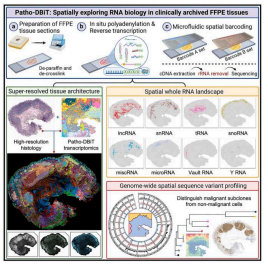
Jan 07, 2025

Spatial Multi-omics Sequencing for Formalin-Fixed Paraffin Embedded Tissue via DBiT-seq



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

DOI
https://dx.doi.org/10.17504/protocols.io.261ger2wwl47/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.261ger2wwl47/v1

External link: https://www.cell.com/cell/fulltext/S0092-8674(24)01019-5

Protocol Citation: Archibald Enninful, Rong Fan, Negin Farzad 2025. Spatial Multi-omics Sequencing for Formalin-Fixed Paraffin Embedded Tissue via DBiT-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261ger2wwl47/v1

Manuscript citation:
Bai et al., 2024, Cell 187, 6760–6779

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

Created: January 06, 2025

Last Modified: January 07, 2025

Protocol Integer ID: 117754

Keywords: cell resolution for spatial transcriptomic, spatial transcriptomic, tissue for spatial omic, rna biology in archival formalin, fixed paraffin embedded tissue via dbit, ffpe tissue sample, embedded tissue, fixed paraffin embedded tissue, exploring rna biology, rna, single cell, spatial omic, combinatorial dna oligo, cell resolution, omics atlas, tissue

Funders Acknowledgements:
SenNet Consortium
Grant ID: 1U54AG076043-01

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 FFPE tissue samples. This approach uses a microfluidic-based method to introduce combinatorial DNA oligo barcodes directly to the cells in a tissue section on a glass slide. This technique does not directly resolve single cells but can achieve a near-single-cell resolution for spatial transcriptomics.

This protocol is adapted from the Patho-DBiT publication - Spatially exploring RNA biology in archival formalin fixed paraffin-embedded tissues (Bai et al., 2024, Cell 187, 6760–6779)

Image Attribution

Image from graphical abstract of this publication - Bai et al., 2024, Cell 187, 6760–6779

Materials

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

	REAGENT or RESOURCE	SOURCE	IDENTIFIER
	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

	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

	Category	Barcode	Specificity	Clone	Barcode Sequence
	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.**

	A	B
	Barcode A-1	/5Phos/AGGCCAGAGCATTCGAACGTGATTTTTTTTTTTTTTTTV N
	Barcode A-2	/5Phos/AGGCCAGAGCATTCGAAACATCGTTTTTTTTTTTTTTTTV N
	Barcode A-3	/5Phos/AGGCCAGAGCATTCGATGCCTAATTTTTTTTTTTTTTTTV N
	Barcode A-4	/5Phos/AGGCCAGAGCATTCGAGTGGTCATTTTTTTTTTTTTTTTV N
	Barcode A-5	/5Phos/AGGCCAGAGCATTCGACCACTGTTTTTTTTTTTTTTTTV N
	Barcode A-6	/5Phos/AGGCCAGAGCATTCGACATTGGCTTTTTTTTTTTTTTTTV N
	Barcode A-7	/5Phos/AGGCCAGAGCATTCGCAGATCTGTTTTTTTTTTTTTTTTV N
	Barcode A-8	/5Phos/AGGCCAGAGCATTCGCATCAAGTTTTTTTTTTTTTTTTV N
	Barcode A-9	/5Phos/AGGCCAGAGCATTCGCGCTGATCTTTTTTTTTTTTTTTTV N
	Barcode A-10	/5Phos/AGGCCAGAGCATTCGACAAGCTATTTTTTTTTTTTTTTTV N
	Barcode A-11	/5Phos/AGGCCAGAGCATTCGCTGTAGCCTTTTTTTTTTTTTTTTV N
	Barcode A-12	/5Phos/AGGCCAGAGCATTCGAGTACAAGTTTTTTTTTTTTTTTTV N
	Barcode A-13	/5Phos/AGGCCAGAGCATTCGAACAACCATTTTTTTTTTTTTTTTV N
	Barcode A-14	/5Phos/AGGCCAGAGCATTCGAACCGAGATTTTTTTTTTTTTTTTV N
	Barcode A-15	/5Phos/AGGCCAGAGCATTCGAACGCTTATTTTTTTTTTTTTTTTV N
	Barcode A-16	/5Phos/AGGCCAGAGCATTCGAAGACGGATTTTTTTTTTTTTTTTV N
	Barcode A-17	/5Phos/AGGCCAGAGCATTCGAAGGTACATTTTTTTTTTTTTTTTV N
	Barcode A-18	/5Phos/AGGCCAGAGCATTCGACACAGAATTTTTTTTTTTTTTTTV N

