Supplementary Information

Improved ClickTags Enable Live-cell Barcoding for Highly Multiplexed Single-cell Sequencing

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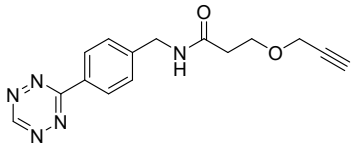
Methods

Synthesis and quality control of ClickTags.

5'-amine modified oligonucleotides (NH_2-oligos) were used to synthesize ClickTags. NH_2-oligos (Sangon Biotech) were resuspended to a concentration of 1 mM in nuclease-free water. NHS-Tz (item No. 1143; Xi'an Confluore Biological Technology, Xi'an, China) were resuspended to a concentration of 100 mM in dry DMSO. All the stock solutions were kept in -20 ℃ until use. For Tz-oligo synthesis, 5 μL of 10× BBS buffer (1.5 M NaCl, 0.15 M sodium borate, pH 7.4), 10 μL of NH_2-oligo (1 mM), 10 μL of DMSO, 31 μL nuclease-free water and 1 μL of NHS-Tz (100 mM) were mixed together. After 15, 30, 45 and 60 min, additional 1 μL of NHS-Tz were added. High resolution LC-MS (Waters, Xevo G2-XS QTof) was used to monitor the reaction with 0.2 mL/min flow rate and the following gradient: 0-2 min sustaining 5% solvent B, 2-5 min ramping solvent B 5% - 95%, 5-7 min sustaining 95% solvent B, 7-7.5 min decreasing solvent B 95% - 5%, and 7.5-10 min sustaining 5% solvent B (solvent A: 10 mM ammonium formate in water; solvent B: 100% methanol). The product, Tz-oligo was purified by silica membrane-based ion exchange DNA adsorption columns according to the manufacturer's protocol (item No. ALH105; Baiaolaibo, Beijing, China). Finally, the Tz-oligo was then characterized by LC-MS.

Flow cytometry analysis.

Cell surface barcoded ClickTags could be probed by fluorescently labelled complementary oligos to screen barcoding conditions. For example, 100,000 Jurkat cells were resuspended in 1× DPBS buffer with 25 μM NHS-TCO and Tz-oligos (diluted to the concentration as indicated) and reacted at room temperature in the dark for 15 minutes. Afterwards, 10 μl of quenching buffer (300 μM alkyne-Tz in FBS) were added and incubated for 5 minutes to terminate the labelling reaction. Fluorophore-conjugated ClickTag-complementary ssDNA oligos (Sangon Biotech, Shanghai, China) were then added to a final concentration of 1 μM and stained for 15 minutes. All reactions were washed with 1× DPBS buffer for three times. Finally, the labelling efficiency of ClickTag on Jurkat cells were analyzed on an Agilent NovoCyte Quanteon flow cytometer. Unlabelled cells were chosen as negative controls. Alkyne-Tz is purchased from Xi'an Confluore Biological Technology (item No. BCE-62; the exact structure is shown below). Jurkat cells were obtained from ATCC.



Mouse primary cell preparation.

Mouse bone marrow (BM) cells and testicle cell were used. BM cells were isolated from femurs of 6-week-old C57BL/6 (B6) mice. After RBC lysis, cells were washed twice with DBPS and subjected to ClickTag labelling and multiplexed scRNA-seq. Testicles of adult B6 mice (6-week-old) were collected aseptically in serum-free Dulbecco's modified Eagle's medium (DMEM, high glucose formulation). After mechanical grinding and trypsinization, testicle cells were washed twice with 1× DPBS and subjected to ClickTag labelling and multiplexed scRNA-seq. Animal handling, housing, and experimentation were carried out in strict accordance with the Institutional

Animal Care and Use Committee (IACUC) and under the guidelines set forth by Nanjing University (IACUC-2012003).

Cell barcoding, multiplexing and scRNA-seq.

