

Integration of Imaging and RNA-Seq for High-Content Pooled Perturbation Analysis of Single Cells

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ABSTRACT

Single-cell RNA sequencing (scRNA-seq) has revolutionised perturbation analysis, providing an understanding of gene functions and drug mechanisms. Multi-omics approaches are expanding the scope toward encompassing entire cellular landscapes, extending beyond just transcriptomics. However, it remains challenging for current RNA-seq and multi-omics methods to seamlessly capture the cytological phenotype.

We introduce a simple integrated analysis method that links cytologic phenotypic behaviour observed in microscopic imaging with single-cell RNA sequencing (scRNA-seq) by utilising a combination of optical indices from cells and hydrogel beads. With our method, the combinations, referred to as joint colour codes, enable the link *via* matching the optical combinations measured by conventional epi-fluorescence microscopy with the concatenated DNA molecular barcodes created by cell-hydrogel bead pairs and sequenced by next-generation sequencing.

We validated our approach by demonstrating an accurate link between the cell image and scRNA-seq with mixed species experiments, longitudinal cell tagging by electroporation and lipofection, and gene expression analysis. Furthermore, we extended our approach to multiplexed chemical transcriptomics, which enabled us to identify distinct phenotypic behaviours in HeLa cells treated with various concentrations of paclitaxel and determine the corresponding gene regulation associated with the formation of a multipolar spindle.

To enhance the scalability of our multiplexed system, we employed a signal unmixing algorithm to mitigate fluorescence spillover, optimised library preparation protocols, and developed a precise molecular system for bead colour coding with machine learning-based decoding. This approach expands the multiplexing capability of imaging readouts, detected genes, pooled samples, and single-cell analysis, facilitating deeper exploration of the heterogeneity within sample pools.

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1 Introduction

1.1 Background

Advances in next-generation sequencing (NGS) and microfluidics technology have revolutionised single-cell analysis, making it possible to analyse RNA expression across the entire transcriptome of individual cells (Klein, A. M. *et al.*, *Cell*, 2015; Macosko, E. Z. *et al.*, *Cell*, 2015). Single-cell RNA sequencing (scRNA-seq) allows researchers to uncover cellular heterogeneity within populations, which reflects difference of cell type, cell cycle, and cell functions.

Recently, the development of scRNA-seq-based perturbation analysis methods has expanded substantially (Cheng, J. *et al.*, *Adv. Sci.*, 2021; Cheng, J. *et al.*, *Adv. Sci.*, 2023). Perturbation analysis serves as a crucial technique for causal inference and examines cellular responses to genetic and chemical perturbations. This understanding enables the elucidation of gene functions and the mechanisms of action (MOAs) of drugs.

Ongoing advances in scRNA-seq perturbation analysis contribute to a comprehensive understanding of perturbation responses. (Ji, Y. *et al.*, *Cell Syst.*, 2021; Gavrilidis, G. I. *et al.*, *Comput. Struct. Biotechnol. J.*, 2024; Rood, J. E. *et al.*, *Cell*, 2024). However, conducting comprehensive scRNA-seq-based perturbation studies remains resource-intensive, requiring numerous trials and data, thus necessitating technical improvements for efficient data accumulation (Cleary, B. *et al.*, *arXiv*, 2020). Pooling techniques improve data processing efficiency and reduce costs by enabling the simultaneous analysis of multiple samples or conditions, while the technical challenges continue to drive ongoing technological innovation.

In addition, recent perturbation analyses are integrating scRNA-seq with other omics data types, including proteomics, epigenomics, and image-based analyses. This multi-omics approach enables the acquisition of multidimensional information such as cellular functions, molecular cascades, and cytological phenotypes that are not accessible with scRNA-seq alone, thereby unlocking new potentials in perturbation analysis.

Given this context, this chapter explores the further development of perturbation analysis methods using scRNA-seq, focusing on pooling technologies and the possibilities offered by integrative multi-dimensional analyses, especially those combining image-based profiling with RNA-seq. These advancements are expected to contribute to a more precise biological understanding within this field.

1.2 Pooled perturbation analysis based on single-cell transcriptomics

In perturbation analysis using scRNA-seq, cells are subjected to specific perturbations and labelled with perturbation-identifying base sequences. Single-cell analysis platforms isolate cells with reverse transcription primers bearing unique molecular barcodes, referred to as cell barcodes, and endogenous mRNA and perturbation-identifying barcodes are linked through a common cell barcode. Given the high information density of base barcodes readable by next-generation sequencing (NGS), this method enables the identification of highly diverse perturbation sequences, allowing a large number of perturbations to be assayed simultaneously.

Current single-cell perturbation analyses are broadly classified into two categories: genetic perturbation analysis and chemical perturbation analysis. Genetic perturbation analysis involves modifying multiple genes within cells, such as by knock-out or knock-down techniques, pooling the modified cells, and examining changes in gene expression via NGS. This approach provides insights into gene functions and regulatory mechanisms by assessing RNA expression changes resulting from these genetic modifications. Chemical perturbation analysis, on the other hand, involves treating cells with various chemical compounds, pooling these treated cells, and using NGS to observe gene expression changes. This approach facilitates the study of the MOAs of different chemical compounds by analysing their impact on gene expression patterns. In the following sections, we discuss the current state and challenges of genetic perturbation analysis and chemical perturbation analysis using scRNA-seq.

1.2.1 Genetic pooled perturbation analysis based on scRNA-seq

Genetic pooled perturbation analysis leverages CRISPR-based technologies. These CRISPR-based gene editing technologies operate by expressing RNA-guided DNA endonuclease enzymes, such as Cas9, along with single-guide RNA (sgRNA) in cells to selectively edit DNA sequences. In scRNA-seq-based genetic perturbation analysis, lentiviruses carrying vectors that encode sgRNA are randomly introduced to cells, generating pools of genetically deficient cells.

To identify gene perturbations using NGS, these methods incorporate mechanisms within vector designs that allow for sgRNA sequence identification alongside mRNA analysis. Early techniques involved encoding perturbation-identifying barcodes within the vectors (Adamson, B. *et al.*, *Cell*, 2016; Dixit, A. *et al.*, *Cell*, 2016; Jaitin, D. A. *et al.*, *Cell*, 2016). These barcodes, carrying poly-A tails, were co-expressed with the sgRNA, enabling the simultaneous readout of perturbations and mRNA expression in each cell. The first trials of genetic perturbation analysis target three genes interference or 15 genes knock out per run, In subsequent advancements, a poly-A tail (Datlinger, P. *et al.*, *Nat. Methods*, 2017) or a designated capture sequence (Replogle, J. M. *et al.*, *Nat. Biotechnol.*, 2020) was directly connected to the sgRNA, enabling targeting 32 genes knock out and 104 genes knockout respectively by direct sgRNA sequence readout. These innovations have reduced readout errors (Adamson, B. *et al.*, *bioRxiv*, 2018; Hill, A. J. *et al.*, *Nat. Methods*, 2018) and allowed for stable expression variations with simpler designs (Replogle, J. M. *et al.*, *Nat. Biotechnol.*, 2020). As a result, the scalability of perturbation analysis has steadily improved (Cheng, J. *et al.*, *Adv. Sci.*, 2023), making genome-scale perturbation (targeting 9,866 genes of ~ 2 million cells) analysis increasingly feasible (Replogle, J. M. *et al.*, *Cell*, 2022). By analysing the resulting data, it is possible to deconvolute and identify transcriptomic changes induced by each genetic perturbation, further distinguishing co-regulated and co-functional genes based on the similarities in their expression changes (Rood, J. E. *et al.*, *Cell*, 2024).

The scalability of pooled genetic perturbation analysis, however, is limited by the high cost of scRNA-seq (Replogle, J. M. *et al.*, *Cell*, 2022). To address this challenge, one strategy involves selectively enriching the sequencing library to reduce sequencing

costs (Schraivogel, D. *et al.*, *Nat. Methods*, 2020). However, additional refinements are still necessary to further enhance cost efficiency and scalability.