	A	B
	Barcode A-19	/5Phos/AGGCCAGAGCATTCGACAGCAGATTTTTTTTTTTTTTTV N
	Barcode A-20	/5Phos/AGGCCAGAGCATTCGACCTCCAATTTTTTTTTTTTTTTV N
	Barcode A-21	/5Phos/AGGCCAGAGCATTCGACGCTCGATTTTTTTTTTTTTTTV N
	Barcode A-22	/5Phos/AGGCCAGAGCATTCGACGTATCATTTTTTTTTTTTTTTV N
	Barcode A-23	/5Phos/AGGCCAGAGCATTCGACTATGCATTTTTTTTTTTTTTTV N
	Barcode A-24	/5Phos/AGGCCAGAGCATTCGAGAGTCAATTTTTTTTTTTTTTTV N
	Barcode A-25	/5Phos/AGGCCAGAGCATTCGAGATCGCATTTTTTTTTTTTTTTV N
	Barcode A-26	/5Phos/AGGCCAGAGCATTCGAGCAGGAATTTTTTTTTTTTTTTV N
	Barcode A-27	/5Phos/AGGCCAGAGCATTCGAGTCACTATTTTTTTTTTTTTTTV N
	Barcode A-28	/5Phos/AGGCCAGAGCATTCGATCCTGTATTTTTTTTTTTTTTTV N
	Barcode A-29	/5Phos/AGGCCAGAGCATTCGATTGAGGATTTTTTTTTTTTTTTV N
	Barcode A-30	/5Phos/AGGCCAGAGCATTCGCAACCACATTTTTTTTTTTTTTTV N
	Barcode A-31	/5Phos/AGGCCAGAGCATTCGGACTAGTATTTTTTTTTTTTTTTV N
	Barcode A-32	/5Phos/AGGCCAGAGCATTCGCAATGGAATTTTTTTTTTTTTTTV N
	Barcode A-33	/5Phos/AGGCCAGAGCATTCGCACTTCGATTTTTTTTTTTTTTTV N
	Barcode A-34	/5Phos/AGGCCAGAGCATTCGCAGCGTTATTTTTTTTTTTTTTTV N
	Barcode A-35	/5Phos/AGGCCAGAGCATTCGCATACCAATTTTTTTTTTTTTTTV N
	Barcode A-36	/5Phos/AGGCCAGAGCATTCGCCAGTTCATTTTTTTTTTTTTTTV N
	Barcode A-37	/5Phos/AGGCCAGAGCATTCGCCGAAGTATTTTTTTTTTTTTTTV N

	A	B
	Barcode A-38	/5Phos/AGGCCAGAGCATTCGCCGTGAGATTTTTTTTTTTTTTTV N
	Barcode A-39	/5Phos/AGGCCAGAGCATTCGCCTCCTGATTTTTTTTTTTTTTTV N
	Barcode A-40	/5Phos/AGGCCAGAGCATTCGCGAACTTATTTTTTTTTTTTTTV N
	Barcode A-41	/5Phos/AGGCCAGAGCATTCGCGACTGGATTTTTTTTTTTTTTV N
	Barcode A-42	/5Phos/AGGCCAGAGCATTCGCGCATACATTTTTTTTTTTTTTV N
	Barcode A-43	/5Phos/AGGCCAGAGCATTCGCTCAATGATTTTTTTTTTTTTTV N
	Barcode A-44	/5Phos/AGGCCAGAGCATTCGCTGAGCCATTTTTTTTTTTTTTV N
	Barcode A-45	/5Phos/AGGCCAGAGCATTCGCTGGCATATTTTTTTTTTTTTTV N
	Barcode A-46	/5Phos/AGGCCAGAGCATTCGGAATCTGATTTTTTTTTTTTTTV N
	Barcode A-47	/5Phos/AGGCCAGAGCATTCGCAAGACTATTTTTTTTTTTTTTV N
	Barcode A-48	/5Phos/AGGCCAGAGCATTCGGAGCTGAATTTTTTTTTTTTTTV N
	Barcode A-49	/5Phos/AGGCCAGAGCATTCGGATAGACATTTTTTTTTTTTTTV N
	Barcode A-50	/5Phos/AGGCCAGAGCATTCGGCCACATATTTTTTTTTTTTTTV N

Table S2B. List of DNA Barcode A Sequences.

	A	B
	Barcode B-1	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACGTGATATCCAC GTGCTTGAG
	Barcode B-2	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAAACATCGATCCAC GTGCTTGAG
	Barcode B-3	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNATGCCTAAATCCAC GTGCTTGAG

	A	B
	Barcode B-4	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGTGGTCAATCCAC GTGCTTGAG
	Barcode B-5	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACCACTGTATCCAC GTGCTTGAG
	Barcode B-6	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACATTGGCATCCAC GTGCTTGAG
	Barcode B-7	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAGATCTGATCCAC GTGCTTGAG
	Barcode B-8	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCATCAAGTATCCAC GTGCTTGAG
	Barcode B-9	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGCTGATCATCCAC GTGCTTGAG
	Barcode B-10	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACAAGCTAATCCAC GTGCTTGAG
	Barcode B-11	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTGTAGCCATCCAC GTGCTTGAG
	Barcode B-12	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGTACAAGATCCAC GTGCTTGAG
	Barcode B-13	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACAACCAATCCAC GTGCTTGAG
	Barcode B-14	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACCGAGAATCCAC GTGCTTGAG
	Barcode B-15	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAACGCTTAATCCAC GTGCTTGAG
	Barcode B-16	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAAGACGGAATCCAC GTGCTTGAG
	Barcode B-17	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAAGGTACAATCCAC GTGCTTGAG
	Barcode B-18	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACACAGAAATCCAC GTGCTTGAG
	Barcode B-19	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACAGCAGAATCCAC GTGCTTGAG
	Barcode B-20	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACCTCCAAATCCAC GTGCTTGAG
	Barcode B-21	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACGCTCGAATCCAC GTGCTTGAG
	Barcode B-22	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACGTATCAATCCAC GTGCTTGAG

