002392#mmc2

PROTOCOL CITATION

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MATERIALS TEXT

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TotalSeqTM antibodies	Biolegend	See Table 1
Chemicals, Peptides, and Recombinant Proteins		
Maxima H Minus Reverse Transcriptase (200 U/L)	Thermo Fisher Scientific	EP0751
dNTP mix	Thermo Fisher Scientific	R0192
RNase Inhibitor	Enzymatics	Y9240L
SUPERase• InTM RNase Inhibitor	Thermo Fisher Scientific	AM2694
T4 DNA Ligase	New England Biolabs	M0202L
KAPA Pure Beads	Kapa Biosystems	KK8002
DynabeadsTM MyOneTM Streptavidin C1	Thermo Fisher Scientific	65001
Proteinase K, recombinant, PCR grade	Thermo Fisher Scientific	EO0491
Kapa HiFi Hotstart ReadyMix PCR Kit	Kapa Biosystems	KK2601
Formaldehyde solution	Sigma	F8775-25ML
NEBuffer 3.1	New England Biolabs	B7203S
T4 DNA Ligase Reaction Buffer	New England Biolabs	B0202S
Evagreen Dye, 20X in water	Biotium	31000-T
Oligonucleotides		
Primers, Ligation linkers, DNA barcodes	IDT	See Table 2
Critical Commercial Assays		
DNA Clean & Concentrator with Zymo-Spin I Columns	Research Products International	ZD4004
Nextera XT DNA Preparation Kit	Illumina	FC-131-1024
Other		
Multiplate PCR Plate, 96-well, clear	BioRad	MLL9601
50 mL Falcon Tube	Corning	352070
BioDot Pure 8 – Strip PCR tubes w/ Optically Clear Flat Caps	Dot Scientific Inc.	403-8PCR
SYLGARDTM 184 Silicone Elastomer Base and Curing Agent	Dow Corning	4019862
SuperChipTM Poly-L-Lysine Slides	Electron Microscopy Sciences	63478-AS
RNase*Zap*TM RNase Decontamination Solution	Thermo Fisher Scientific	AM9780
Silicon wafer	WaferPro	C04004
Photoresist SU-8 2010	MicroChem Laboratory	SU-8 2010

Citation: Graham Su, archibald.enninful, Yang Liu, Rong Fan Spatial Multi-omics Sequencing for Fixed Tissue via DBiT-seq https://dx.doi.org/10.17504/protocols.io.3byl4j2kolo5/v1

Category	Barcode	Specificity	Clone	Barcode Sequence
TotalSeqTM-A	12	CD117 (c-kit)	2B8	TGCATGTCATCGGTG
TotalSeqTM-A	78	CD49d	R1-2	CGCTTGGACGCTTAA
TotalSeqTM-A	96	CD45	30-F11	TGGCTATGGAGCAGA
TotalSeqTM-A	104	CD102	3C4 (MIC2/4)	GATATTCAGTGCGAC
TotalSeqTM-A	115	FcεRIα	MAR-1	AGTCACCTCGAAGCT
TotalSeqTM-A	118	NK-1.1	avas12	GTAACATTACTCGTC
TotalSeqTM-A	119	Siglec H	551	CCGCACCTACATTAG
TotalSeqTM-A	122	TER-119/Erythroid Cells	TER-119	GCGCGTTTGTGCTAT
TotalSeqTM-A	130	Ly-6A/E (Sca-1)	D7	TTCCTTTCCTACGCA
TotalSeqTM-A	232	MAdCAM-1	MECA-367	TTGGGCGATTAAGAA
TotalSeqTM-A	381	Panendothelial Cell Antigen	MECA-32	CGTCCTAGTCATTGG
TotalSeqTM-A	415	P2RY12	S16007D	TTGCTTATTTCCGCA
TotalSeqTM-A	439	CD201 (EPCR)	RCR-16	TATGATCTGCCCTTG
TotalSeqTM-A	442	Notch 1	HMN1-12	TCCGGTCACTCAGTA
TotalSeqTM-A	443	CD41	MWReg30	ACTTGGATGGACACT
TotalSeqTM-A	449	CD326 (Ep-CAM)	G8.8	ACCCGCGTTAGTATG
TotalSeqTM-A	552	CD304 (Neuropilin-1)	3E12	CCAGCTCATTCAACG
TotalSeqTM-A	553	CD309 (VEGFR2, Flk-1)	Avas12	ATAAGAGCCCACCAT
TotalSeqTM-A	558	CD55 (DAF)	RIKO-3	ATTGTTGTCAGACCA
TotalSeqTM-A	559	CD63	NVG-2	ATCCGACACGTATTA
TotalSeqTM-A	564	Folate Receptor β (FR-β)	10/FR2	CTCAGATGCCCTTTA
TotalSeqTM-A	596	ESAM	1G8/ESAM	TATAGTTTCCGCCGT

Table S1. **TotalSeq^{TM}Antibodies, related to Step 9.**

Oligo Name	Sequence
Primer 1	CAAGCGTTGGCTTCTCGCATCT
Primer 2	AAGCAGTGGTATCAACGCAGAGT
Primer 3 (cite-seq)	CCTTGGCACCCGAGAATT*C*C
Ligation Linker	CGAATGCTCTGGCCTCTCAAGCACGTGGAT
Template Switch Primer	AAGCAGTGGTATCAACGCAGAGTGAATrGrG+G
P5 oligo	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
P5 oligo (cite-seq)	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTTGGCACCCGAGAATTCCA
P7 oligo (701)	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAGCGTTGGCTTCTCGCATCT
P7 oligo (702)	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAGCGTTGGCTTCTCGCATCT
P7 oligo (703)	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAGCGTTGGCTTCTCGCATCT
P7 oligo (704)	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAGCGTTGGCTTCTCGCATCT
P7 oligo (705)	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAGCGTTGGCTTCTCGCATCT
P7 oligo (706)	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAGCGTTGGCTTCTCGCATCT

Table S2A. **List of PCR Oligo.**

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https://dx.doi.org/10.17504/protocols.io.3byl4j2kolo5/v1