1.2.2 Chemical pooled perturbation analysis based on scRNA-seq

In scRNA-seq-based chemical perturbation analysis, cells treated with different compounds in separate arrays are pooled and subsequently analysed using NGS. Chemical perturbation analysis employs externally applied DNA molecular barcodes to label the cells in each array to identify specific drug perturbations.

These external tagging label cells with DNA barcodes using oligo-conjugated antibodies (Stoeckius, M. *et al.*, *Genome Biol.*, 2018), oligo-conjugated lipids/cholesterols (McGinnis, C. S. *et al.*, *Nat. Methods*, 2019), transient transfection (Shin, D. *et al.*, *Sci. Adv.*, 2019), and fixation (Srivatsan, S. R. *et al.*, *Science*, 2020). These barcoding methods need to be selected based on the specific experimental design (Mylka, V. *et al.*, *Genome Biol.*, 2022). Oligo-conjugated antibodies demonstrate high specificity in samples with high antigen expression on the cell surface. Oligo-conjugated lipids/cholesterols, which are not dependent on antigen expression, are more suitable for universal applications.

These methods discern MOAs of compounds based on the transcriptomic profiles corresponding to each perturbation (McFarland, J. M. *et al.*, *Nat. Commun.*, 2020; Srivatsan, S. R. *et al.*, *Science*, 2020). sciPlex (Srivatsan, S. R. *et al.*, *Science*, 2020) is a notable method that has investigated the response to 188 chemical compounds across three cancer cell lines, with four doses and two replicates in a single experiment. This method characterise gene expression changes across varying concentrations of histone deacetylase (HDAC) inhibitors, revealing that their MOAs in cell growth inhibition via acetate sequestration in chromatin, disrupts acetyl-CoA metabolism.

The scalability of pooled chemical perturbation analysis is also constraint by the cost of scRNA-seq. Additionally, current chemical pooled perturbation analyses require processing samples across multiple arrays, making the process labour-intensive and

further limiting scalability. Continued improvements are needed to overcome these challenges.

1.3 Multiomics for pooled perturbation analysis

Perturbation analysis introduced in the previous section measures transcriptomic responses within cells to various perturbations using scRNA-seq. Integrating RNA-seq with other layers of omics, such as the proteome or epigenome, enables the study of relationships between different molecular layers. This integration clarifies cellular heterogeneity that is often obscured by transcriptome profiling alone, such as differences in cell function or transcriptional activity (Hao, Y. *et al.*, *Cell*, 2021). To achieve a more comprehensive understanding of cellular perturbation responses, this section summarizes conventional multiomics approaches and their potential applications in perturbation analysis.

1.3.1 Linking transcriptome with proteome

Studies have demonstrated discrepancies between RNA and protein expression (Wang, D. *et al.*, *Mol. Syst. Biol.*, 2019; Nusinow, D. P. *et al.*, *Cell*, 2020; Reimegard, J. *et al.*, *Commun. Biol.*, 2021). Integrating scRNA-seq with protein profiling can address the origins of these discrepancies.

NGS-based single-cell proteomics utilises antibody-conjugated DNA molecular barcodes to quantify the expression. Depending on their target, CITE-seq (Stoeckius, M. *et al.*, *Nat. Methods*, 2017) and REAP-seq (Peterson, V. M. *et al.*, *Nat. Biotechnol.*, 2017) measure the expression of cell surface proteins, inCITE-seq (Chung, H. *et al.*, *Nat. Methods*, 2021) targets nuclear proteins. These methods able to link protein expression with RNA expression.

Although these antibody-based protein profiling methods enable high-throughput single-cell protein analysis, their multiplexing capacity is limited by antibody cross-reactivity and they cannot measure proteins that do not have specific antibodies available

(Bennett, H. M. *et al.*, *Nat. Methods*, 2023). Consequently, further technological advancements are needed to achieve more comprehensive and unbiased protein profiling at the single-cell level.

These techniques, for example, enable the clarification of the functional differences among immune cell types and link transcriptome-wide profiling (Hao, Y. *et al.*, *Cell*, 2021), emphasizing the importance of integrated analysis of protein and RNA expression.

1.3.2 Linking transcriptome with chromatin accessibility

Chromatin accessibility indicates the extent to which other molecules and proteins can physically access DNA, and it is a crucial characteristic involved in regulating RNA transcription (Klemm, S. L. *et al.*, *Nat. Rev. Genet.*, 2019). ATAC-seq (Buenrostro, J. D. *et al.*, *Nat. Methods*, 2013) and scATAC-seq (Buenrostro, J. D. *et al.*, *Nature*, 2015; Cusanovich, D. A. *et al.*, *Science*, 2015) is a technique that uses Tn5 transposase to cutout and assess accessible chromatin regions across the genome. Advances in this field have introduced methods that combine ATAC-seq with RNA-seq through droplet sequencing (Chen, S. *et al.*, *Nat. Biotechnol.*, 2019) or combinatorial indexing (Cao, J. *et al.*, *Science*, 2018), allowing for the simultaneous profiling of chromatin accessibility and gene expression at a single-cell level. These approaches enable the joint profiling of chromatin and transcriptome state at single cell level. Additionally, these methods enable the assessment of RNA expression of transcription factors and the profiling of open chromatin regions, thereby improving the accuracy of Gene Regulatory Network (GRN) descriptions by mapping transcription factor binding sites within accessible chromatin regions (Badia, I. M. P. *et al.*, *Nat. Rev. Genet.*, 2023).

1.3.3 Profiling transcriptome across the expression process

Recent advances in RNA research have introduced innovative methods that allow scientists to examine RNA processing in unprecedented detail, providing deep insights

into the underlying regulatory mechanisms. One such development includes techniques capable of distinguishing newly synthesised RNA from pre-existing RNA (Erhard, F. *et al.*, *Nat. Rev. Methods Primers*, 2022), enabling selective identification of new RNA molecules to reveal dynamic processes in transcription. Another method, SINC-seq, profiles RNA within both nuclear and cytoplasmic compartments in the same cell, before and after nuclear export (Abdelmoez, M. N. *et al.*, *Genome Biol.*, 2018; Oguchi, Y. *et al.*, *Sci. Adv.*, 2021), using microfluidic platforms. Additionally, identification of pre- and post-splicing RNA offer insights into the splicing kinetic of individual cells, referred as to RNA velocity, inferring the future state (La Manno, G. *et al.*, *Nature*, 2018; Bergen, V. *et al.*, *Nat. Biotechnol.*, 2020). Together, these cutting-edge methods provide a more comprehensive view of RNA processing and regulation across different cellular stages, advancing our understanding of gene expression mechanisms.

1.3.4 Multiomics for pooled perturbation analysis

Researchers are employing multiomics approaches to advance genetic perturbation analysis. ECCITE-seq (Mimitou, E. P. *et al.*, *Nat. Methods*, 2019) simultaneously analyses single-guide RNAs (sgRNAs), antibody-conjugated primers, and mRNA, combining surface protein profiling to reveal the molecular mechanisms driving programmed cell death ligand 1 (PD-L1) expression in T cells (Papalexi, E. *et al.*, *Nat. Genet.*, 2021). This technology also uses DNA molecular barcodes for sample multiplexing, which enhances analytical efficiency by allowing the simultaneous processing of multiple experimental samples. Perturb-CITE-seq (Frangieh, C. J. *et al.*, *Nat. Genet.*, 2021) has expanded the range of sgRNAs used for knockout analysis to 744, enabling a thorough study of 248 genes related to immune checkpoint resistance (ICR). Researchers have identified that CD58 loss enables cancer cells to escape immune surveillance by T cells and NK cells. This immune evasion occurs independently of the well-known IFN- γ -JAK/STAT pathway, providing critical insights into mechanisms underlying resistance to immune therapy.