In general, one sample was barcoded with ClickTags by the following procedure: for each ClickTag preparation, 0.5 μl of NHS-TCO (1 mM in DMSO) was added to 20 μl of 25 μM Tz-oligo and immediately pipetted into the sample after thorough mixing. Cell barcoding proceeded for 15 min in the dark at room temperature on a rotating platform and then quenched by 10 μl of quenching buffer (300 μM alkyne-Tz in FBS) for 5 min. After washing with 200 μl of DPBS buffer for three times and detecting the cell number and cell viability, cells were pooled and loaded to a microwell chip targeting 5,000 cells on Singleron Matrix® (GEXSCOPE Single Cell RNA-seq Kit, Singleron Biotechnologies, Nanjing, China). The scRNA-seq libraries were preparation according to the manufacturer's instructions (Singleron Biotechnologies, Nanjing, China). After amplification, cDNA and ClickTags were separated by SPRI size-selection with 0.6× and 1.4× SPRI, respectively. ClickTag libraries were quantified (Qubit, Invitrogen) and amplified using primer SGR-beads-1/SGR-tag-1 (Table S2) and indexed by additional PCR with primer SGR-beads-2/SGR-tag-2 (Table S2). Final ClickTag libraries and transcriptome libraries were analyzed on a BioAnalyzer high-sensitivity DNA kit (Agilent) and sequenced on Illumina NovaSeq 6000. Other multiplexed scRNA-seq experiments with different cells and ClickTags were conducted following the same procedure. Specially, two-dimensional barcoding was enabled by mixing two different Tz-oligos (10 μL, 50 μM for each ClickTag). Cells lines used for proof-of-concept sample multiplexing in scRNA-seq, namely, CCRF-CEM, NB4, THP-1 and U937 cell lines were purchased from National Collection of Authenticated Cell Cultures, Shanghai, China.

Bladder cancer TIL isolation and preparation.

Single-cell suspensions were obtained after collagenase digestion and grinding of bladder tumor specimens and then cryopreserved in FBS containing 10% DMSO in liquid nitrogen until FACS sorting. For FACS sorting, samples were thawed at 37 ℃ and then stained by anti-hCD45 fluorescent antibody (HI30; BioLegend, USA, 304006) for 30 min on ice. BD FACS Aris II was used to sort the hCD45+ populations and resuspended in 1× DPBS buffer. Cells from three patients were labelled with unique ClickTag pairs respectively, and pooled to perform scRNA-seq as described previously. This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital (2021-394-01). Informed consents were obtained from human participants in this study.

Data analysis.

Raw sequenced reads were processed using CeleScope pipeline (v1.3.1) with default parameter (https://github.com/singleron-RD/CeleScope). ClickTag libraries were processed with a new feature barcode processing plug-in ("teg") of CeleScope inspired by previous scRNA-seq multiplexing algorithm[1,2]. Gene expression matrices were then analyzed using R language.