A	B
Barcode A-1	/5Phos/AGGCCAGAGCATTCGAACGTGATTTTTTTTTTTTTTTTVN
Barcode A-2	/5Phos/AGGCCAGAGCATTCGAAACATCGTTTTTTTTTTTTTTTTVN
Barcode A-3	/5Phos/AGGCCAGAGCATTCGATGCCTAATTTTTTTTTTTTTTTTVN
Barcode A-4	/5Phos/AGGCCAGAGCATTCGAGTGGTCATTTTTTTTTTTTTTTTVN
Barcode A-5	/5Phos/AGGCCAGAGCATTCGACCACTGTTTTTTTTTTTTTTTTVN
Barcode A-6	/5Phos/AGGCCAGAGCATTCGACATTGGCTTTTTTTTTTTTTTTTVN
Barcode A-7	/5Phos/AGGCCAGAGCATTCGCAGATCTGTTTTTTTTTTTTTTTTVN
Barcode A-8	/5Phos/AGGCCAGAGCATTCGCATCAAGTTTTTTTTTTTTTTTTVN
Barcode A-9	/5Phos/AGGCCAGAGCATTCGCGCTGATCTTTTTTTTTTTTTTTTVN
Barcode A-10	/5Phos/AGGCCAGAGCATTCGACAAGCTATTTTTTTTTTTTTTTTVN
Barcode A-11	/5Phos/AGGCCAGAGCATTCGCTGTAGCCTTTTTTTTTTTTTTTTVN
Barcode A-12	/5Phos/AGGCCAGAGCATTCGAGTACAAGTTTTTTTTTTTTTTTTVN
Barcode A-13	/5Phos/AGGCCAGAGCATTCGAACAACCATTTTTTTTTTTTTTTTVN
Barcode A-14	/5Phos/AGGCCAGAGCATTCGAACCGAGATTTTTTTTTTTTTTTTVN
Barcode A-15	/5Phos/AGGCCAGAGCATTCGAACGCTTATTTTTTTTTTTTTTTTVN
Barcode A-16	/5Phos/AGGCCAGAGCATTCGAAGACGGATTTTTTTTTTTTTTTTVN
Barcode A-17	/5Phos/AGGCCAGAGCATTCGAAGGTACATTTTTTTTTTTTTTTTVN
Barcode A-18	/5Phos/AGGCCAGAGCATTCGACACAGAATTTTTTTTTTTTTTTTVN
Barcode A-19	/5Phos/AGGCCAGAGCATTCGACAGCAGATTTTTTTTTTTTTTTTVN
Barcode A-20	/5Phos/AGGCCAGAGCATTCGACCTCCAATTTTTTTTTTTTTTTTVN
Barcode A-21	/5Phos/AGGCCAGAGCATTCGACGCTCGATTTTTTTTTTTTTTTTVN
Barcode A-22	/5Phos/AGGCCAGAGCATTCGACGTATCATTTTTTTTTTTTTTTTVN
Barcode A-23	/5Phos/AGGCCAGAGCATTCGACTATGCATTTTTTTTTTTTTTTTVN
Barcode A-24	/5Phos/AGGCCAGAGCATTCGAGAGTCAATTTTTTTTTTTTTTTTVN
Barcode A-25	/5Phos/AGGCCAGAGCATTCGAGATCGCATTTTTTTTTTTTTTTTVN
Barcode A-26	/5Phos/AGGCCAGAGCATTCGAGCAGGAATTTTTTTTTTTTTTTTVN
Barcode A-27	/5Phos/AGGCCAGAGCATTCGAGTCACTATTTTTTTTTTTTTTTTVN
Barcode A-28	/5Phos/AGGCCAGAGCATTCGATCCTGTATTTTTTTTTTTTTTTTVN
Barcode A-29	/5Phos/AGGCCAGAGCATTCGATTGAGGATTTTTTTTTTTTTTTTVN
Barcode A-30	/5Phos/AGGCCAGAGCATTCGCAACCACATTTTTTTTTTTTTTTTVN
Barcode A-31	/5Phos/AGGCCAGAGCATTCGGACTAGTATTTTTTTTTTTTTTTTVN
Barcode A-32	/5Phos/AGGCCAGAGCATTCGCAATGGAATTTTTTTTTTTTTTTTVN
Barcode A-33	/5Phos/AGGCCAGAGCATTCGCACTTCGATTTTTTTTTTTTTTTTVN
Barcode A-34	/5Phos/AGGCCAGAGCATTCGCAGCGTTATTTTTTTTTTTTTTTTVN
Barcode A-35	/5Phos/AGGCCAGAGCATTCGCATACCAATTTTTTTTTTTTTTTTVN
Barcode A-36	/5Phos/AGGCCAGAGCATTCGCCAGTTCATTTTTTTTTTTTTTTTVN
Barcode A-37	/5Phos/AGGCCAGAGCATTCGCCGAAGTATTTTTTTTTTTTTTTTVN
Barcode A-38	/5Phos/AGGCCAGAGCATTCGCCGTGAGATTTTTTTTTTTTTTTTVN
Barcode A-39	/5Phos/AGGCCAGAGCATTCGCCTCCTGATTTTTTTTTTTTTTTTVN
Barcode A-40	/5Phos/AGGCCAGAGCATTCGCGAACTTATTTTTTTTTTTTTTTTVN
Barcode A-41	/5Phos/AGGCCAGAGCATTCGCGACTGGATTTTTTTTTTTTTTTTVN
Barcode A-42	/5Phos/AGGCCAGAGCATTCGCGCATACATTTTTTTTTTTTTTTTVN
Barcode A-43	/5Phos/AGGCCAGAGCATTCGCTCAATGATTTTTTTTTTTTTTTTVN
Barcode A-44	/5Phos/AGGCCAGAGCATTCGCTGAGCCATTTTTTTTTTTTTTTTVN
Barcode A-45	/5Phos/AGGCCAGAGCATTCGCTGGCATATTTTTTTTTTTTTTTTVN
Barcode A-46	/5Phos/AGGCCAGAGCATTCGGAATCTGATTTTTTTTTTTTTTTTVN
Barcode A-47	/5Phos/AGGCCAGAGCATTCGCAAGACTATTTTTTTTTTTTTTTTVN
Barcode A-48	/5Phos/AGGCCAGAGCATTCGGAGCTGAATTTTTTTTTTTTTTTTVN
Barcode A-49	/5Phos/AGGCCAGAGCATTCGGATAGACATTTTTTTTTTTTTTTTVN
Barcode A-50	/5Phos/AGGCCAGAGCATTCGGCCACATATTTTTTTTTTTTTTTTVN

Table S2B. List of DNA Barcode A Sequences.

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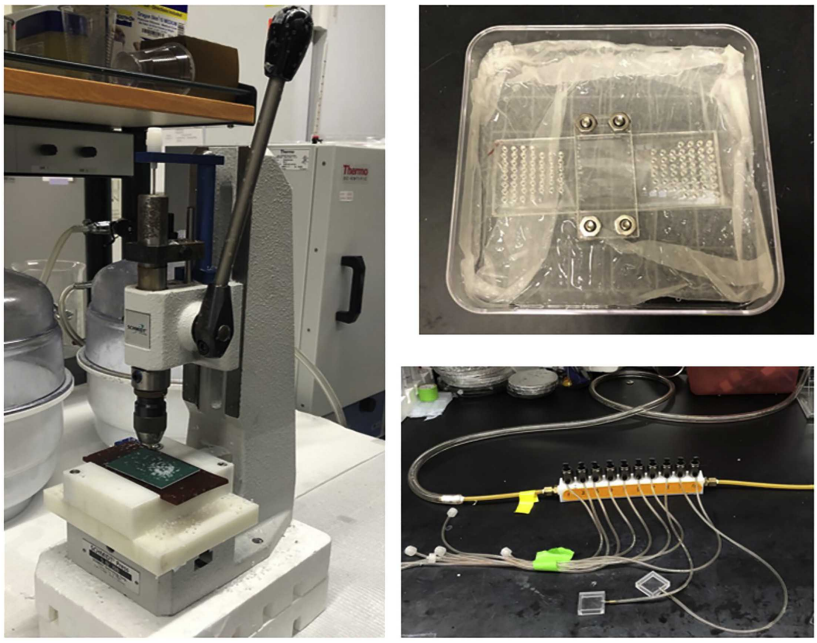
https://dx.doi.org/10.17504/protocols.io.3byl4j2kolo5/v1