1.4 Opportunity of pooled perturbation analysis linked with image-based profiling

In modern screening analyses, image-based methods have become essential tools. With multiplexed fluorescent imaging, high-content imaging techniques have emerged, enabling multiparametric analysis to study multiple cellular responses to various perturbations (Lang, P. *et al.*, *Nat. Rev. Drug Discov.*, 2006). Image-based data provide distinct information that reflects aspects of cellular behaviour such as protein localization, cellular morphology, enzymatic activity, and metabolic states, which is not captured by molecular profiling methods such as RNA-seq (Haghighi, M. *et al.*, *Nat. Methods*, 2022; Way, G. P. *et al.*, *Cell Syst.*, 2022; Moshkov, N. *et al.*, *Nat. Commun.*, 2023). Multiplexed imaging analysis is especially valuable in pooled perturbation analyses. Optical pooled screens (Feldman, D. *et al.*, *Cell*, 2019) serve as a clear example of this utility. This method employs high-content imaging and in situ sequencing to identify sgRNAs in CRISPR-edited cells, linking genetic perturbations to cytologic phenotypes, allowing researchers to map the gene function on cellular phenotypes (Funk, L. *et al.*, *Cell*, 2022; Carlson, R. J. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2023).

1.4.1 Integrated single-cell analysis of image-based profiling and gene expression

In the field of scRNA-seq technology, advancements have extended beyond multiomics approaches to include methods that integrate image-based profiling with scRNA-seq for comprehensive gene expression analysis. For example, SCOPE-seq (Yuan, J. *et al.*, *Genome Biol.*, 2018; Liu, Z. *et al.*, *Sci. Rep.*, 2020) uses a microwell array to trap cells along with DNA-barcoded poly dT beads, and then uses fluorescence microscopy to capture cell images and read out the unique DNA barcode sequences using FISH. The dT sequences capture cellular mRNA, and the DNA barcode sequences are read through NGS. By referencing the barcode sequences obtained from the images, this method enables the integration of image data and RNA expression information at the single-cell level.

Similarly, μ CB-seq (Chen, T. N. *et al.*, *Lab Chip*, 2020) and μ DamID (Altemose, N. *et al.*, *Cell Syst.*, 2020) utilise microfluidic devices to trap cells, capture images, and construct sequencing libraries with cell-specific barcode sequences, thereby linking

image information with RNA expression data. Additionally, another method cultivates cells within microfluidic channels and creates sequencing libraries in situ, allowing for the integration of live imaging data with RNA expression profiles (Lane, K. *et al.*, *Cell Syst.*, 2017).

There are also approaches that link image information and RNA expression data on a single-cell basis using robotic systems (Jin, J. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2023). Despite their significant potential, these methods often come with challenges such as the requirement for specialized devices, complex experimental workflows, and intricate microfluidic setups, presenting substantial technical obstacles for implementation for single-cell perturbation analysis.

1.5 Objective and organisation of the thesis

In this thesis, we present the development of opto-combinatorial indexing, a novel and simple method that integrates image-based analysis and gene expression profiling by NGS for perturbation analysis. Additionally, we demonstrated the application of this technology to chemical perturbation analysis and outlined strategies for achieving higher-content imaging and more scalable single-cell analysis.

The major contributions of this dissertation are as follows:

- Design and validation of a simple system for the single-cell integration analysis
- Demonstration of utility for the chemical perturbation analysis
- Development of strategies to scale the system, enabling multiplexed imaging for cytological phenotyping, expanded gene detection, and increased cell throughput, surpassing system-imposed limitations

The thesis comprises four main chapters. In chapter two, we describe the methodology in detail and validate it through species-mixing experiments. We further investigate the molecular mechanisms underlying paclitaxel-induced multispindle formation using this method. In chapter three, we focus on system scaling through the use of an unsupervised unmixing algorithm, enhancements in library preparation, and

improvements to the bead decoding system. In chapter four, we conclude our findings throughout the thesis and present recommendations for future research work.

2 Opto-combinatorial indexing for the integration of transcriptome and image analysis

2.1 Introduction

The latest single-cell RNA-seq (scRNA-seq) allows assaying thousands of cells per experiment by combining compartmentalisation of cells with microfluidics and tagging cDNA with cell barcodes to profile gene expression of single cells (Klein, A. M. *et al.*, *Cell*, 2015; Macosko, E. Z. *et al.*, *Cell*, 2015; Gierahn, T. M. *et al.*, *Nat. Methods*, 2017; Hughes, T. K. *et al.*, *Immunity*, 2020). The tagging approach has been extended for profiling other omics layers including, surface proteins (Peterson, V. M. *et al.*, *Nat. Biotechnol.*, 2017; Stoeckius, M. *et al.*, *Nat. Methods*, 2017), nuclear proteins (Chung, H. *et al.*, *Nat. Methods*, 2021), and chromatin accessibility (Chen, S. *et al.*, *Nat. Biotechnol.*, 2019). However, most of the omics approaches are still incapable of linking the measured molecular profile to cellular phenotypes, such as morphology and molecular localisation (Abdelmoez, M. N. *et al.*, *Genome Biol.*, 2018; Oguchi, Y. *et al.*, *Sci. Adv.*, 2021).

Single cell optical phenotyping and expression (SCOPE-seq and SCOPE-seq2) (Yuan, J. *et al.*, *Genome Biol.*, 2018; Liu, Z. *et al.*, *Sci. Rep.*, 2020) is a method for linking scRNA-seq with live cell imaging. SCOPE-seq co-isolates a single cell and bead bearing barcoded DNA in a microwell, images cellular morphology, and captures mRNA from the single cell on the bead for pooled scRNA-seq. SCOPE-seq links the image of the single cell to the scRNA-seq by optically decoding the barcode of each bead using cyclic hybridisation of fluorescently labelled oligonucleotide probes, followed by fluorescence microscopy.

Herein, we propose a novel and simple approach for optical indexing that leverages the combination of cells and hydrogel beads dual-labelled with optical indices and DNA molecular barcodes (we refer to this dual label as “colour code.”) for linking cellular images with scRNA-seq. To link the combinations of optical indices decoded from the imaging to the cell barcodes in scRNA-seq, our approach creates concatenated

fragments of barcoded DNA oligos (DNA tags) derived from the cells and barcoded dT primers derived from the hydrogel beads. The concatenated fragments are sequenced with the scRNA-seq library and provide a look-up table to link the combinations of optical indices and cell barcodes. Our approach is free of automated microfluidic controls and offers fewer on-chip steps, which are advantageous in easily implementing the approach in a standard laboratory setup. We demonstrate our approach with multiplexing up to 256 combinations of colour codes (joint colour codes), using 16 pools of colour-coded cells and 16 pools of colour-coded hydrogel beads, and decoding them with four colours of fluorescence using standard epi-fluorescence microscopy.

2.2 Methods

2.2.1 The concept of opto-combinatorial indexing

Our strategy to link a single-cell image and gene expression leverages joint colour code created by co-isolated single cell and hydrogel bead (Fig. 2.1A). The cells and hydrogel beads are respectively labelled by 16 different colours, which were bright or dim combinations of four fluorescence dyes ($2^4 = 16$), and which also corresponded to 16 different sequences of DNA barcodes, and corresponding DNA molecular barcodes (Fig. 2.1B) that are read out by epi-fluorescence microscopy and next-generation sequencing. The joint colour codes increase the possible unique codes by the combination and enable linking single-cell images and gene expression profiles in the two data pools (Fig. 2.1C).