Supplementary Figures

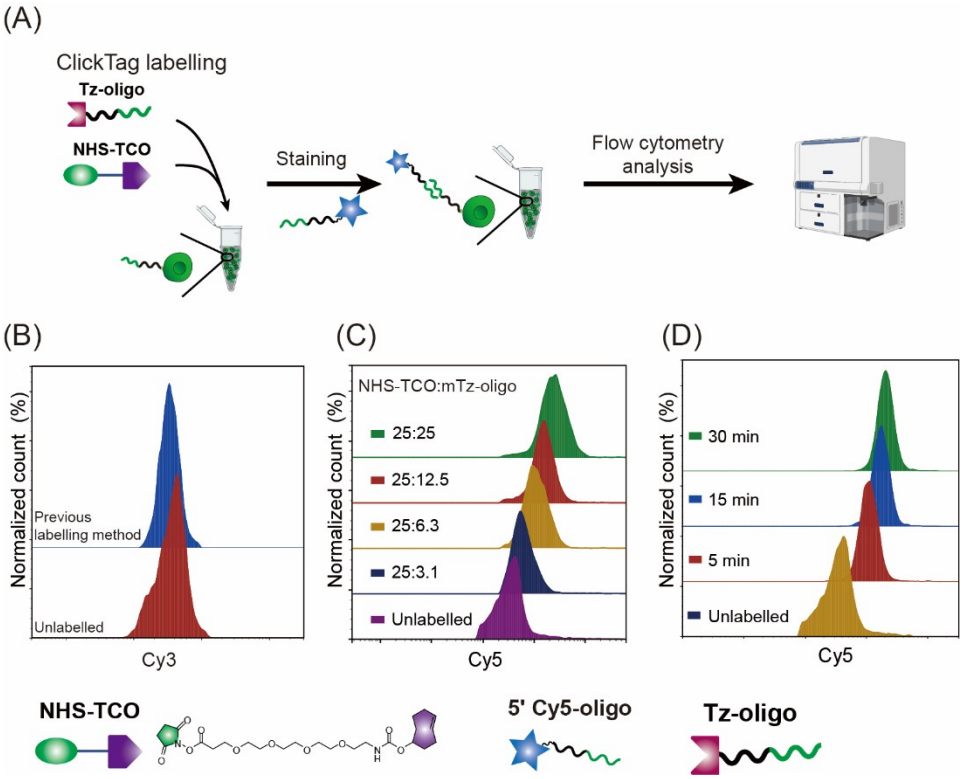


Fig. S1 Optimization of reaction conditions for barcoding live cells with ClickTags by flow cytometry. (A) Schematic illustration of the procedure for ClickTag labelling and flow cytometry analysis. (B) DNA barcoding of Jurkat cells in DPBS buffer following the previous method. For the control group, Jurkat cells were stained with 5' Cy3-poly-T without ClickTag barcoding. (C) DNA barcoding of Jurkat cells under different ratios of NHS-TCO versus Tz-oligos (25 μM NHS-TCO). (D) Time-course analysis of ClickTag barcoding under 25 μM NHS-TCO and 25 μM Tz-oligos.