A	B
Barcode B-1	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACGTGATATCCACGTGCTTGAG
Barcode B-2	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAAACATCGATCCACGTGCTTGAG
Barcode B-3	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNATGCCTAAATCCACGTGCTTGAG
Barcode B-4	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGTGGTCAATCCACGTGCTTGAG
Barcode B-5	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACCACTGTATCCACGTGCTTGAG
Barcode B-6	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACATTGGCATCCACGTGCTTGAG
Barcode B-7	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAGATCTGATCCACGTGCTTGAG
Barcode B-8	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCATCAAGTATCCACGTGCTTGAG
Barcode B-9	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGCTGATCATCCACGTGCTTGAG
Barcode B-10	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACAAGCTAATCCACGTGCTTGAG
Barcode B-11	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTGTAGCCATCCACGTGCTTGAG
Barcode B-12	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGTACAAGATCCACGTGCTTGAG
Barcode B-13	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACAACCAATCCACGTGCTTGAG
Barcode B-14	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACCGAGAATCCACGTGCTTGAG
Barcode B-15	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACGCTTAATCCACGTGCTTGAG
Barcode B-16	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAAGACGGAATCCACGTGCTTGAG
Barcode B-17	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAAGGTACAATCCACGTGCTTGAG
Barcode B-18	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACACAGAAATCCACGTGCTTGAG
Barcode B-19	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACAGCAGAATCCACGTGCTTGAG
Barcode B-20	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACCTCCAAATCCACGTGCTTGAG
Barcode B-21	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACGCTCGAATCCACGTGCTTGAG
Barcode B-22	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACGTATCAATCCACGTGCTTGAG
Barcode B-23	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACTATGCAATCCACGTGCTTGAG
Barcode B-24	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGAGTCAAATCCACGTGCTTGAG
Barcode B-25	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGATCGCAATCCACGTGCTTGAG
Barcode B-26	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGCAGGAAATCCACGTGCTTGAG
Barcode B-27	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGTCACTAATCCACGTGCTTGAG
Barcode B-28	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNATCCTGTAATCCACGTGCTTGAG
Barcode B-29	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNATTGAGGAATCCACGTGCTTGAG
Barcode B-30	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAACCACAATCCACGTGCTTGAG
Barcode B-31	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGACTAGTAATCCACGTGCTTGAG
Barcode B-32	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAATGGAAATCCACGTGCTTGAG
Barcode B-33	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCACTTCGAATCCACGTGCTTGAG
Barcode B-34	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAGCGTTAATCCACGTGCTTGAG
Barcode B-35	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCATACCAAATCCACGTGCTTGAG
Barcode B-36	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCAGTTCAATCCACGTGCTTGAG
Barcode B-37	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCGAAGTAATCCACGTGCTTGAG
Barcode B-38	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCGTGAGAATCCACGTGCTTGAG
Barcode B-39	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCTCCTGAATCCACGTGCTTGAG
Barcode B-40	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGAACTTAATCCACGTGCTTGAG
Barcode B-41	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGACTGGAATCCACGTGCTTGAG
Barcode B-42	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGCATACAATCCACGTGCTTGAG
Barcode B-43	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTCAATGAATCCACGTGCTTGAG
Barcode B-44	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTGAGCCAATCCACGTGCTTGAG
Barcode B-45	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTGGCATAATCCACGTGCTTGAG
Barcode B-46	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGAATCTGAATCCACGTGCTTGAG
Barcode B-47	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAAGACTAATCCACGTGCTTGAG
Barcode B-48	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGAGCTGAAATCCACGTGCTTGAG
Barcode B-49	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGATAGACAATCCACGTGCTTGAG
Barcode B-50	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGCCACATAATCCACGTGCTTGAG

Table S2C. List of DNA Barcode B Sequences.

Citation: Graham Su, archibald.enninful, Yang Liu, Rong Fan Spatial Multi-omics Sequencing for Fixed Tissue via DBiT-seq
https://dx.doi.org/10.17504/protocols.io.3byl4j2kolo5/v1

- This protocol requires using a microfluidic device fabricated with PDMS using soft lithography.
- A hole punching machine (SCHMIDT® Manual Press) is needed to punch the inlet and outlet holes in the PDMS chip after its fabrication.
- The tissue of interest should be placed on the center of a poly-L-lysine coated glass slide. (CatLog no. 63478-AS, electron microscopy sciences).
- A custom-designed acrylic clamp with screws is needed to hold the PDMS device and the glass slide together firmly.
- The silicon wafer used for fabricating the PDMS mold is purchased from WaferPro (CatLog. No. C04004).
- The photoresist is purchased from MicroChem Laboratory (CatLog. No. SU-8 2010).
- A homemade laboratory vacuum system(See Figure Below) is needed to applying suction to move fluid in the microfluidic channels.
- Microscopy – The tissue of interest can be scanned and imaged using EVOS (Thermo Fisher EVOS fl), typically at a magnification of 10×. Any suitable optical microscope can be used.
- A Laser Engraver Cutter is used to cut out the acrylic barcode and lysis clamps.
- A "humidified chamber" (See Figure Below) to prevent reagent evaporation during incubation.



Key equipment used in DBiT-seq

Hole punch machine, the key device for DBiT-seq, and the house vacuum lines. (Left) SCHMIDT® Manual Press used for hole punching 2-mm diameter holes for the inlets and outlets of Chip-A and Chip-B. (Top Right) Example of a "humidified chamber" to prevent reagent evaporation during the incubation steps. (Bottom Right) House vacuum used for pulling reagents through the microchannels.

Recipes

Critical: Prepare the RT mix, template switch mix, ligation mix, PCR mix, 1X lysis solution, flow wash buffer, PBS-RI, 80% ethanol on the same day as usage. 2X lysis buffer, 1X B&W Buffer with 0.05% Tween-20, and 2X B&W Buffer can be stored for up to 6 months at room temperature.

PBS-RI

Reagent	Volume (µL)
1X PBS	5000
RNase Inhibitor (40 U/µL) (Enzymatics)	7
Total	**5007**

80% Ethanol

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Reagent	Volume(ml)
RNase-free water	1
100% Ethanol	4
Total	**5**

RT Mixture

Reagent	Volume (µL)
5X Maxima RT buffer	50
RNase-free water	32.8
RNase Inhibitor (Enzymatics)	1.6
Superase In RNase Inhibitor (Ambion)	3.2
dNTPs (10 mM stock)	12.5
Maxima H Minus Reverse Transcriptase	25
PBS-RI	100
Total	**225.1**

Ligation Mix

Reagent	Volume (µL)
10X T4 Ligase Buffer	27
1X NEB buffer 3.1 with 1% RI (Enzymatics)	115.8
5% Triton-X100	5.4
RNase Inhibitor (Enzymatics)	2.2
RNase-free water	69.5
SuperaseIn RNase Inhibitor (Ambion)	0.7
T4 DNA Ligase (400 U/µL)	11
Total	**231.6**

Template switch mix

Reagent	Volume (µL)
20% Ficoll PM-400	44
5X Maxima RT buffer	44
dNTPs (10 mM stock)	22
RNase Inhibitor (Enzymatics)	5.5
Maxima H Minus Reverse Transcriptase	11
Template Switch Primer (100 µM stock)	5.5
RNase-free water	88
Total	**220**

PCR mix

Reagent	Volume (µL)
2X Kapa Hifi HotStart master mix	110
Primer1 BC_0062 (10µM)	8.8
Primer2 BC_0108 (10µM)	8.8
RNase-free water	92.4
Total	**220**

Flow Wash Buffer

Reagent	Volume (mL)
1X PBS	4
10 % Triton X-100	0.04
Superase In RNase Inhibitor	0.01
Total volume	**4.05**

1X Lysis Solution

Reagent	Volume (µL)
1X PBS	50
2X Lysis Buffer	50
Proteinase K (20mg/mL) (Thermo)	10
Total	**110**

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2X Lysis Buffer

Reagent	Stock Concentration	Final Concentration (2X)	Volume (mL)
Tris, pH 8.0	1 M	20 mM	0.5
NaCl	5 M	400 mM	2
EDTA, pH 8.0	0.5 M	100 mM	5
SDS	10%	4.4 %	11
RNase-free Water	NA	NA	6.5
Final Volume			**25**

1X B&W Buffer with 0.05% Tween-20

Reagent	Volume
1M Tris-HCl pH 8.0	100 µL
EDTA, 0.5M	20 µL
5M NaCl	4 mL
Tween 20 10%	100 µL
RNase-free water	15.78 mL
Total	**20 mL**

2X B&W Buffer

Reagent	Volume
1M Tris-HCl pH 8.0	500 µL
EDTA, 0.5M	100 µL
5M NaCl	20 mL
RNase-free water	29.4 mL
Total	**50 mL**

CRITICAL: Handle all in an RNase-free area.