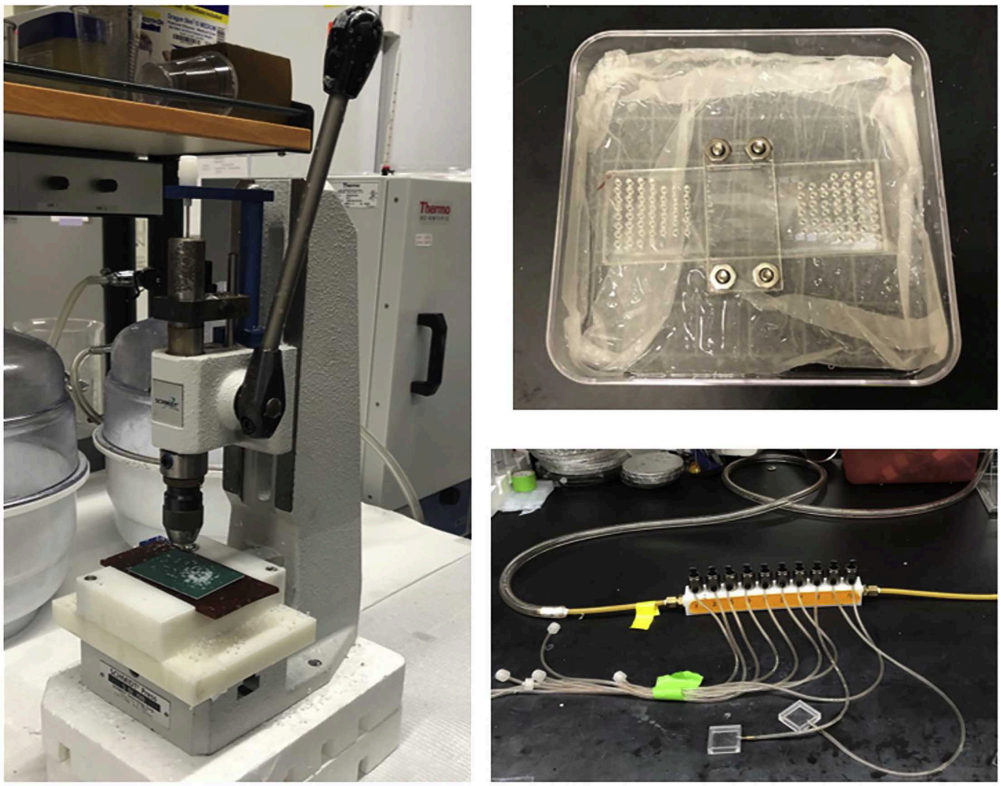
	A	B
	Barcode B-23	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNACTATGCAATCCAC GTGCTTGAG
	Barcode B-24	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGAGTCAAATCCAC GTGCTTGAG
	Barcode B-25	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGATCGCAATCCAC GTGCTTGAG
	Barcode B-26	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGCAGGAAATCCAC GTGCTTGAG
	Barcode B-27	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNAGTCACTAATCCAC GTGCTTGAG
	Barcode B-28	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNATCCTGTAATCCAC GTGCTTGAG
	Barcode B-29	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNATTGAGGAATCCAC GTGCTTGAG
	Barcode B-30	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAACCACAATCCAC GTGCTTGAG
	Barcode B-31	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGACTAGTAATCCAC GTGCTTGAG
	Barcode B-32	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAATGGAAATCCAC GTGCTTGAG
	Barcode B-33	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCACTTCGAATCCAC GTGCTTGAG
	Barcode B-34	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAGCGTTAATCCAC GTGCTTGAG
	Barcode B-35	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCATACCAAATCCAC GTGCTTGAG
	Barcode B-36	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCAGTTCAATCCAC GTGCTTGAG
	Barcode B-37	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCGAAGTAATCCAC GTGCTTGAG
	Barcode B-38	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCGTGAGAATCCAC GTGCTTGAG
	Barcode B-39	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCCTCCTGAATCCAC GTGCTTGAG
	Barcode B-40	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGAACTTAATCCAC GTGCTTGAG
	Barcode B-41	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGACTGGAATCCAC GTGCTTGAG

	A	B
	Barcode B-42	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCGCATACAATCCAC GTGCTTGAG
	Barcode B-43	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTCAATGAATCCAC GTGCTTGAG
	Barcode B-44	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTGAGCCAATCCAC GTGCTTGAG
	Barcode B-45	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCTGGCATAATCCAC GTGCTTGAG
	Barcode B-46	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGAATCTGAATCCAC GTGCTTGAG
	Barcode B-47	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNCAAGACTAATCCAC GTGCTTGAG
	Barcode B-48	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGAGCTGAAATCCAC GTGCTTGAG
	Barcode B-49	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGATAGACAATCCAC GTGCTTGAG
	Barcode B-50	/5Biosg/CAAGCGTTGGCTTCTCGCATCTNNNNNNNNNNGCCACATAATCCAC GTGCTTGAG

Table S2C. List of DNA Barcode B Sequences.

- 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

	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

	Reagent	Volume (µL)
	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**

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 start

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 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 50Prepare chrome photomasks of the microfluidics chip by printing the high-resolution CAD files onto a glass substrate (Figure 1).

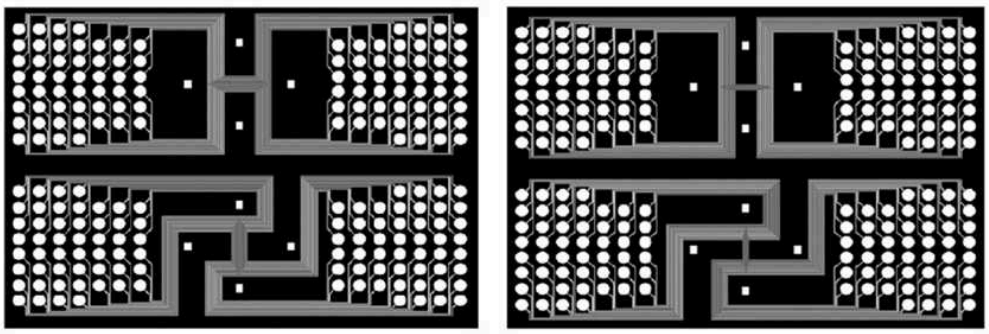


Figure 1: Microfluidic Photomask Design.
(Left) AutoCAD design for the 25 micron-width 50 channel Chip-A and Chip-B. (Right) AutoCAD design for 10 micron-width channel Chip-A and Chip-B.