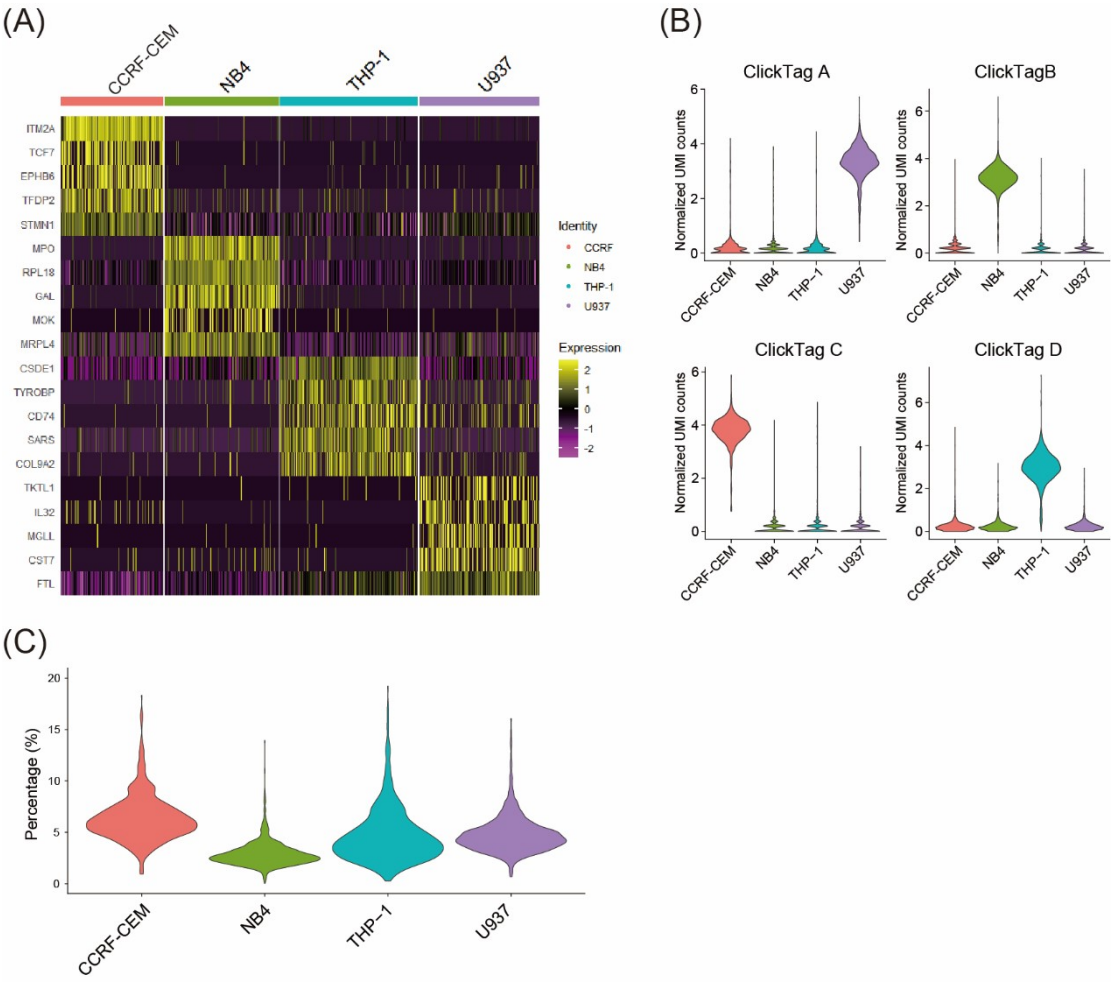


Fig. S2 Marker genes and ClickTag barcoding efficiency for the cell-line barcoding experiment. (A) Heatmap of marker genes for the cell-line barcoding experiment. Related to Fig. 3B. (B) Violin plots describing scaled (z-score) normalized UMI counts of ClickTags in the four cell-line samples. (C) Violin plots describing barcode UMI counts percentage of the transcriptome.

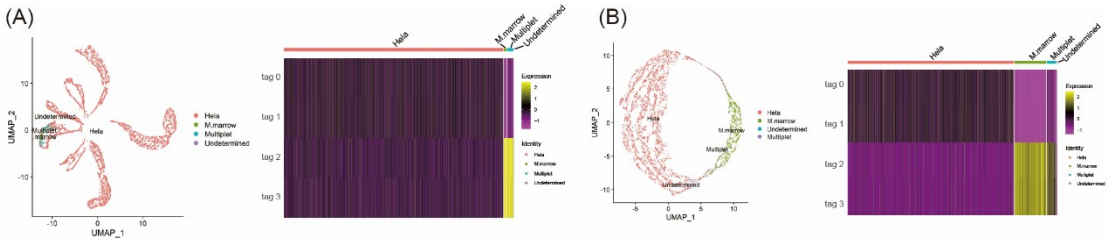


Fig. S3 Comparison of previous method and optimized method for multiplexing of HeLa cells and mouse bone marrow cells in aqueous phase. ClickTag-based UMAP embedding (left) and heatmap of scaled (z-score) normalized ClickTag values (right) following the previous method (A) and the optimized method (B).