BEFORE STARTING

The protocol below describes the reagents, equipment, and specific experimental steps for using the DBiT-seq platform on frozen fixed tissue slides. The 10µm, 25µm, 50µm microfluidic channel width were designed and

Citation: Graham Su, archibald.enninful, Yang Liu, Rong Fan Spatial Multi-omics Sequencing for Fixed Tissue via DBiT-seq https://dx.doi.org/10.17504/protocols.io.3byl4j2kolo5/v1

validated to provide spatial profiling at different resolutions.

CRITICAL: Work in an RNase-free environment when the microfluidic device is not on the tissue slide. Use RNaseZap^{TM} or other commercially available cleaner solution and filter-tips. Clean surfaces and gloves with RNaseZap^{TM}.

CRITICAL: Keep reagents on ice at all times.

Fabricating the Silicon Wafer Device Mold 12h

1 Prepare a high-resolution computer-aided-design (CAD) file with the desired microfluidics chip design. A CAD file is also available in the link provided. (https://ars.els-cdn.com/content/image/1-s2.0-S2666166721002392-mmc4.zip)

2 Prepare chrome photomasks of the microfluidics chip by printing the high-resolution CAD files onto a glass substrate (Figure 1).

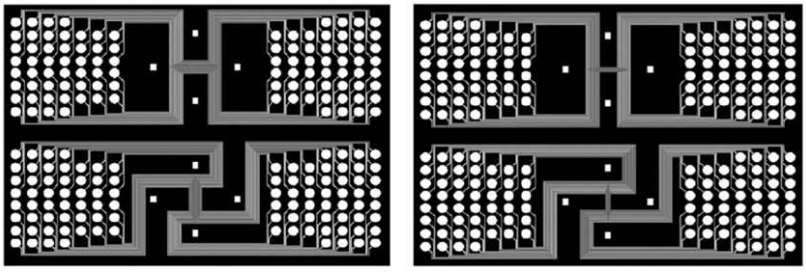

Figure 1: Microfluidic Photomask Design.

(Left) AutoCAD design for the 25 micron-width fifty channel Chip-A and Chip-B. (Right) AutoCAD design for 10 micron-width channel Chip-A and Chip-B.

NOTE: We have outsourced this step (Front Range Photomask, USA).

Using these masks, prepare a replica mold as follows:

3 Start the process by cleaning a 4-inch silicon wafer with 100% acetone (Aldrich) and then 100% isopropanol (Aldrich), then dry with compressed air.

NOTE: Acetone and isopropanol are mildly toxic. Use proper PPE when handling and discard waste in the appropriate containers.

4 Bake the wafer at 180°C for 10 min on a hot plate to dry it out.

5 Use a spin coater to evenly spread SU-8 2010 photoresist (MicroChem) for 10 µm device or SU-8 2025 (MicroChem) for 25 µm and 50 µm device onto the wafer at 500 rpm for 5 seconds followed by 1100 rpm (10 µm device), 3500 rpm (25 µm device) or 1750 rpm (50 µm device) for 40 seconds.

6 Soft bake the wafer for 3 min at 65°C (25 µm and 50 µm device) and 4 min (10 µm device) or 6 min (25 µm and 50 µm device) at 95°C.

7 Expose the SU-8 on the wafer through the photomask using Mask Aligner with a dose of 150 mJ/cm2 UV.

8 For the post exposure bake, bake the wafer for 1 min at 65°C (25 µm and 50 µm device) and 5 min (10 µm and 25 µm device) or 6 min (50 µm device) at 95°C.

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9 Develop the SU-8 for 4 min (10 µm and 25 µm device) or 5 min (50 µm device) in a bath of ~50 mL SU-8 developer (MicroChem).

10 Rinse the wafer with 100% isopropanol and dry with compressed air.

11 Perform a hard bake by baking the wafer for 10 min at 180°C.

CRITICAL: This process needs to be carried out in a microelectronics cleanroom.

Pause Point: The replica mold can now be stored and reused indefinitely.

Creating the Acrylic Clamps 30m

12 Prepare the pattern and dimensions for the barcoding clamp and lysis clamp.

13 Peel covering of acrylic sheet and place it in the laser cutting machine.

14 Select the program dimensions and cut two pieces for the top and bottom of the barcoding clamp.

15 Place the top blank piece into the laser cutter and select the pattern to cut out the four holes in the corner for the screws and nuts.

16 Remove cut-out scraps with a pipet tip or a similar pointed tool.

17 Repeat steps 15 and 16 for the bottom blank piece.

18 Repeat steps 13 to 17 for the lysis clamp using the correct dimensions and pattern with an additional hole in the center of the top piece (Example shown in Figure 2).

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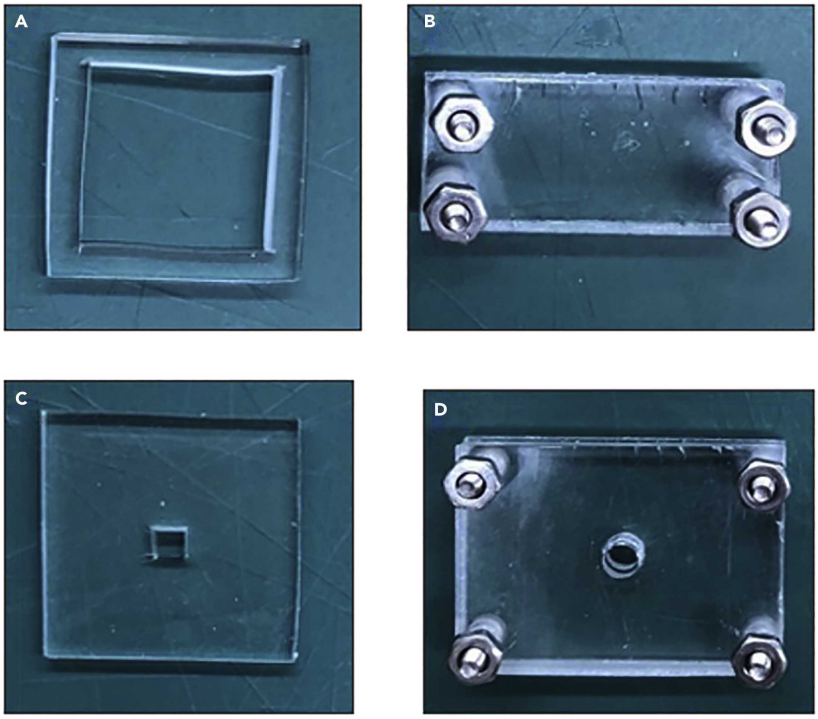

Figure 2. Key parts for setup. PDMS reservoirs and acrylic clamps used in DBiT-seq. (A) Inlet Reservoir. (B) Barcoding Clamp. (C) Lysis Reservoir. (D) Lysis Clamp.

NOTE: The acrylic clamps can be reused indefinitely.