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.

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).

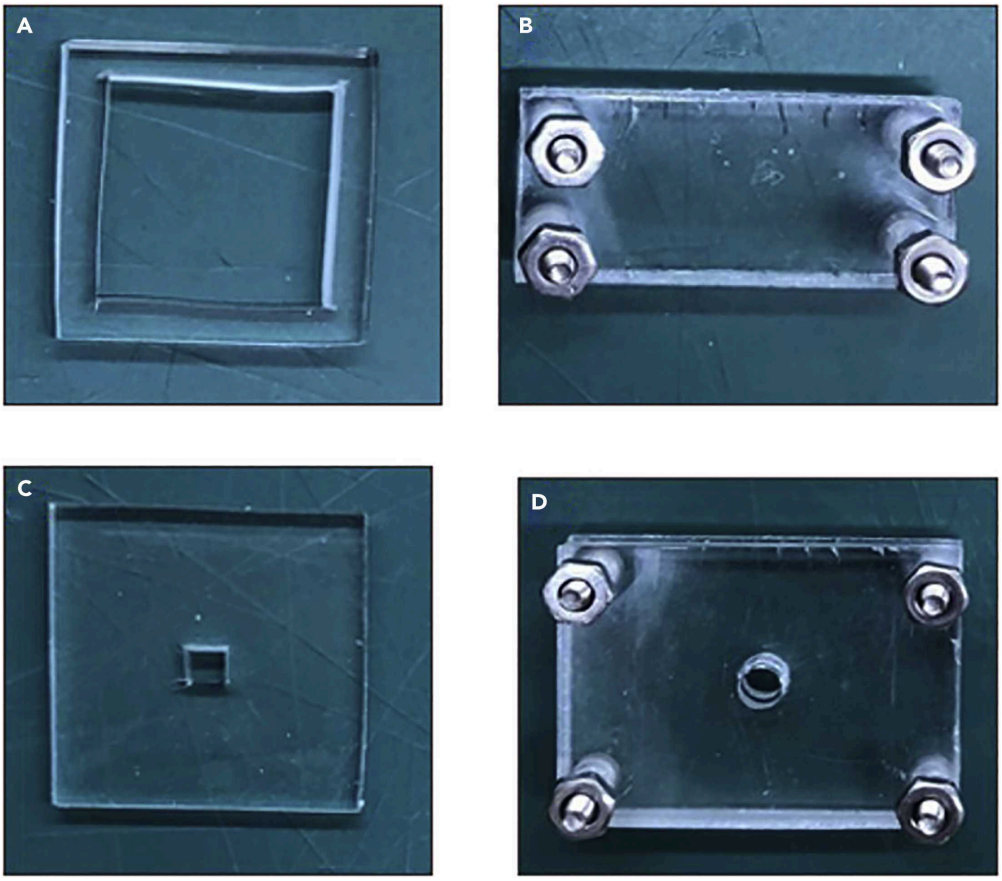


Figure 2. Key parts for setup. PDMS reservoirs and acrylic clamps used in DBiT-seq. (A) Inlet Reservoir. (B) Barcoding Clamp. (C) Lysis Reservoir. (D) Lysis Clamp.

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).

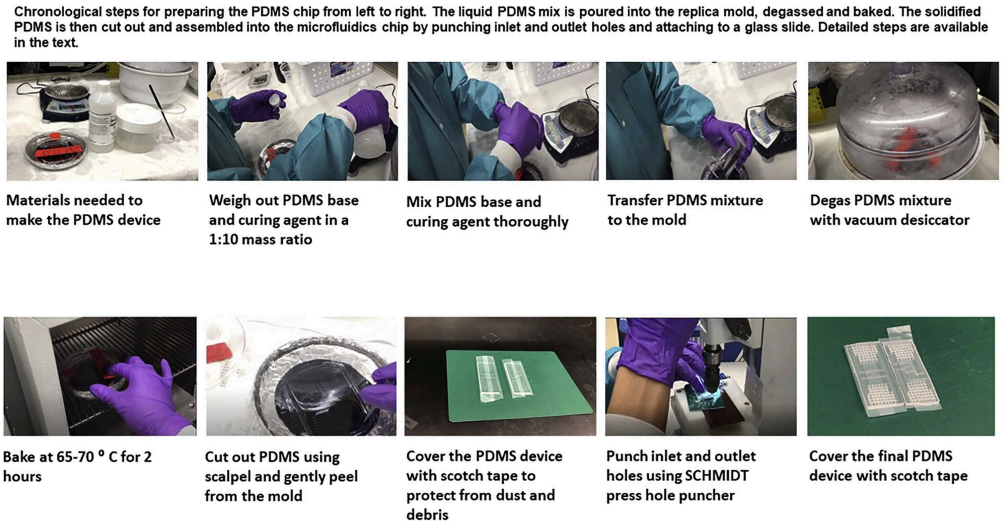


Figure 3: Step-by-step visual guide for making Chip-A and Chip-B.

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.

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 25×25×5mm PDMS pieces.

26 For the barcoding reservoir, cut out a 20×20mm 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 (1×1mm for the 10µm channel device, 2.5×2.5mm 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 Barcodes

1h

28 Thoroughly mix the ligation linker with each barcodes $A_{1\text{-}50}$ $B_{1\text{-}50}$ 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 FFPE tissue sections were cut into sections with a thickness < 10 um and were placed at the center of a poly-L-lysine glass slide. Sections were stored at -80 ˚C until use.