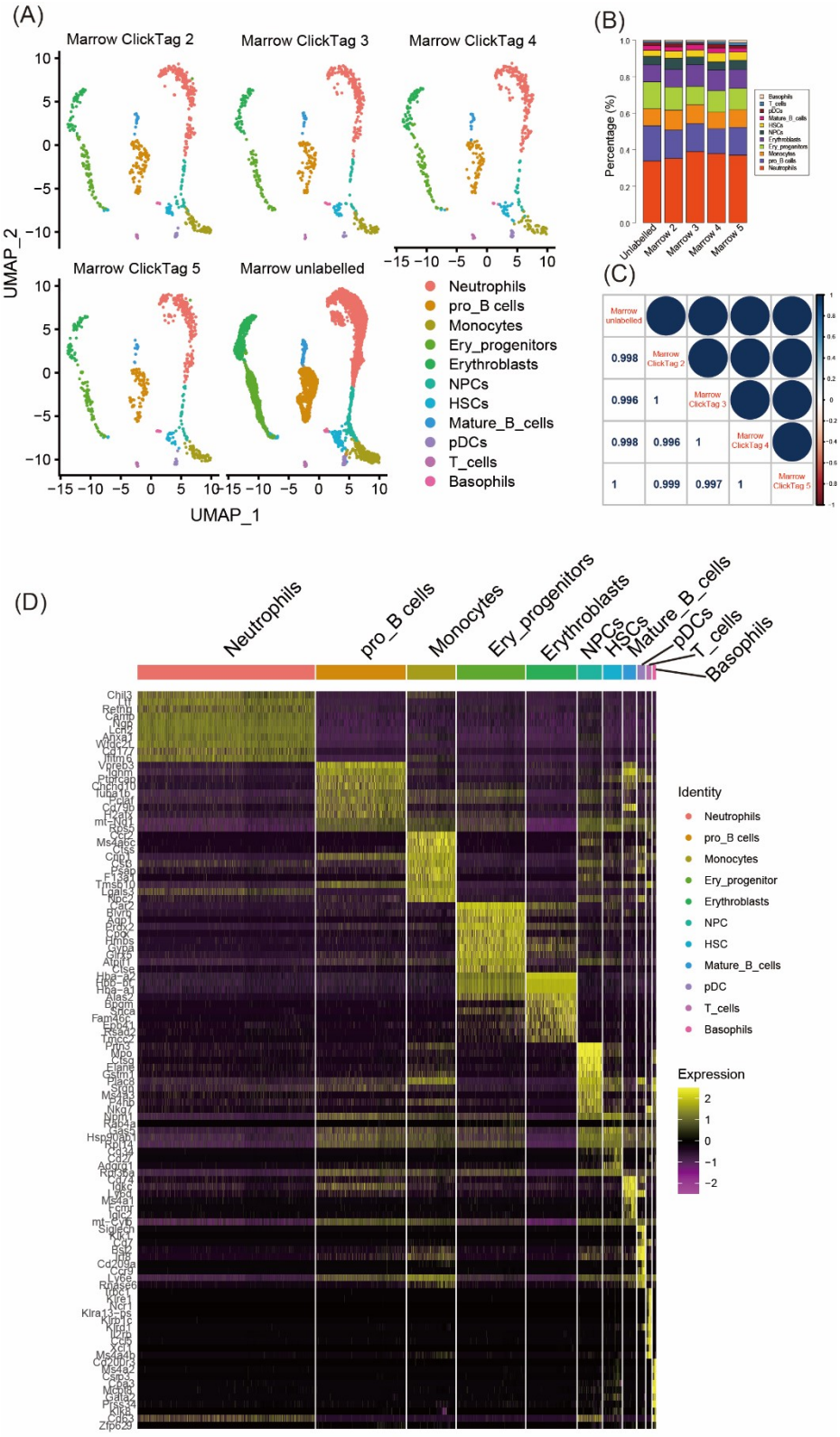


Fig. S4 Application of ClickTags for multiplexing of mouse bone marrow cells. (A) UMAP analysis of four mouse bone marrow samples (Marrow ClickTag1, Marrow ClickTag2, Marrow ClickTag3, Marrow ClickTag4) and unlabelled sample (Marrow unlabelled). Cell types was annotated by transcriptome-based clustering. (B) Cell types proportions for each sample. (C) Gene expression correlation between all the five samples. (D) Heatmap of marker genes describing markers utilized for cell type annotation.