Preparing the Microfluidic Device and reagent reservoirs 3h

19 Thoroughly mix polydimethylsiloxane (PDMS) elastomer base and curing agent (these come together) at a 10:1 ratio (See Figure 3).

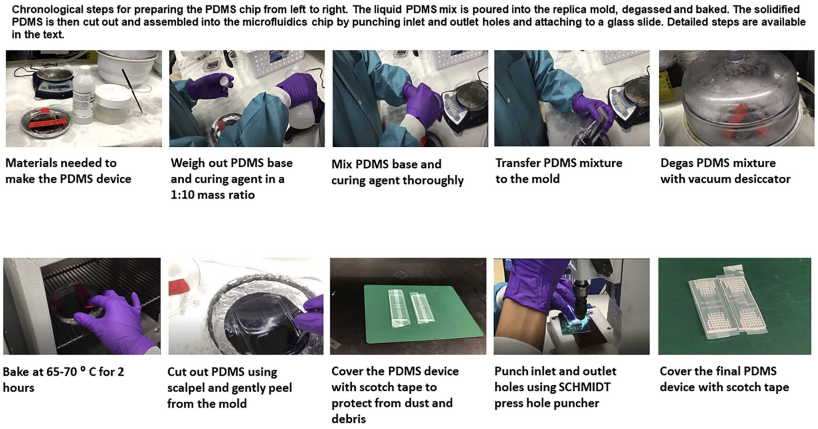

Figure 3: Step-by-step visual guide for making Chip-A and Chip-B.

20 Pour the mixture into the silicon device mold.

NOTE: Aim for a chip height of about 5mm.

21 Place in a vacuum desiccator until all bubbles dissipate from the mixture about 30-60 min.

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22 Cure in an oven at 65-70˚C for a minimum of 2 hours and up to overnight.

NOTE: Make sure to place on an even surface to prevent uneven curing!

23 Cut out the cured device and hole punch each of the 50 inlets and outlets for the channels.

NOTE: Cut both Chip-A and Chip-B in the approximate size of a glass slide.
NOTE: Make sure there are no PDMS pieces left in the inlets or outlets.

24 Thoroughly clean the surface of the device with scotch tape.

25 Pour mixture in a container large enough to cut out two roughly 25x25x5mm PDMS pieces.

26 For the barcoding reservoir, cut out a 20x20mm piece in the center, barely large enough to surround the inlets of the chips when placed above.

27 For the lysis reservoir, depending on the device size used, cut out the center to barely surround the barcoded region of the tissue (1x1mm for the 10µm channel device, 2.5x2.5mm for the 25µm channel device).

NOTE: The reservoirs are reusable. Thoroughly wash with 70% ethanol after each use.

CRITICAL: Be careful when cutting out the Chips A and B. Make sure not to cut across any patterned areas of the silicon wafer mold, otherwise it will need to be replaced and re-fabricated.

Preparing Barcode B 1h

28 Thoroughly mix the ligation linker with each barcode $B_{1\text{-}50}$ at a 1:1 ratio.

29 Place the 50 mixes in a thermal cycle and heat to 97˚C to anneal.

30 Slowly cool to room temperature at a rate of -0.1˚C/sec.

31 Store at -20˚C for up to 6 months.

Preparing the Tissue Slide 1d

32 Fix in 4% paraformaldehyde (PFA) in PBS.

NOTE: PFA is moderately toxic and should be handled in a chemical fume hood with proper PPE. It should be disposed of in the proper waste container. This step is optional.

33 25% sucrose overnight bath.

34 Embed in OCT frozen on dry ice.

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35 Section at ~10 µm thickness for the 25µm channel device and ~5 µm thickness for the 10 µm channel device onto Poly-L-lysine slides.

36 Store slides at -80˚C for up to 6 months until use.

CRITICAL: Ensure that the tissue is sectioned as evenly as possible, otherwise the risk of cross-flow between the microfluidic channels will be increased. Tissue section should be placed at the center of a glass slide to the greatest extent.

Tissue Preparation 1h

37 Remove stored sections from the freezer and allow to warm to room temperature for 10 minutes.

38 Clean sections by pipetting 2 mL of PBS-RI across the tissue.

39 If sections are not yet fixed, then fix here with 1 mL 4% PFA by applying on top of the tissue and incubate for 20 min at room temperature.

40 Wash the slides 3 times with 1 mL of PBS and then dip the slide in a 50 mL falcon tube with DI water.

41 Dry the section with gentle air flow.

42 Take a full pre-scan image of the section at the desired optical resolution (Figure 4A).

Recommended 10X resolution.

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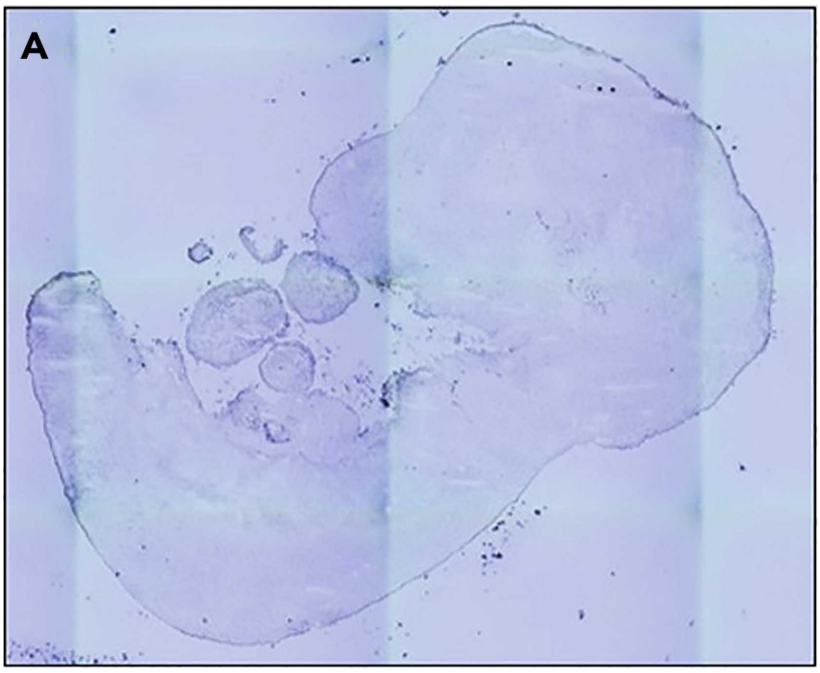

Figure 4 : Example scanning of the tissue slide.
(A) Initial full scan of the tissue section prior to DBiT-seq.

43 Block the tissue slide with 1% BSA and 1% RNase Inhibitor in 1X PBS for 30 minutes at 4˚C.

44 Remove the blocking buffer and briefly airdry.

45 Add a cocktail of the desired DNA-antibody conjugates + blocking buffer and incubate for 30 minutes at 4˚C.

46 Wash the slide with 1X PBS three times.

47 Dip in a 50 mL falcon tube with DI water and gently dry with air flow.

48 Attach Chip-A as shown in Figure 5 centered on the tissue and take another scan of the channels laying on the tissue (Figure 4C).