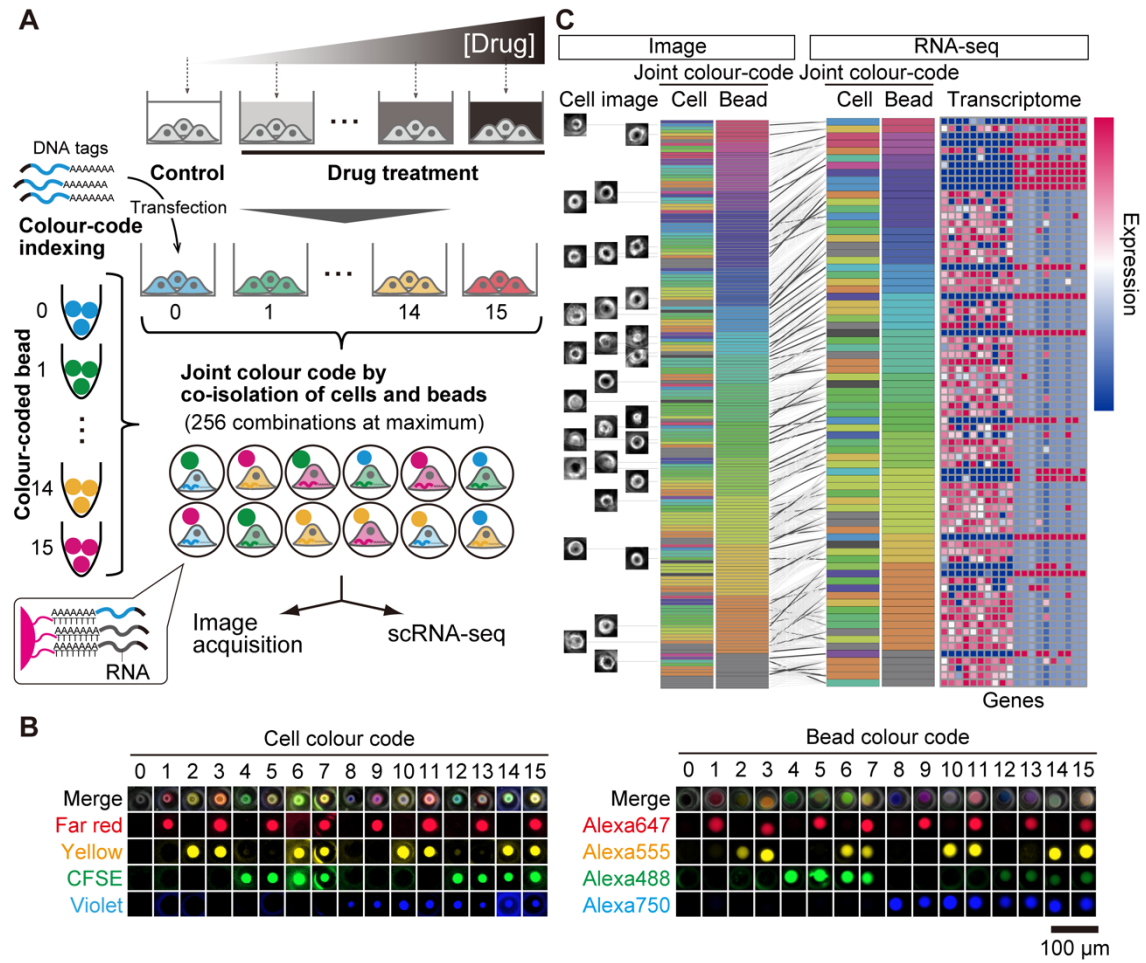


Figure 2.1 Linking a cellular image to single-cell RNA-seq with the combination of colour codes.

A. Cells were colour-coded with matching DNA tags and a set of fluorophores as per the 16 different conditions. Hydrogel beads were also colour-coded with matching barcode sequences and fluorophores. Colour-coded cells and hydrogel beads were co-isolated in microwells. We imaged fluorescence combinations of a cell and a hydrogel bead, followed by the generation of the concatenated DNA fragments from the DNA tag of the cell and the barcode sequence of the hydrogel bead, along with reverse transcription of cDNA from mRNA. Cell images and transcriptome data were linked by matching the fluorescence combinations from imaging with the library of the joint colour code generated from the concatenated DNA fragments. B. Representative fluorescence images of colour-coded cells and beads in microwells. Scale bar = 100 μm. C. The single cells

with unique joint colour code combinations resulted in linked datasets of cellular morphology and gene expression.

2.2.2 Synthesis of colour-coded hydrogel beads

To allow optical readout of the bead colour codes, we designed the branched DNA that hybridises with the bead colour code and converts the nucleotide sequence to a bright or dim combination of four fluorophores by hybridising four readout oligos with or without fluorophores, creating $2^4=16$ different colour combinations (Fig 2.2, Table S1). This approach minimizes the number of readouts oligos labelled with fluorophores and significantly reduces the cost of synthesizing them. We synthesised the polyacrylamide hydrogel beads with poly(dT) sequences through two rounds of split-pool ligation (Zilionis, R. *et al.*, *Nat. Protoc.*, 2017). Briefly, we generated droplets of acrylamide premix with 50 μ M acrydited primer (5'-Acryd/AAGCAGTGGTATCAACGCAGAGTACGACGCTCTT-3') using a simple coflow microfluidic device. The final bead size was $\sim 40 \mu$ m. We then ligated the first barcode fragments containing the bead colour code and the first part of the cell barcode (Table S1, Stem_CC00_ID01–Stem_CC15_ID25). In the second round of ligation, we added fragments with the second part of the cell barcode, UMI and poly(T) (NNNNNNNNTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN) (Table S1, ID00_dT–ID32_dT). The combination of the first and second parts of the cell barcode created 6400 unique barcodes.

To stain beads with colour codes, we pooled the beads with cell barcodes in a single tube and combined 3×10^4 beads, a mixture of 6 μ M branch oligos (Table S1, Branch_00_NNNN–Branch_15_BGPR), a mixture of 12 μ M readout oligos (with Alexa 488, Alexa 555, Alexa 647, and Alexa 750, Table S1, Readout_Alexa647–Readout_Alexa750), 12 μ M oligo without fluorophore (Table S1, Readout_R0–Readout_B0 to fill the sequence in branch oligo for dim beads) and 0.1 mg/mL salmon sperm DNA in hybridisation buffer (5 mM Tris-HCl (pH 8.0), 1 M KCl, 5 mM EDTA, 0.05% (vol/vol) Tween-20). We incubated the mixture at 94°C for 5 min and cooled it by

5°C every 5 min to 25°C and then kept it at 4°C. Excess probes were washed three times with an ice-cold hybridisation buffer.

We reasoned the hybridisation-based staining of hydrogel beads has an insignificant effect on the synthesis of cDNA and the amplification with PCR, because the branch oligo hybridises downstream of the cDNA extension during reverse transcription, and the concentration of the branch oligo in PCR is estimated at 3.52 nM per colour code while that of the PCR primer is at 240 nM. Further, the melting temperature of the branch oligo is lower at 66.4°C than that of the PCR primer at 77.5°C (under conditions of 50 mM Na⁺ and 3 mM Mg²⁺ as an example), while the annealing temperature for PCR is at 65°C.