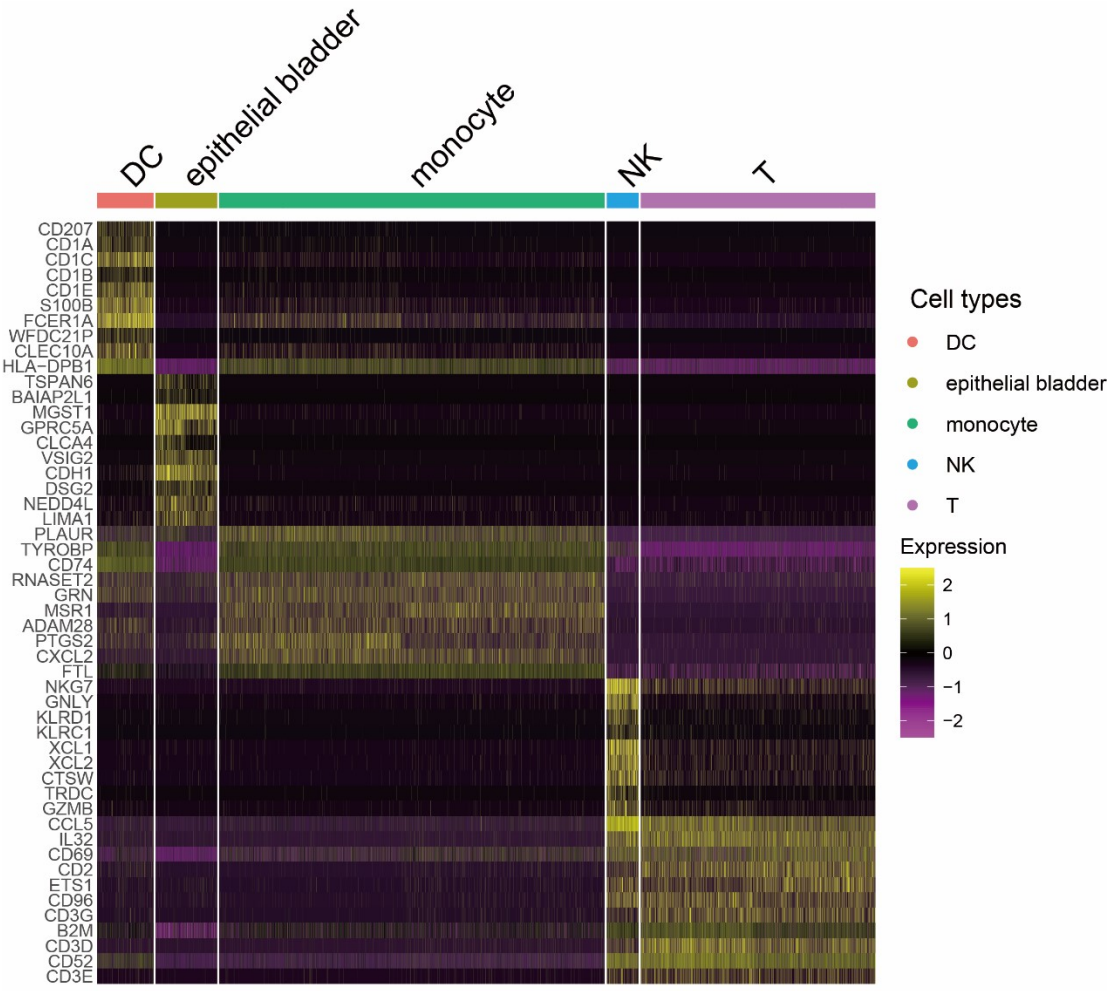


Fig. S5 Heatmap of marker genes used for cell type annotation in TILs sample multiplexing. Related to Fig. 5B.

Supplementary Tables

Table S1 Oligonucleotide sequences and modifications

entry	sequence	modification
NH_2-oligo A	TGTCAAGATGCTACCGTTCAGAGACCAATGGCGCATG GAAAAAAAAAAAAAAAAAAA*A*A	All oligos are modified with 5' end NH_2 and phosphorothioate modification at *.
NH_2-oligo B	TGTCAAGATGCTACCGTTCAGAGGTTATGCGACCGCGA AAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo C	TGTCAAGATGCTACCGTTCAGAGATACGCAGGGTCCG AAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo D	TGTCAAGATGCTACCGTTCAGAGTCGCCAGCCAAGTCT AAAAAAAAAAAAAAAAAAA*A*A	
□		□
NH_2-oligo0	TGTCAAGATGCTACCGTTCAGAGCGTGTTAGGGCCGAT AAAAAAAAAAAAAAAAAAA*A*A	All oligos are modified with 5' end NH_2 and phosphorothioate modification at *.
NH_2-oligo1	TGTCAAGATGCTACCGTTCAGAGGAGTGGTTGCGCCAT AAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo2	TGTCAAGATGCTACCGTTCAGAGAAGTTGCCAAGGGC CAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo3	TGTCAAGATGCTACCGTTCAGAGTAAGAGCCCGGCAA GAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo4	TGTCAAGATGCTACCGTTCAGAGTGACCTGCTTCACGC AAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo5	TGTCAAGATGCTACCGTTCAGAGGAGACCCGTGGAAT CAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo6	TGTCAAGATGCTACCGTTCAGAGGTTATGCGACCGCGA AAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo7	TGTCAAGATGCTACCGTTCAGAGATACGCAGGGTCCG AAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo8	TGTCAAGATGCTACCGTTCAGAGAGCGGCATTTGGGA CAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo9	TGTCAAGATGCTACCGTTCAGAGTCGCCAGCCAAGTCT AAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo10	TGTCAAGATGCTACCGTTCAGAGACCAATGGCGCATG GAAAAAAAAAAAAAAAAAAA*A*A	