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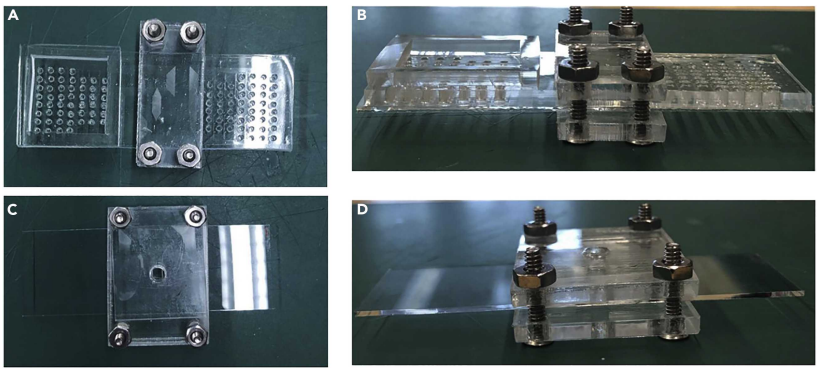

Figure 5. Assembly to create the setup for the PDMS reservoir and acrylic clamps. (A) Chip-A with the inlet reservoir and barcoding clamp. (B) Sideview of Chip-A. (C) The lysis reservoir with the lysis clamp on top of the barcoded tissue. (D) Sideview of the lysis setup.

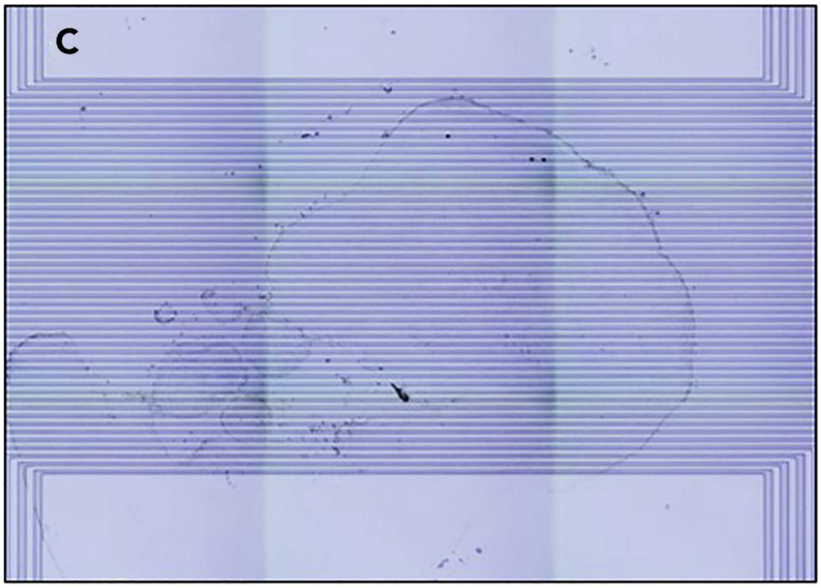

Figure 4. Example Scanning of the Tissue Slide.
(**C**) Scan of Chip-A channels placed on the tissue.

49 Permeabilize the tissue section.

CRITICAL: These steps should be done in an RNA-free area.

49.1 Firmly attach the barcoding clamp across the center of the device & slide.

49.2 Place the inlet reservoir above the inlets.

49.3 Add ~1 mL 0.5% Triton X-100 in PBS to the reservoir
- Use a 10 µL pipet tip to pipet up and down inside the inlets to remove bubbles.

49.4 Attach the vacuum head to the outlets as shown in Figure 6 and pull the solution through the 50 channels.

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NOTE: Make sure all channels are filled with solution.

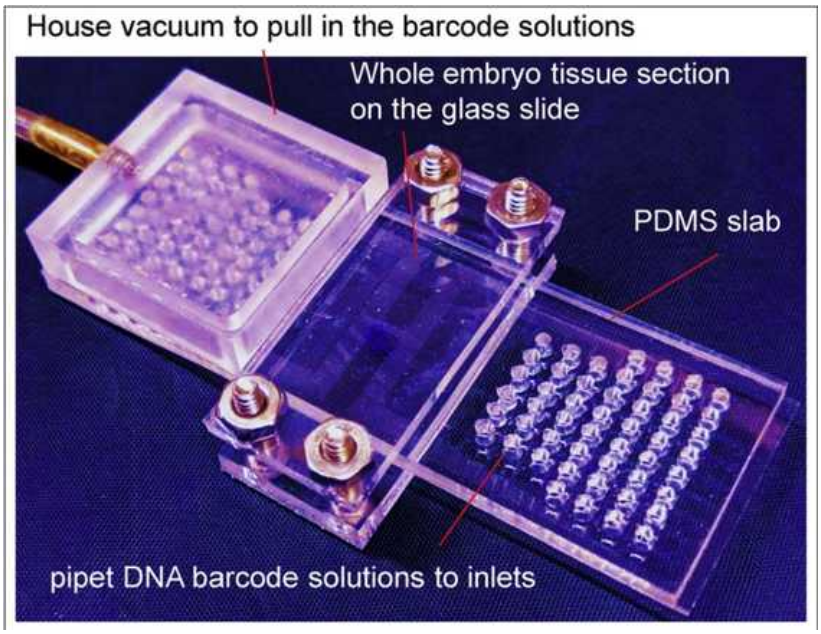

Figure 6. Chip-A with Acrylic Clamp and House Vacuum attached.

49.5 Stop the vacuum and incubate for 20 min at room temperature.

49.6 Remove remaining permeabilization solution from the reservoir and the inlets/outlets.

49.7 Add ~1 mL 0.5X PBS-RI to the reservoir and flow for about 10 min to wash and clean the channels of Chip-A.

In-Tissue Reverse Transcription 2h 30m

50 Prepare the RT mixture.

50.1 For one experiment, prepare 50 µL of 5X RT Buffer, 32.8 µL of RNase-free water, 1.6 µL Enzymatics RNase inhibitor, 3.2 µL SUPERase• InTM RNase Inhibitor, 12.5 µL of 10 mM dNTPs each, 25 µL of Maxima H Minus Reverse Transcriptase, and 100 µL 0.5X PBS-RI.

51 Prepare barcode solution.

51.1 In a 96-well PCR plate, add 4 µL of the RT mixture in each of 50 wells.

51.2 Add 1 µL of each 25 µM Barcode A1-50 to the wells and mix thoroughly.

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52 Introduce final barcode A mixture to the tissue.

52.1 Pipet each barcode A mixture directly into the inlets following the sequence as indicated in Figure 7. This sequence corresponds to the ordering of the channels across the center region of interest (for example, channels from top to bottom of Chip A correspond to 1-50 in the pipetting order).

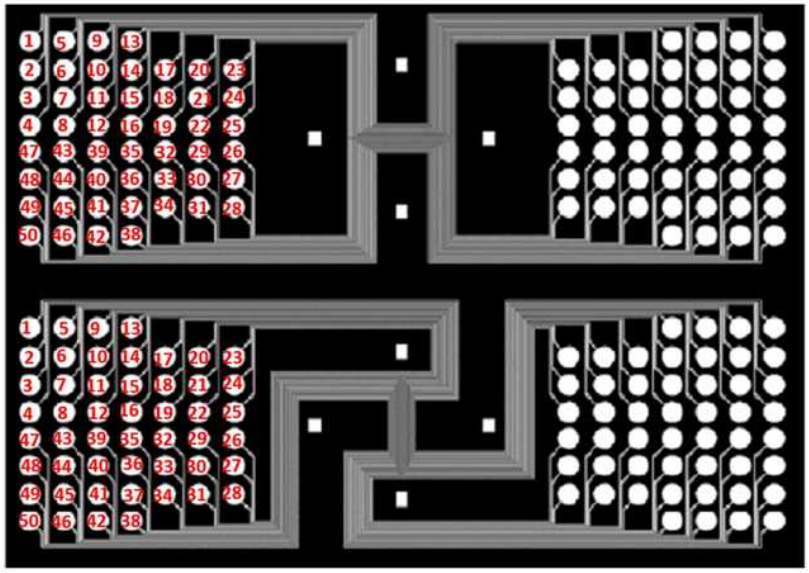

Figure 7. Sequential numbering of the inlet holes and pipetting order for Barcodes A_{1-50} and B_{1-50}.