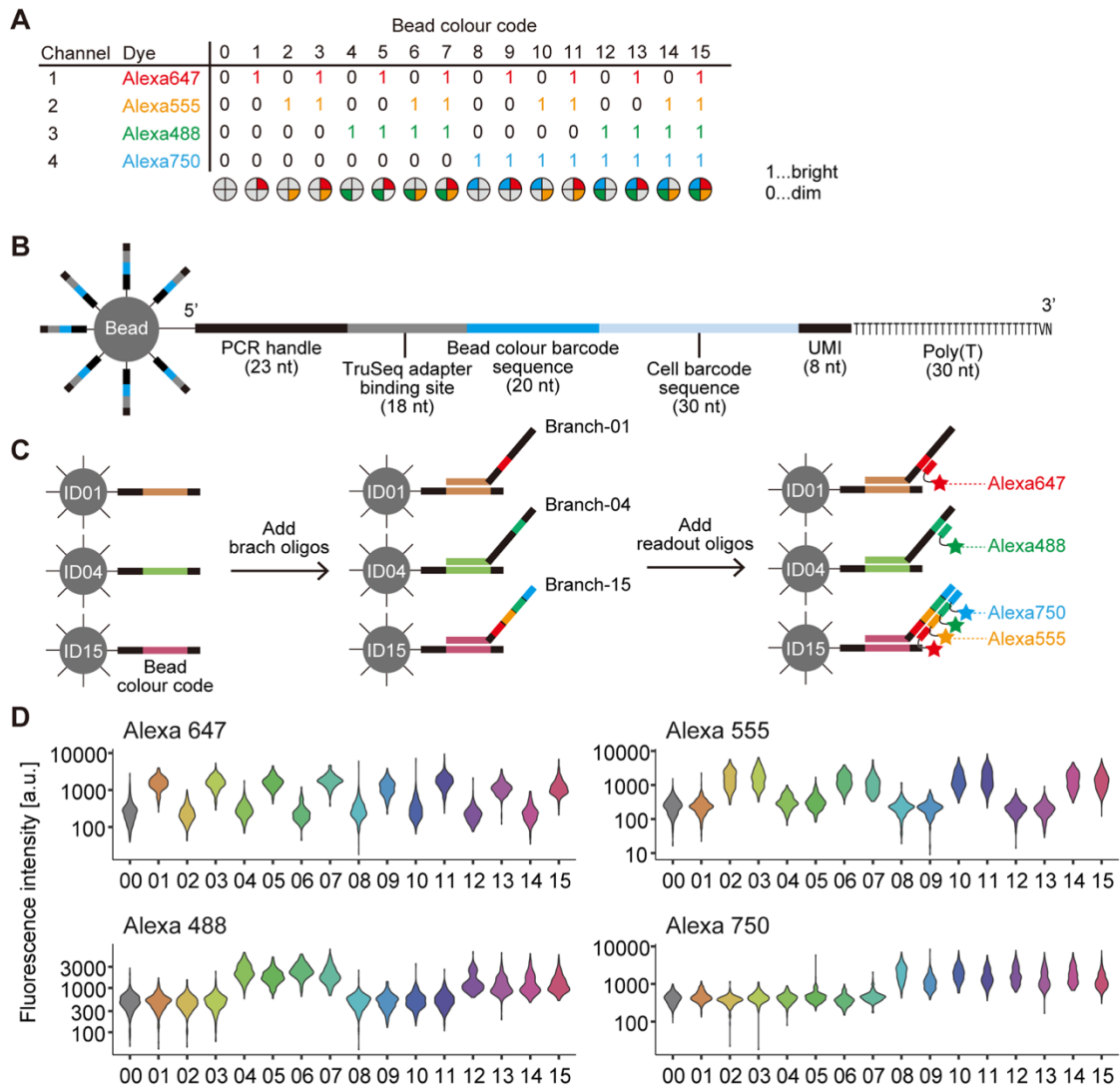


Figure 2.2 DNA-barcoded hydrogel beads with 16 colour codes.

A. Combination of four fluorophores for 16 colour codes. 1 and 0 respectively indicate bright and dim. B. Schematic images of barcoded primers attached to hydrogel beads. C. Example schematic images of colour code staining. The designated fluorophores are attached to beads via branch DNAs hybridising to the sequence of bead colour code. D. Mean fluorescence intensities of colour-coded beads.

2.2.3 Preparation of colour-coded cells

We labelled cells with a combination of four different dyes (CellTrace Violet, CFSE, Yellow, and Far Red from Thermo Fisher Scientific) (Fig.2.3) and corresponding oligonucleotide DNA molecules, referred to as DNA tags (Table S2), via transfection. These DNA tags contained an 8-nucleotide barcode, a poly A sequence, and a PCR handle. The protocols to label cells with colour code differed slightly depending on specific experimental requirements, as follows.

We cultured HeLa (RCB0007, RIKEN BRC) and NIH/3T3 cells (RCB2767, RIKEN BRC) cells in Dulbecco's Modified Eagle Medium (DMEM, 08456-65, Nacalai Tesque) containing 10% fetal bovine serum (FBS, 26140-079, gibco) and 1% penicillin-streptomycin (P/S, P4333-100ML, Sigma-Aldrich).

For the species-mixing experiment, we respectively seeded the HeLa cells and NIT/3T3 cells at a concentration of 2.0×10^5 cells/mL each separately in a 100 mm dish and cultured them for 1 day. After trypsinisation, we aliquoted cells equally into eight sub-pools per cell type in 16 tubes and individually stained with the 16 different combinations of four types of CellTrace (5 μ M Violet, 5 μ M CFSE, 5 μ M Yellow, and 1 μ M Far Red, Invitrogen™) at the concentration of 1.0×10^6 cells/mL. We then individually suspended the stained cells in Gene Pulser® Electroporation Buffer (Bio-Rad) with 100 nM of DNA tag (Table S2) corresponding with the respective fluorescence colour. Immediately after the electroporation by Gene Pulser Mxcell™ Electroporation System (Bio-Rad, voltage: 250 V, capacitance: 2000 μ F, resistance: ∞ Ohm, duration: 20 ms for HeLa cell, and 400 V, 950 μ F, ∞ Ohm, 20 ms for NIT/3T3 cell), we added five-fold volume of culture medium and incubated them for 1 h at 37°C in 5% CO₂. We washed the cells three times with 1 $\times$ phosphate-buffered saline (PBS, 14249-24, Nacalai tesque), and combined the 16 sub-pools in the loading buffer (1% polyvinylpyrrolidone in 1 $\times$ PBS).

For the experiment of tag retention assay, we seeded the HeLa cells at a concentration of 5.0×10^4 cells/mL for one day, followed by lipofection with 20 nM DNA tags and cultured for 4 h. In addition, we performed electroporation on 1.0×10^6 cells/mL

HeLa cells in Gene Pulser® Electroporation Buffer with 100 nM DNA tag under the same conditions described above. We then cultured the cells in a 24-well plate for varying durations up to 48 h and stained them with six different combinations of three CellTrace (5 μ M CFSE, 5 μ M Yellow, and 1 μ M Far Red). After three PBS washes, we pooled all of the cells at the same concentration and resuspended them in the loading buffer.

For the experiments of paclitaxel treatments, we seeded the HeLa cells at the concentration of 5.0×10^4 cells/mL in eight wells of a 24-well plate and cultured them for one day. We transfected cells individually with different types of DNA tags using Lipofectamine® 3000 reagents (Invitrogen™) according to the manufacturer's protocol (incubation for 4 h at 20 nM DNA tag concentration). After the lipofection, we washed the cells three times with a culture medium. We stained the cells individually in each well with eight combinations of three CellTrace (5 μ M CFSE, 5 μ M Yellow, and 1 μ M Far Red). We cultured cells in a culture medium containing paclitaxel (163-28163, FUJIFILM) at different concentrations for one day. We washed the cells three times with $1 \times$ PBS. After trypsinisation, we pooled the eight sub-pools of the cells at the equal cell concentrations. Subsequently, the cells were stained with 10 μ g/mL Hoechst 33342 and resuspended in the loading buffer.

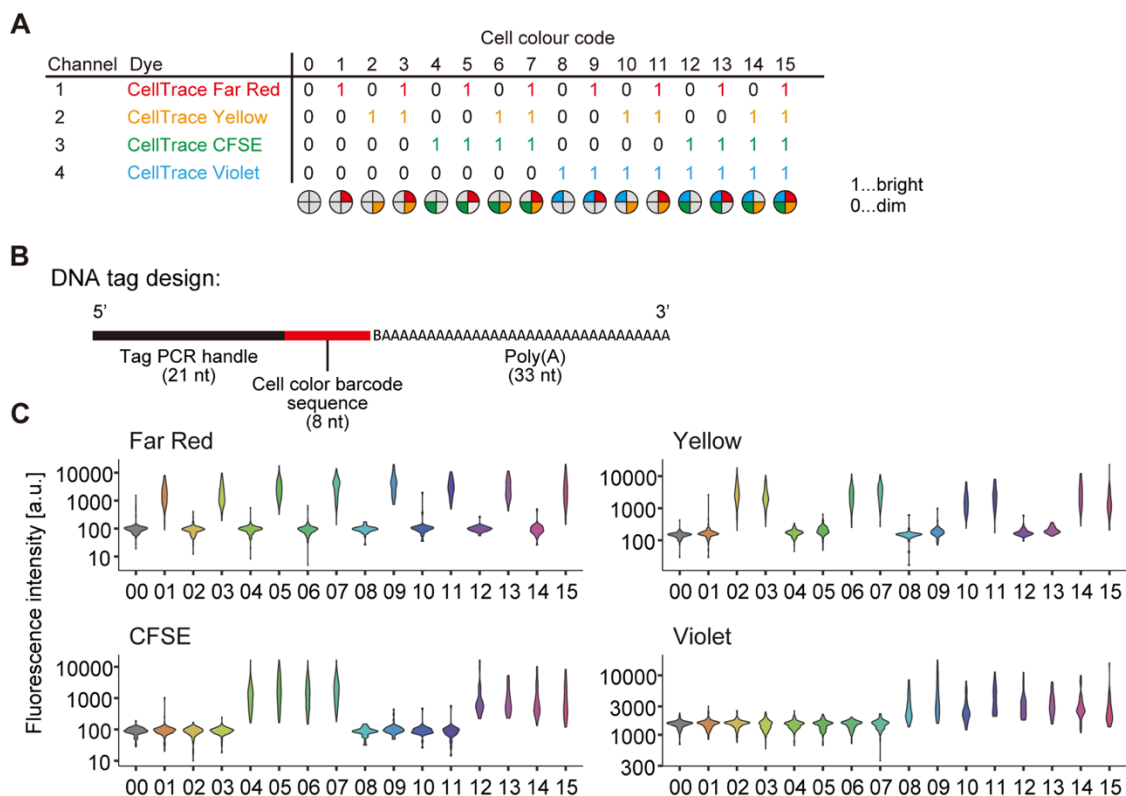


Figure 2.3 Design of 16 cell colour codes.