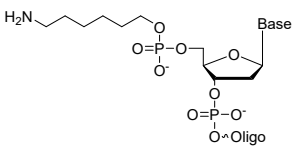
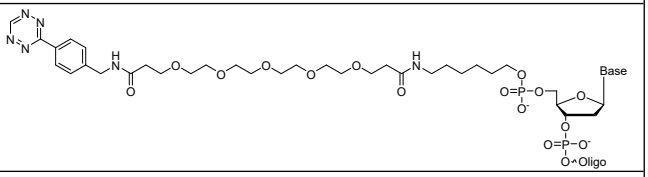
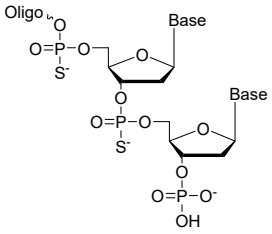
NH_2-oligo11	TGTCAAGATGCTACCGTTCAGAGTCCTCCTAGCAACCCAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo12	TGTCAAGATGCTACCGTTCAGAGGGCCGATACTTCAGCAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo14	TGTCAAGATGCTACCGTTCAGAGCCGTTCGACTTGGTGAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo15	TGTCAAGATGCTACCGTTCAGAGCGCAAGACACTCCACAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo16	TGTCAAGATGCTACCGTTCAGAGCTGCAACAAGGTCGCAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo17	TGTCAAGATGCTACCGTTCAGAGGTACCACTGCGATCGAAAAAAAAAAAAAAAAAAA*A*A	
NH_2-oligo18	TGTCAAGATGCTACCGTTCAGAGCCCTGTCTGGTGACAAAAAAAAAAAAAAAAAAA*A*A	
poly-dT-Cy5	TTTTTTTTTTTTTTTTTTTTTTTT	5' end Cy5 modification
anti-oligo 0-Cy3	CGTGTTAGGGCCGAT	5' end Cy5 modification
anti-oligo 1-Cy5	GAGTGGTTGCGCCAT	5' end Cy3 modification

Table S2 Primers for ClickTag library preparation

entry	sequence
SGR-beads-1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC
SGR-tag-1	TGGAGTTCAGACGTGTGCTCTTCCGATCTGTTGTCAAGATGCTACCGTTCAGAG
SGR-beads-2	AATGATACGGCGACCACCGAGATCT
SGR-tag-2	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCAGACGTGTGCTC

Table S3 Exact chemical structures of some oligos with modifications* in Table S1

abbreviation	full name	chemical structure

NH_2-oligo	5' end NH_2 modified oligo	
Tz-oligo	5' end Tz modified oligo	
phosphorothioate	phosphorothioate	

* Structures of oligos 5’ end functionalized with Cy5 or Cy3 are not provided by manufacturer.

References

1 M. Stoeckius, S. Zheng, B. Houck-Loomis, S. Hao, B. Z. Yeung, W. M. Mauck, P. Smibert and R. Satija, *Genome Biol.*, 2018, **19**, 224.

2 J. Gehring, J. Hwee Park, S. Chen, M. Thomson and L. Pachter, *Nat. Biotechnol.*, 2020, **38**, 35–38.