52.2 Apply the vacuum and pull the solution until all channels are full.

NOTE: May take as short as 10 sec or as long as 2 min depending on channel dimensions (smaller channels may take longer to fill).

52.3 Incubate in the humidified chamber at room temperature for 30 min followed by 42˚C for 90 min for in-cell reverse transcription (Figure 8).

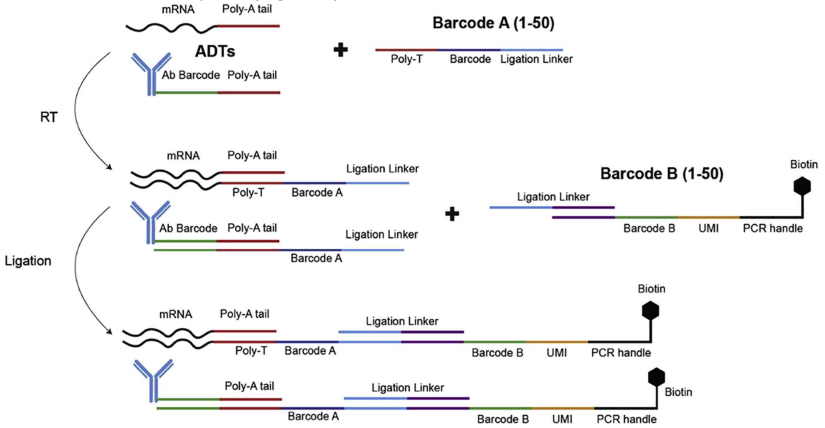

Figure 8: Deterministic Tissue Barcoding Chemistry Workflow.

53 Aspirate remaining barcode A mixture from inlets/outlets.

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54 Clean the channels by flushing 1X NEB buffer 3.1 with 1% Enzymatics RNase Inhibitor for 10 min.

55 Peel off Chip-A and briefly dip the slide in a 50mL falcon tube with DI water and gently dry with air flow.

In-Tissue Ligation 1h

56 Attach Chip-B centered on the tissue.

56.1 Scan the channels over the tissue to ensure proper placement (Figure 4D).

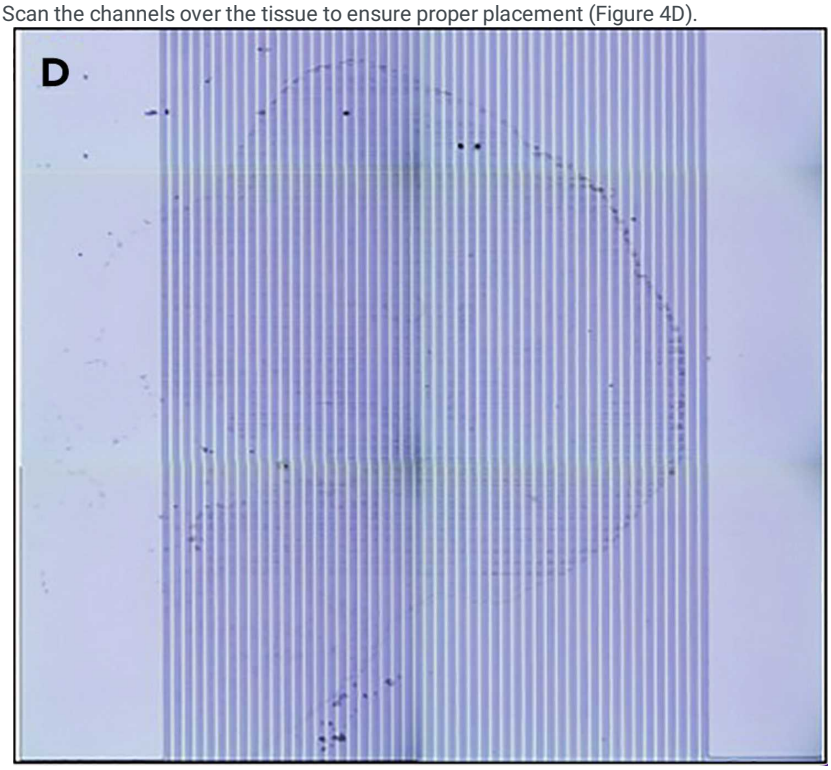

Figure 4 : Example scanning of the tissue slide.
(D) Scan of Chip-B channels across the tissue.

56.2 Firmly clamp as before.

57 Prepare the ligation mixture.

57.1 For one experiment, prepare 69.5 µL RNase-free water, 27 µL 10X T4 Ligation buffer (NEB), 11 µL T4 DNA Ligase (400 U/µL, NEB), 2.2 µL RNase inhibitor (40 U/µL, Enzymatics), 0.7 µL SUPERase• InTM RNase Inhibitor (20 U/µL, Ambion), and 5.4 µL of 5% Triton X-100. Mix with 115.8 µL 1X NEB buffer 3.1 with 1% RNase Inhibitor (Enzymatics).

58 Prepare barcode B mixture.

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58.1 In a 96-well PCR plate, add 4 µL of the ligation mixture in each of 50 wells.

58.2 Add 1 µL of each 25 µM Barcode $B_{1\text{-}50}$ to the wells and mix thoroughly.

59 Introduce final barcode B mixture to the tissue.

59.1 Pipet each barcode B mixture directly into the inlets.

59.2 Apply the vacuum and pull the solution until all channels are full. May take as short as 10 sec or as long as 2 min.

CRITICAL: Do not over vacuum! Make sure the inlets do not run out of barcode B mixture which will cause the channels to lose the mixture and fill with air.

59.3 Incubate in the humidified chamber at 37˚C for 30 min for in-cell ligation of barcode B (Figure 8).

60 Clean the channels with wash buffer for 10 min.

60.1 Prepare wash buffer by mixing 4 mL of 1X PBS, 40 µL of 10% Triton X-100, and 10 µL of SUPERase• InTM RNase Inhibitor.

61 Peel off Chip-B and dip the slide into a 50mL falcon tube with DI water and gently dry with air flow.

62 Take a final post-scan of the tissue section (Figure 4B).

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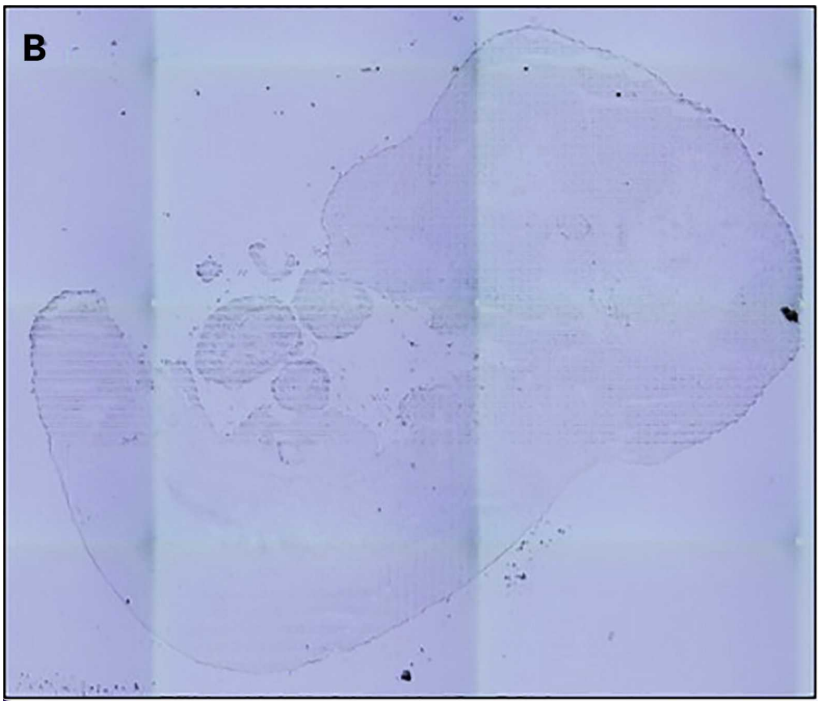

Figure 4. Example Scanning of the Tissue Slide.
(B) Final scan after barcoding and removing the device.