33 Tissue sections were taken out from the freezer and allowed to come up to room temperature for **10 min** until all the moisture disappears.

Tissue Preparation

1h

34 Bake slides at 60 degrees Celsius for 2 hours.

35 Perform deparaffinization as follows:
* **100% Xylene 5 minutes**
* **100% Xylene 5 minutes**

*** 100% Ethanol for 5 minutes**
*** 90% Ethanol for 5 minutes**
*** 70% Ethanol for 5 minutes**
*** 50% Ethanol for 5 minutes**
*** Deionized water for 5 minutes**

36 5. Antigen-retrieval using 1X Tris-EDTA pH 9
* Allow to boil
* Incubate slide for
~ 10 minutes
* Allow to cool to room temperature (Can incorporate cooling on ice to speed up the process)

37 **Permeabilization:** 500µL **0.5% Triton X-100 in PBS** was added on top of the tissue, and tissues were permeabilized for **20 min** at room temperature.

38 To stop permeabilization, **0.5X PBS-RI** was used to clean up the tissue sections.

39 Take a full pre-scan image of the section at the desired optical resolution

Recommended 10X resolution.

In situ polyadenylation

40 Reagents
Poly(A) wash buffer: 20µL 5X Poly(A) Polymerase Reaction Buffer, 2µL RNase Inhibitor, 78µl nuclease-free water.

	A	B	C	D
	Reagent name	Concentration	For 1 chip	For 2 chips
	Nuclease-free Water		47.6µL	95.3µL
	5X Poly(A) Reaction Buffer	5X	15µL	30µL
	Poly(A) Polymerase	600U/µL	3µL	6µL
	ATP	100mM	0.38µL	0.75µL

	A	B	C	D
	SuperaseIn RNase Inhibitor	20U/µL	6µL	12µL
	RNase Inhibitor	40U/µL	3µL	6µL

Table 1: Poly(A) mix preparation

41 The tissue section was equilibrated by adding 100µL of Poly(A) wash buffer, incubated for **5 min** at room temperature, followed by air drying.

42 Next, attach the PDMS reservoir, add 75µL of Poly(A) Mix to the reaction chamber, and seal with
parafilm. Incubate at 37˚C for **25 min**, in a wet box.

43 After incubation, **0.5X PBS-RI** was used to clean up the tissue sections.

Bulk Tissue Reverse Transcription

1h

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

45 Primer sequences: 15T Biotin RT Primer:
/5Phos/CATCGGCGTACGACTNNNNNNNNNN/iBiodT/TTTTTTTTTTTTTTTVN

46 Reverse transcription mix

	A	B	C	D
	Reagent name	Concentration	For 1 chip	For 2 chips
	0.5X PBS-RI		20µL	40µL
	5X Maxima RT buffer	5X	12µL	24µL
	30T Biotin RT Primer	25uM	10µL	20µL
	Maxima H Minus RT	200U/µL	6µL	12µL

	A	B	C	D
	Nuclease-free Water		8µL	16µL
	dNTPs (10mM)	10mM	3µL	6µL
	SuperaseIn RNase Inhibitor	20U/µL	0.8µL	1.6µL
	RNase Inhibitor	40U/µL	0.4µL	0.8µL

Table 2: Reverse Transcription (RT) mix preparation

1X NEB buffer 3.1: 100µL 10X NEB buffer + 900µL Nuclease-free Water.

47 The 60µL RT Mix was loaded on the sample with PDMS reservoir and sealed with parafilm. Then, incubated at room temperature for **30 min**, and 42˚C for **90 min**, in a wet box.

48 After RT reaction, samples were flow washed with 1 mL **Nuclease-free Water** for 5 times, then incubated in 500µL **1X NEB buffer 3.1** for **5 min** at room temperature, followed by air drying.

Ligation of Barcodes

49 Reagents
Ligation mix

	A	B	C	D
	Reagent name	Concentration	For 1 chip	For 2 chips
	1X NEB buffer 3.1	1X	100µL	200µL
	10X T4 Ligase buffer	10X	26µL	52µL
	Nuclease-free Water		61.3µL	122.6µL
	T4 DNA Ligase	400U/µL	15µL	30µL
	5% Triton-X100		5µL	10µL
	RNase Inhibitor	40U/µL	2µL	4µL
	SuperaseIn RNase Inhibitor	20U/µL	0.7µL	1.4µL

Table 3: Preparation of ligation mix

50 Then, in a 96-well plate, add 4µL of the above Ligation Mix for each well.

51 For each well, 1µL of **25uM of prepared Barcode A with Linker1** (A1-A50) was added and mixed with the Ligation Mix.

52 Prepare the wash buffer as follows:
Wash buffer: 2mL PBS+20µL 10% Triton X-100+5µL SuperaseIn RNase Inhibitor.

Attaching PDMS chips and loading of ligation mix

1h

53 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).