A. Combination of four fluorophores for 16 colour codes. 1 and 0 respectively indicate bright and dim. B. Design of DNA tag transfected to the cells. C. Mean fluorescence intensities of colour-coded cells.

2.2.4 Chip fabrication

In order to lyse the cells and capture mRNA and DNA tags using co-encapsulated beads within the microwells, we adopted a method to enclose the microwells by sealing them with a track-etched membrane (Gierahn, T. M. *et al.*, *Nat. Methods*, 2017; Hughes, T. K. *et al.*, *Immunity*, 2020). The fabrication of a chip specifically designed for this purpose is based on a protocol reported in previous work (Gierahn, T. M. *et al.*, *Nat. Methods*, 2017; Hughes, T. K. *et al.*, *Immunity*, 2020). We fabricated microwell arrays from polydimethylsiloxane (PDMS, SILPOT 184, Dow Corning) by soft lithography using a SU-8 mould. To hydrophilize the microwell array and to perform efficient sealing

in cell lysis and hybridisation step, we functionalized the array with the same protocol as the previously reported (Hughes, T. K. *et al.*, *Immunity*, 2020).

2.2.5 On-chip experiment procedure

We placed a PDMS slab with the microwell array superstructure onto a glass-based dish (3961-035, IWAKI), dispensed PBS over the microwells, and kept it under vacuum for 15 min to remove bubbles in the microwells. We then dropped pooled cells suspended in the loading buffer onto the microarray and incubated them for 5 min at room temperature to allow the cells to settle. After washing the microwell array with PBS, we added 2 mL of DMEM without phenol red (08490-05, Nacalai tesque) and acquired the scanned images of the microwell array containing the cells. In every experiment, we adjusted the exposure times to effectively use the full dynamic range of the camera and used the same setting for the entire chip. This approach ensures robust performance even when brightness variations occur due to batch effects, as the settings can be flexibly adjusted to maintain consistency. We dropped 20 μ L of the colour-coded bead suspension at the concentration of 1.0×10^6 beads/mL onto the microwell array and incubated them for 10 min to capture the beads in microwells, followed by tapping and resting for 1 min at 37°C, which was repeated five times. To seal the microwells with a track-etched membrane with nanopores of 10 nm in diameter (Sterlitech), which was pre-treated with atmospheric plasma (BD-20, Electro-Technic Products) for 60 s and then hydrated in the PBS, we pressed the membrane and the PDMS slab by placing a glass slide (8 mm square per side, S7214, MATSUNAMI) and a 100-g weight at 37°C for 30 min. We removed the glass side by adding 3 mL of PBS. We lysed the cells by dispensing 3 mL of a cell lysis buffer (5 M Guanidine Thiocyanate, 1 mM EDTA, 0.50% Sarkosyl, 1.0% 2-Mercaptoethanol) and agitating them in a microplate shaker at 5~60 rpm for 20 min. Next, we washed the microwell array with 3 mL of hybridisation buffer (2 M NaCl, 0.64% PEG8000, 0.52 $\times$ PBS) to hybridise the released mRNA and DNA tag with the primers on the hydrogel beads by agitation at 5~60 rpm for 40 min. After removing the membrane over the microwell array, we acquired the images of the microwell array containing the

hydrogel beads with the adjusted exposure times. To collect beads, we placed the microwell array directly into a 200 μ L tube and flushed with 200 μ L of wash solution 1 (2 M NaCl, 3 mM MgCl₂, 20 mM Tris-HCl (pH 8.0), 0.64% PEG8000, 0.1% tween20). We exchanged wash solution 1 for wash solution 2 (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM MgCl₂, 0.4 U/ μ L Recombinant RNase inhibitor (2313A, Takara), 0.1% Tween 20) by repeating centrifugation (3000 g, 4°C, 3 min) and the buffer exchange twice.

2.2.6 Library preparation

To construct cDNA and DNA-tag libraries (Fig 2.4), we performed the reverse transcription (RT) and polymerase chain reaction (PCR) according to the protocol of the CITE-seq (Stoeckius, M. *et al.*, *Nat. Methods*, 2017) with modifications. We added 10 μ L of RT mix (1xFirst-Strand Buffer, 4.8 μ M biotinylated template switching oligonucleotide (TSO, Qiagen), 2 mM dNTP mix, 4 mM DTT, 2 U/ μ L Recombinant RNase inhibitor (2313A, Takara), 20 U/ μ L SMARTScribe Reverse Transcriptase (Takara) to 10 μ L of suspended beads and incubated them in a thermal cycler at 42°C for 90 min to obtain first-strand cDNA by reverse transcription and then heated at 70°C for 10 min to stop the reaction. To remove excess RT primers, we added 2 μ L of 2.5 U/ μ L Exonuclease I (2650A, Takara) and incubated them at 37°C for 50 min, followed by inactivation, heating at 80°C for 20 min.

The first-strand cDNA was amplified by PCR in a 50 μ L reaction containing 0.24 μ M primer2, 9 nM additive primer, 1xSeqAmp PCR Buffer and 0.025 U/ μ L SeqAmp DNA Polymerase (Takara) (Table S2) using the following program: 95°C for 1 min; 16-18 cycles of 98°C for 10 s and 65°C for 30 s; 68°C for 4 min; and 72°C for 10 min. We purified the mRNA-derived cDNAs (long cDNA) and the DNA tag-derived cDNAs (<200 bp short cDNA) by size fractionation using SPRIselect beads (B23318, Beckman Coulter). We mixed the cDNA product with 0.6 $\times$ SPRIselect beads and placed them on the DynaMagTM- Spin Magnet (InvitrogenTM) to capture the beads. We then transferred the first supernatant containing the DNA-tagged product to a new tube and further purified it with 1.4 $\times$ SPRI beads. The magnetic beads were respectively washed

three times (long) and twice (short) respectively on a magnetic stand with 0.2 ml of fresh 80% (vol./vol.) ethanol and air-dried for 2.5 min at room temperature. The cDNAs derived from mRNA and DNA tag were eluted with 13 and 11 μ L of elution buffer (10 mM Tris-HCl, pH 8.5), respectively.

For the mRNA-derived cDNA, we examined the yield, quality, and size distribution respectively using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and a quantitative real-time PCR (qPCR) targeting *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Hs02758991_g1, Thermo Fisher Scientific) and with an Agilent High Sensitivity DNA Kit using Bioanalyzer 2100 (Agilent). We then performed the tagmentation of 600 pg of cDNA using a Nextera XT DNA Library Prep Kit (Illumina) and PCR with custom indexing primers. We then cleaned up the PCR products with 0.6 $\times$ SPRIselect beads and eluted them with 6.5 μ L of Resuspension Buffer (RSB, Illumina).