Lysis and Sub-library Generation 2h 30m

63 Place the lysis reservoir directly around the barcoded tissue region.

NOTE: Try to cover as little extra tissue as possible.

64 Tightly apply the lysis clamp.

65 Add lysis solution to lysis reservoir depending on channel dimensions (~20 µL for the 25 µm channels, ~10 µL for the 10 µm channels).

65.1 Lysis solution is made with 50 µL 1X PBS, 50 µL of 2X lysis buffer, and 10 µL of proteinase K solution (20mg/mL).

65.2 2X lysis buffer is made up of 20 mM Tris (pH 8.0), 400 mM NaCl, 100 mM EDTA (pH 8.0), and 4.4% SDS.

66 Place in the humidified chamber and tightly wrap the chamber with parafilm and incubate at 55˚C for 2 hours.

67 Collect the lysate into a 1.5 mL centrifuge tube. Add the same amount of extra lysis solution to wash out the reservoir and retrieve as much lysate as possible.

68 Immediately store at -80˚C until next step.

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Pause Point: Stop here for the first day. The lysate should be stable at -80˚C for up to 2 weeks. The **Methods Video S1** shows up to this step.

cDNA Purification 1h 30m

69 The cDNA is purified and bound to Dynabeads™ MyOne™ Streptavidin C1 beads in this step.

Prepare 40 µL DynabeadsTM MyOneTM Streptavidin C1 beads (Thermofisher) per sub-library.

69.1 Wash 3 times with 800 µL of 1X B&W buffer with 0.05% Tween-20.

69.2 Resuspend beads in 100 µL 2X B&W buffer + 2 µL SUPERase• InTM RNase Inhibitor.

70 Purify lysate following the DNA Clean & Concentrator with Zymo-spin IC Columns (RPI Research Products) protocol. Use 100 µL water to elute DNA.

71 Add 100 µL of resuspended DynabeadsTM MyOneTM Streptavidin C1 magnetic beads to each lysate.

72 Rotate at 30 RPM and room temperature for 60 min for binding to occur.

73 Wash beads.

73.1 Wash twice with 400 µL of 1X B&W buffer with 5 min rotation after resuspending beads.

73.2 Wash once more with 400 µL 10 mM Tris containing 0.1% Tween-20 with 5 min rotation after resuspension.

Template Switch 2h

74 This step performs template switching and adds the second PCR handle.

Resuspend the streptavidin beads bound with cDNA in solution containing 44 µL of 5X Maxima RT buffer (ThermoFisher), 44 µL of 20% Ficoll PM-400 solution, 22 µL of 10 mM dNTPs each (ThermoFisher), 5.5 µL RNase Inhibitor (Enzymatics), 11 µL of Maxima H Minus Reverse Transcriptase (ThermoFisher), 5.5 µL of 100 µM of template switch primer, and 88 µL of RNase-free water.

75 Rotate beads at 30 RPM and room temperature for 30 min followed by rotation at 42˚C for 90 min.

PCR 2h

76 Wash beads and resuspend.

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76.1 Wash once with 400 µL of 10 mM Tris and 0.1% Tween-20 solution and once more with 400 µL of RNase-free water.

76.2 Resuspend in the PCR mix solution containing 110 µL of 2X Kapa HiFi HotStart Master Mix (Kapa Biosystems), 8.8 µL each of 10 µM stocks of primers 1 and 2, and 92.4 µL of RNase-free water. Transfer 200 µL to four PCR tubes with 50 µL in each.

77 Perform PCR to detach cDNA from beads.

Steps	Temperature	Time	Cycles
Initial Denaturation	95˚C	3 min	1
Denaturation	98˚C	20 sec	5
Annealing	65˚C	45 sec	
Extension	72˚C	3 min	

PCR Cycling Conditions

78 Remove the Dynabeads™ from the PCR solution using a magnetic tube holder.

79 Add Evagreen (Biotium) at a 1X concentration.

NOTE: This step is optional.

80 Perform PCR again with the following thermocycling conditions. Cycling can also be halted once the qPCR signal begins to plateau.

Steps	Temperature	Time	Cycles
Initial Denaturation	95˚C	3 min	1
Denaturation	98˚C	20 sec	15 cycles
Annealing	65˚C	20 sec	
Extension	72˚C	3 min	
Final Extension	72˚C	5 min	1
Hold	4˚C	forever	

cDNA Purification 40m

81 Add 80 µL KAPA pure beads for 100 µL of the cDNA sample.

NOTE: Ensure that beads have been equilibrated to room temperature and are fully resuspended before use.

82 Mix thoroughly by pipetting.

83 Incubate the tubes at room temperature for at least 10 min to bind the cDNA to the beads.

84 Place the tubes on a magnet to capture the beads and incubate until the liquid is clear.

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85 Carefully remove and discard the supernatant.

86 Keep the tubes on the magnet and add 200 µL of 80% ethanol.

87 Incubate the tubes at room temperature for at least 30 sec.

88 Carefully remove and discard the ethanol.

89 Repeat steps 86-88.

90 Try to remove all residual ethanol and dry the beads at room temperature for 3-5 min or until all the ethanol has evaporated.

CRITICAL: Over-drying may result in reduced yield.

91 Remove the tubes from the magnet and resuspend the beads in 15 µL of PCR-grade water or elution buffer depending on downstream application.

92 Incubate the tubes at room temperature for at least 10 min to elute the cDNA off the beads.

93 Place the tubes back on the magnet and incubate until the liquid is fully clear.

94 Transfer the clear supernatant to a new tube.

95 Perform Bioanalyzer QC to obtain cDNA length profile and concentration.

Pause Point: Stop here for the second day. Store at 4˚C for 1-2 weeks or up to 6 months at -20˚C.

Library Preparation 1h 30m

96 Dilute 0.75 to 1 ng of purified cDNA in PCR-grade water to a total of 5 µL.

97 Add 10 µL of Tagment DNA buffer and 5 µL of Amplicon Tagment Mix to the cDNA for a total of 20 µL.

98 Mix thoroughly and incubate at 55˚C for 5 min.

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99 Add and mix 5 µL of NT Buffer to the solution and incubate at room temperature for 5 min.

100 In this order, add 15 µL of the PCR master mix, 8 µL of water, and 1 µL of each 10 µM primer (P5 primer and indexed P7 primer) to the mix for a total of 50 µL.

101 Perform PCR with the following thermocycling conditions.

Steps	Temperature	Time	Cycles
Initial Denaturation	95˚C	30 sec	1
Denaturation	95˚C	10 sec	12 cycles
Annealing	55˚C	30 sec	
Extension	72˚C	30 sec	
Final Extension	72˚C	5 min	1
Hold	4˚C	forever	

102 Purify the resulting PCR reaction mix at a 0.7X ratio of KAPA pure beads according to the manufacturer's manual to generate an Illumina-compatible sequencing library (as in steps 81-95).

103 Perform quality control analysis (Bioanalyzer) to obtain more accurate concentration and yield to prepare for sequencing.

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