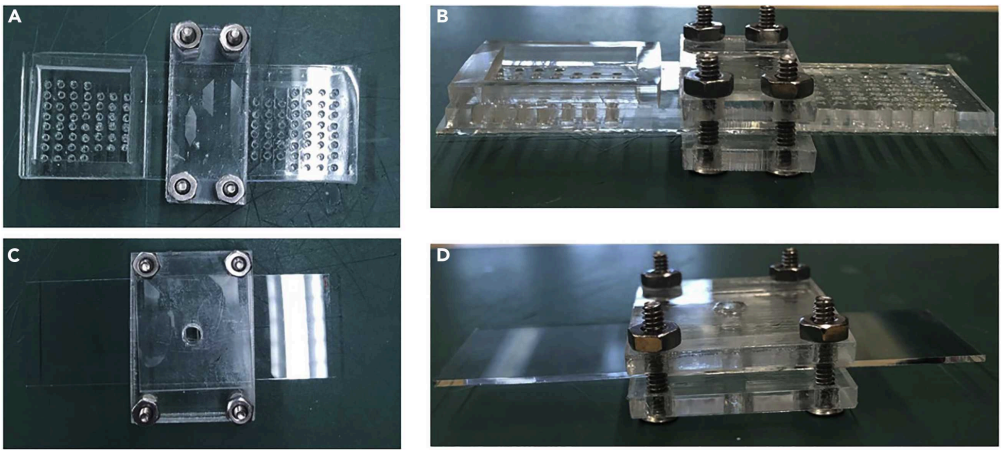


Figure 4. Assembly to create the setup for the PDMS reservoir and acrylic clamps. (A) Chip-A with the inlet reservoir and barcoding clamp. (B) Sideview of Chip-A. (C) The lysis reservoir with the lysis clamp on top of the barcoded tissue. (D) Sideview of the lysis setup.

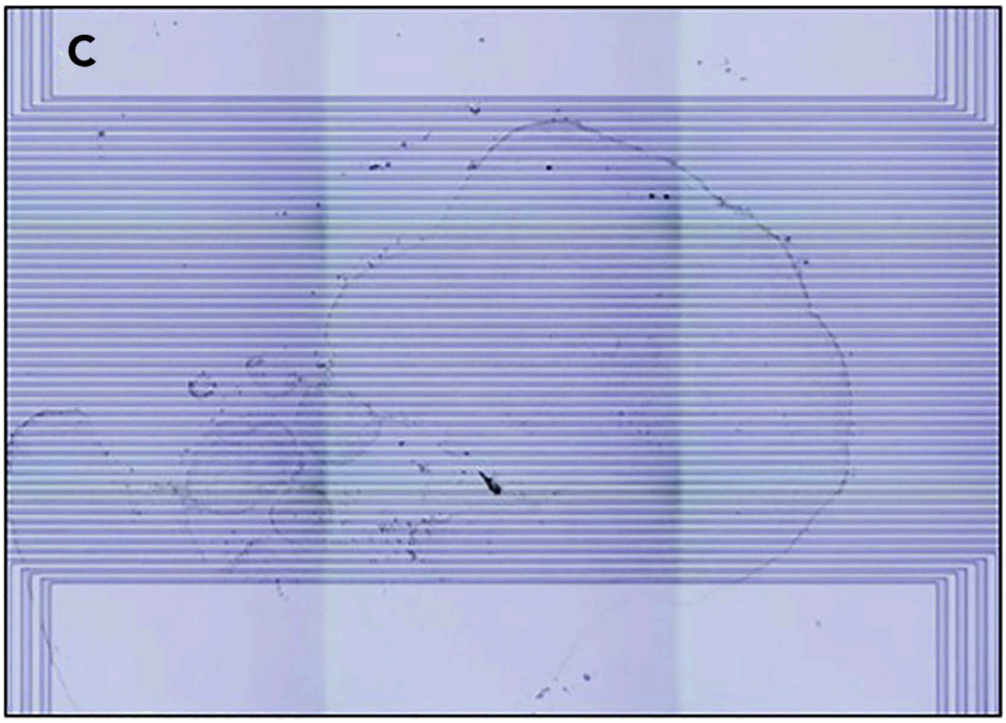


Figure 5. Example Scanning of the Tissue Slide.
(**C**) Scan of Chip-A channels placed on the tissue.

54 Use the acrylic clamp to firmly hold the PDMS chip against the tissue.

54.1 Place the inlet reservoir above the inlets. The final mixture (5µL in total) was injected into each of the 50 inlets of the 1st PDMS and vacuumed through the channels, making sure all the channels were filled.

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

NOTE: Make sure all channels are filled with solution.

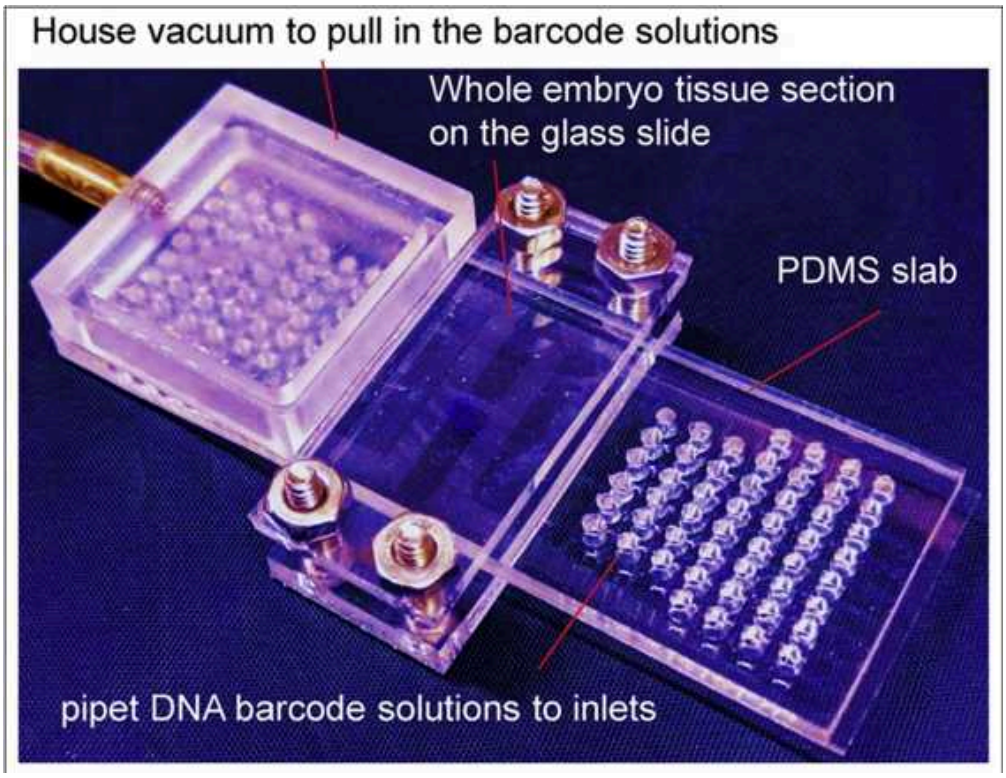


Figure 6. Chip-A with Acrylic Clamp and House Vacuum attached.