To construct the DNA tag library, the short cDNA was amplified in 20 μ L of 1xKAPA Hifi Hotstart Ready Mix (Roche) containing 1.6 μ L of 10-fold diluted templates and 0.25 or 0.5 μ M of indexing primers (Table S2) using the following program: 98°C for 2 min; 2 cycles of 98°C for 20 s and 74°C for 30 s; 12-18 cycles of 98°C for 20 s and 72°C for 30 s; 72°C for 5 min. The library was then purified with 1.5 $\times$ SPRI beads and eluted with 8 μ L of elution buffer. We assessed the yield and length of the library respectively using a Qubit™ dsDNA HS Assay Kit and the Agilent High Sensitivity DNA Kit (Agilent), respectively.

The typical size of the mRNA-derived cDNA library was approximately 500 bp, while the size of DNA tag libraries was 224 bp. Finally, we quantified the library using the KAPA Library Quantification Kits (Roche) according to the manufacturer's protocol. The library was sequenced on a HiSeq X (Illumina) instrument with 2 x 150 bp paired-end reads.

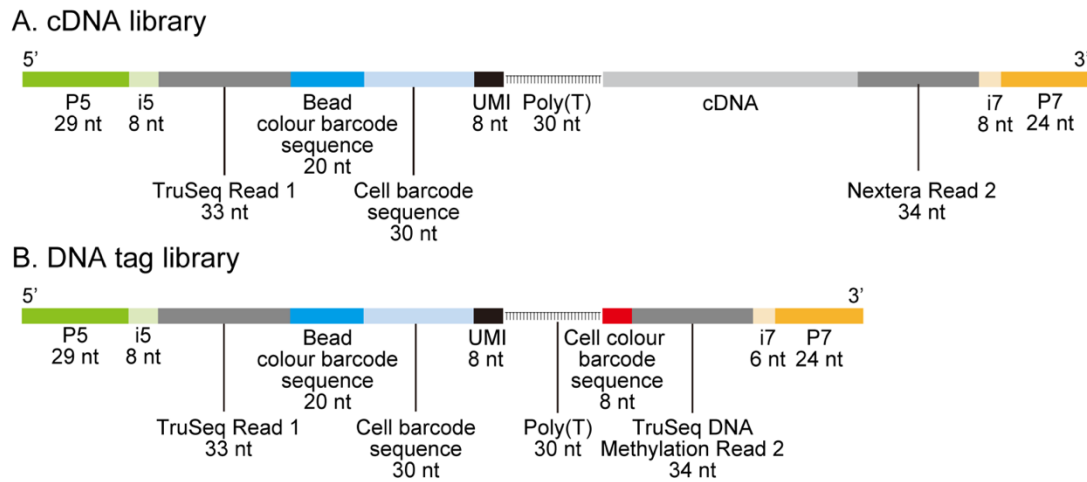


Figure 2.4 Libraries design of cDNA and DNA tag for Illumina sequencing.

A. cDNA library. B. DNA tag library.

2.2.7 Cell and bead image processing

We scanned the entire PDMS chip with the Micro-Magellan (Pinkard, H. *et al.*, *Nat. Methods*, 2016) to image the cells and beads, respectively. We performed flat-field correction on all fluorescence images using a built-in MATLAB function before stitching them together. We visually detected the cells and beads captured in the wells and registered their respective colour codes. To identify cell and bead pairs co-captured in the same wells, we applied an affine transformation to cell images to align the positions of the microwells of cell images to the microwells of bead images. When the centres of the cells were within the radii of the bead-captured wells, we assigned them as co-captured pairs. To compensate for the different focus of the nuclei of paclitaxel-treated cells in the microwells, z-stack images were taken with a 10x lens (10x, UPlanFL N) at 3 μ m intervals from the bottom to the top of the wells. We then ran an extended depth of field algorithm (Forster, B. *et al.*, 2004 *2nd IEEE International Symposium on Biomedical Imaging: Nano to Macro*, 2004) provided by Fiji software. We count the spindle poles using CellProfiler (Stirling, D. R. *et al.*, *BMC Bioinformatics*, 2021) by enhancing the speckles with ‘*EnhanceOrSuppressFeature*’ module and segmenting them into individual poles with ‘*IdentifyPrimaryObjects*’ module.

2.2.8 Single-cell RNA data processing

We demultiplexed the sequence reads derived from cDNA and DNA tags using UMI-tools (Smith, T. *et al.*, *Genome Res.*, 2017) into each cell barcode and each UMI. We demultiplexed DNA-tag UMI counts into each tag type by CITE-seq-Count program. We mapped cDNA reads to reference genomes and transcriptomes of GRCh38 (human, .p12 for the experiment of species-mixing and p13 for the experiments of paclitaxel treatments) and GRCm38.p6 (mouse) by the STAR (version 2.7.10b) mapping program (Dobin, A. *et al.*, *Bioinformatics*, 2013). To remove artifact, we filtered out the cells with less than 800 UMI counts. To link the sequencing data and the image data, we assessed the cosine similarities between the centred log ratio (CLR) of the UMI counts of DNA tag from the sequencing data and dummy variables of the joint colour code from the image data. Here we assumed that cosine similarity is a function of the signal-noise ratio of tag counts expressed by the following equation.

$$(\text{Cosine similarity}) = \frac{\mathbf{c} \cdot \mathbf{e}}{|\mathbf{c}| |\mathbf{e}|} = \frac{s}{1 \cdot \sqrt{s^2 + (k-1)\bar{n}^2}} = \frac{1}{\sqrt{1 + (k-1) \frac{\bar{n}^2}{s^2}}}$$

where $\mathbf{c}$ is a vector of the one-hot encoded joint colour code, $\mathbf{e}$ is a vector of the tag count, which consists of an element of signal s and others of noise n ($\bar{n}$ is the mean value), and k is a pooling number of tags. We optimized the combinations that maximized the sum of the cosine similarities within each chip. This resulted in the inclusion of linked data with small value of similarities. To remove them, we further filtered out the linked data whose signal-noise ratios were less than $\sqrt{5}$. To remove batch effects, we integrate data from different batches with the functions of ‘*SelectIntegrationFeatures*’, ‘*FindIntegrationAnchors*’, and ‘*IntegrationData*’ from Seurat (version 4.3.0.1) (Hao, Y. *et al.*, *Cell*, 2021) package.

2.2.9 Weighted nearest neighbouring analysis for paclitaxel-treated cells

For paclitaxel-treated cells, we filtered out the cells with less than 2000 UMI count, excluded data linked to doublet cells in a well, and genes with low detection rates below

0.2, and mitigated the batch effect as described above. Subsequently, we isolated cells whose unique joint colour code was unique on the chip. We then transformed gene expression into the principal components using ‘*runPCA*’ function from Seurat package. Furthermore, we combined paclitaxel concentrations (log10-transformed with a 0.1 offset) and the number of spindle poles to create principal components using the ‘*prcomp*’ function from the stats package. To incorporate both sets of principal components in subsequent analyses, we performed a weighted nearest neighbouring (WNN) analysis (Hao, Y. *et al.*, *Cell*, 2021) utilising the ‘*FindMultiModalNeighbors*’ function (with a k-nearest neighbors’ parameter of 25) from the Seurat package.

2.2.10 Extraction of transcriptomic response

We clustered cells and projected them on the UMAP based on the neighbouring information from WNN using the ‘*FindCluster*’ and ‘*RunUMAP*’ functions from Seurat package. Using the clusters and UMAP, we performed trajectory analysis with slingshot (Street, K. *et al.*, *BMC Genomics*, 2018) and extracted the transcriptomic response, ψ , with ‘*slingAvgPseudotime*’ function. To determine the differentially expressed genes that share the increase in transcriptomic response within trajectories, we fit the gene expression with the following model using the edgeR (Robinson, M. D. *et al.*, *Bioinformatics*, 2010) package

$$\log(\mu_{gi}) = \beta_0 + \beta_\psi \psi + \log(N_i)$$

where μ_{gi} is an expected expression of gene g in a cell i calculated by edgeR, ψ is the transcriptomic response, N_i is a size factor of cell i . We assessed the significance of β_ψ to derive fold changes and p values with the quasi-likelihood F-test. To determine differentially expressed genes between trajectories, we fit the gene expression with the following model,

$$\log(\mu_{gi}) = \beta_0 + \beta_{\psi,1} t_1 \psi + \beta_{\psi,2} t_2 \psi + \log(N_i)$$

where t_k is a factor denoting the assignment to trajectory k . We assessed the significance of $\beta_{\psi,2} - \beta_{\psi,1}$ to derive fold changes and p values.