54.3 Stop the vacuum and incubate at 37 ˚C for 15 mins. Take out the chips and check if there are
missed channels induced by bubbles. Vacuum again if needed and further incubate for 30 mins.

54.4 After ligation reaction, aspirate remaining barcode A mixture from inlets/outlets.

54.5 Clean the channels by flushing 1mL **Wash buffer** for **5 min** and peel off PDMS and flow wash the chip once with 1 mL **Nuclease-free Water**, followed
by air dry.

In-Tissue Ligation of barcode B

1h

55 Attach Chip-B centered on the tissue.

55.1 Scan the channels over the tissue to ensure proper placement.

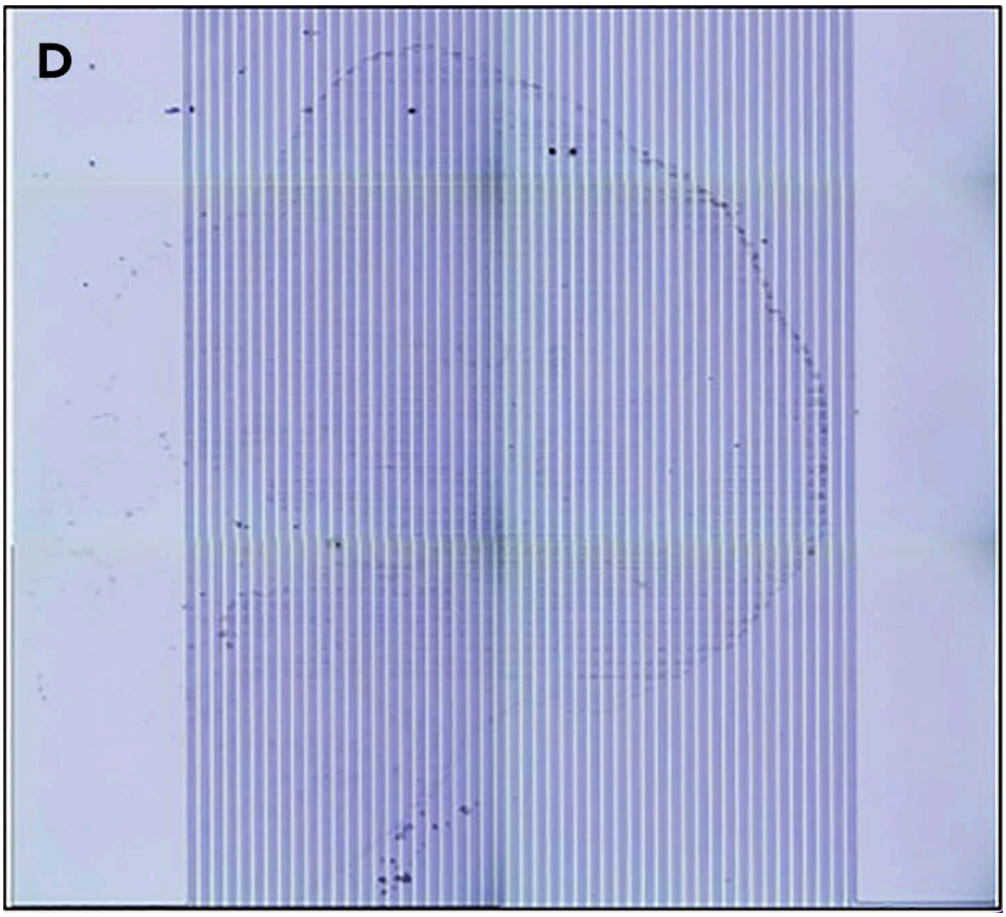


Figure 7: Example scanning of the tissue slide.
(D) Scan of Chip-B channels across the tissue.

55.2 Firmly clamp as before.

56 Prepare the ligation mixture.

57 Prepare barcode B mixture.

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

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

58 Introduce final barcode B mixture to the tissue.

58.1 Pipet each barcode B mixture directly into the inlets.

58.2 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.

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

59 Clean the channels with wash buffer for 10 min.

59.1 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.

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

61 Take a final post-scan of the tissue section.

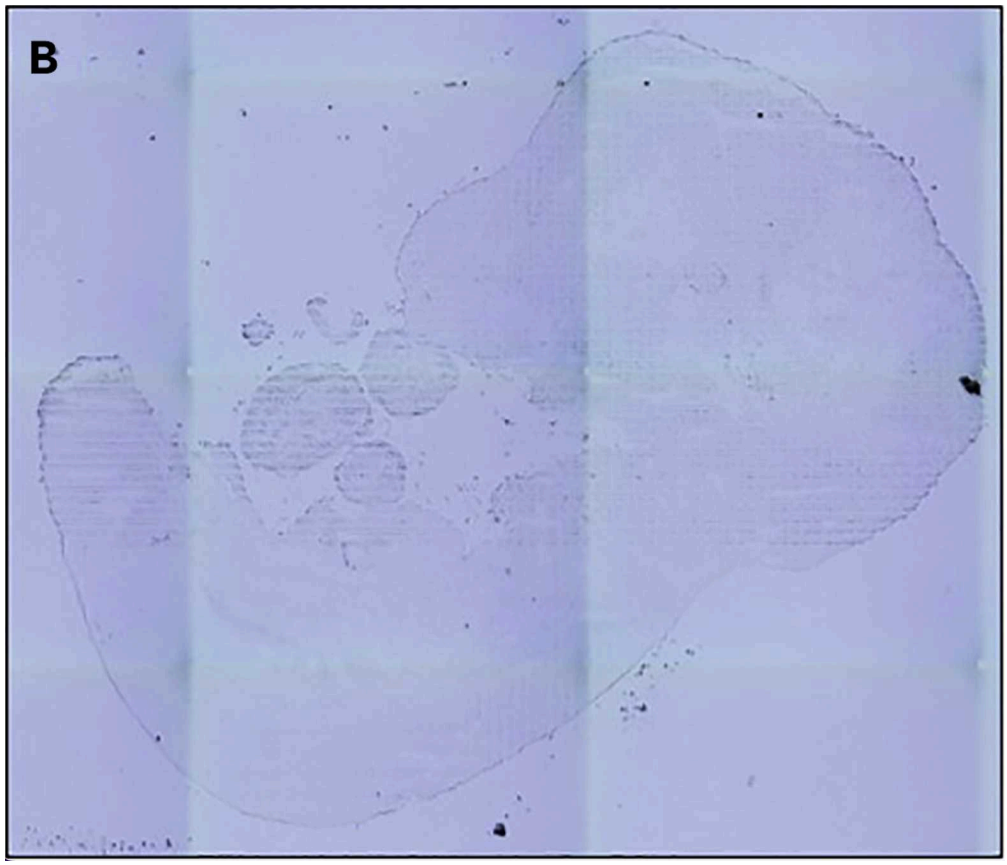


Figure 8. Example Scanning of the Tissue Slide.
(B) Final scan after barcoding and removing the device.

Lysis and Sub-library Generation

2h 30m

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

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

63 Tightly apply the lysis clamp.

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

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

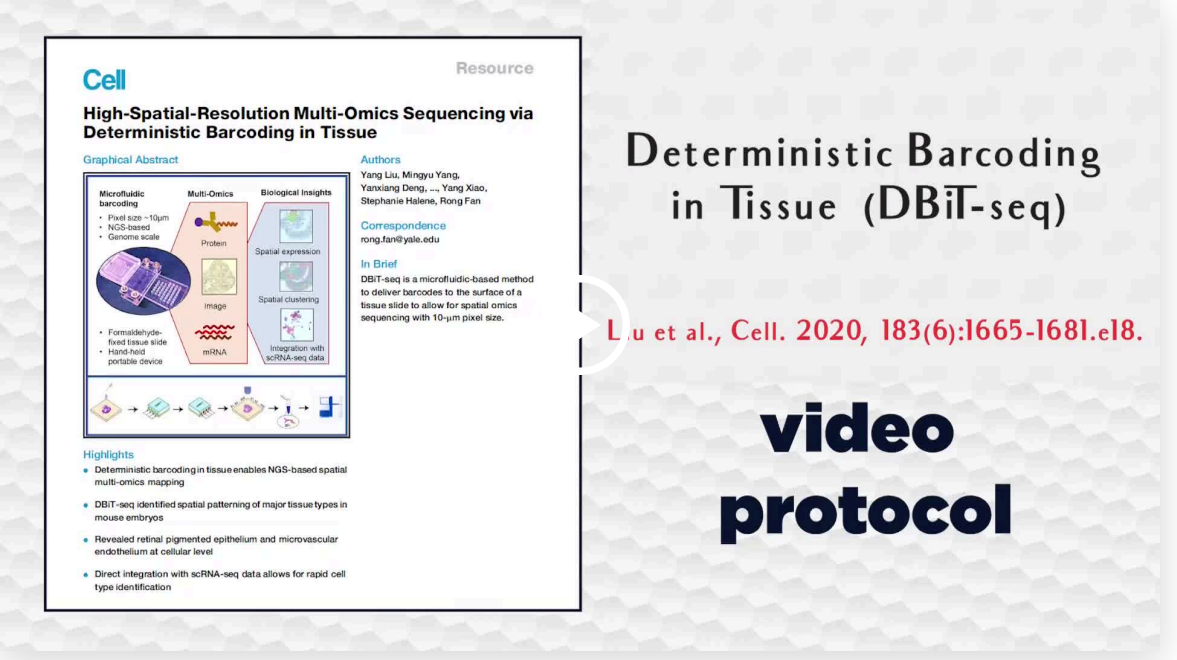
64.2 2X lysis buffer is made up of 20 mM Tris (pH 8.0), 400 mM NaCl, 100 mM EDTA (pH 8.0), and 4.4% SDS.

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

66 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.

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

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

68 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.

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

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

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

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

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

72 Wash beads.

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

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

Template Switch

2h

73 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.

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

PCR

2h

75 Wash beads and resuspend.

75.1 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.

75.2 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.

76 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

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

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

NOTE: This step is optional.

79 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	

Removal of ribosomal RNA

80 rRNA removal using Bio-Rad kit. Link: **SEQuoia RiboDepletion Kit (**17006487)
After rRNA removal, another 10 cycle PCR using 200uL PCR reaction system (100uL 2X Kapa
HiFi) using direct ligation primer and Illumina N70X primer

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.

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 PCR product from previous step can be sequenced.