2.3 Results

2.3.1 Variation of the system through mixed species experiments

To demonstrate our protocol, we performed mixed-species experiments using HeLa cells (human) and NIH/3T3 cells (mouse) (Fig. 2.5A). We prepared a pool of 16 differently colour-coded cells (eight sub-pools each of HeLa and NIH/3T3 cells) that were respectively labelled with colour codes. We then isolated the single cells out of the pool of 16 colour codes in microwells to image them by epi-fluorescence and a bright field (Fig. 2.5B). The hydrogel beads bearing barcoded primers with colour codes were subsequently isolated in the microwells to capture mRNA and DNA tags. To retain the molecules released from the cells within the microwells, we sealed the microwells with a track-etched membrane with nanopores of 10 nm in diameter, chemically lysed the cells in microwells, and captured the mRNA and DNA tags by the hydrogel beads via hybridisation. After peeling off the track-etched membrane, we imaged the fluorescence of the hydrogel beads in the microwells to read out the colour codes (Fig. 2.5B). We registered the images of the single cells with the joint colour codes by integrating the microscopic images of cells and hydrogel beads. We finally transferred the hydrogel beads to a standard PCR tube to synthesise libraries of the scRNA-seq and DNA tag by off-chip reactions (see Methods). The latter library yielded a look-up table that linked the cell barcodes in the cDNA fragments and joint colour codes by creating concatenated fragments of colour codes of hydrogel beads and cells (Fig. 2.4).

We designed 16 colour codes, respectively for cells and hydrogel beads, thus, a maximum of 256 joint colour codes (16×16) could be registered. The expected number of cell-bead pairs with unique joint colour codes per experimental run was predicted to attain a maximum of approximately 94 when assaying 256 single cells on the basis of Poisson distribution, excluding the cells with duplicated joint colour codes (Fig. 2.5C). In this experiment, our microwell chip, which had 2511 wells per chip, captured approximately 149 ± 70 cells and 1326 ± 462 hydrogel beads per run ($n = 13$), and created approximately 137 ± 64 pairs of single cells and hydrogel beads on average. The image-based decoding of the joint colour codes showed that the number of unique joint colour codes matched the theoretical prediction (Fig. 2.5C).

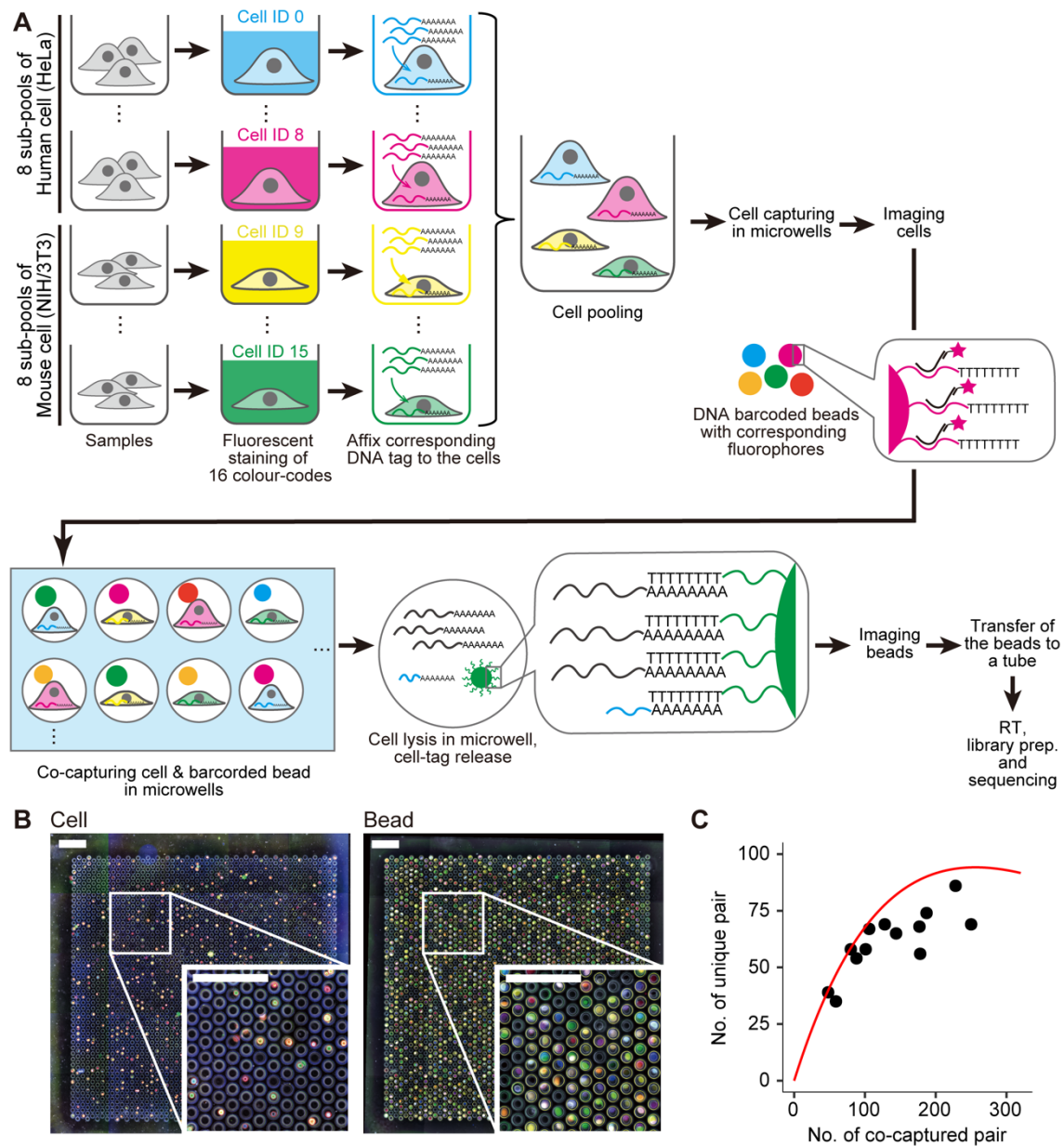


Figure 2.5 Procedures of the species-mixing experiment.

A. We divided human cells (HeLa) and mouse cells (NIH/3T3) into the eight sub-pools respectively for a total of 16 sub-pools. We individually stained with different combinations of fluorescence dyes and transfected with corresponding DNA tags via electroporation. We combined all sub-pools of colour-coded cells into a single tube and captured the cells in a microwell array. After cell imaging, we introduced the colour-coded hydrogel beads into the microwells, creating pairs of a colour-coded cell and bead

in each microwell. Upon cell lysis, released mRNAs and DNA tags hybridise with barcoded dT primers on hydrogel beads. We imaged the hydrogel beads and proceeded to reverse transcription, PCR, and next-generation sequencing. B. The single cells were co-isolated with single hydrogel beads out of a pool of those bearing 16 different colour codes in microwells. Cells are outlined with red borders, while bead-captured wells are outlined with yellow borders. The isolated single cells were processed to yield a scRNA-seq library and a joint colour code library that read out the gene expression and the colour code combination, respectively. Scale bar = 300 μm . C. The expected number of unique joint colour codes per experimental run follows the Poissonian distribution.

2.3.2 Development of a computational framework to link image and sequence data

To link the cell barcode in scRNA-seq to single-cell images via joint colour codes, we devised a framework that optimises pairs of cell barcode and single-cell image by maximizing the sum of the similarity between the decoded colour code from the images and counts of DNA tag (Fig. 2.6). We benchmarked the framework in terms of the accuracy and number of linked datasets using the mixed-species data, computing with various metrics of similarity and normalisation approaches for the DNA tag counts. The result showed that the cosine similarity in combination with the centred log ratio (CLR per feature) for normalisation of DNA tags performed the best among those tested (Fig. 2.6.C). The framework with the cosine similarity and CLR yielded a consistency of 91% for species at a threshold of 0.5 for the cosine similarity. We employed the same framework throughout this study.

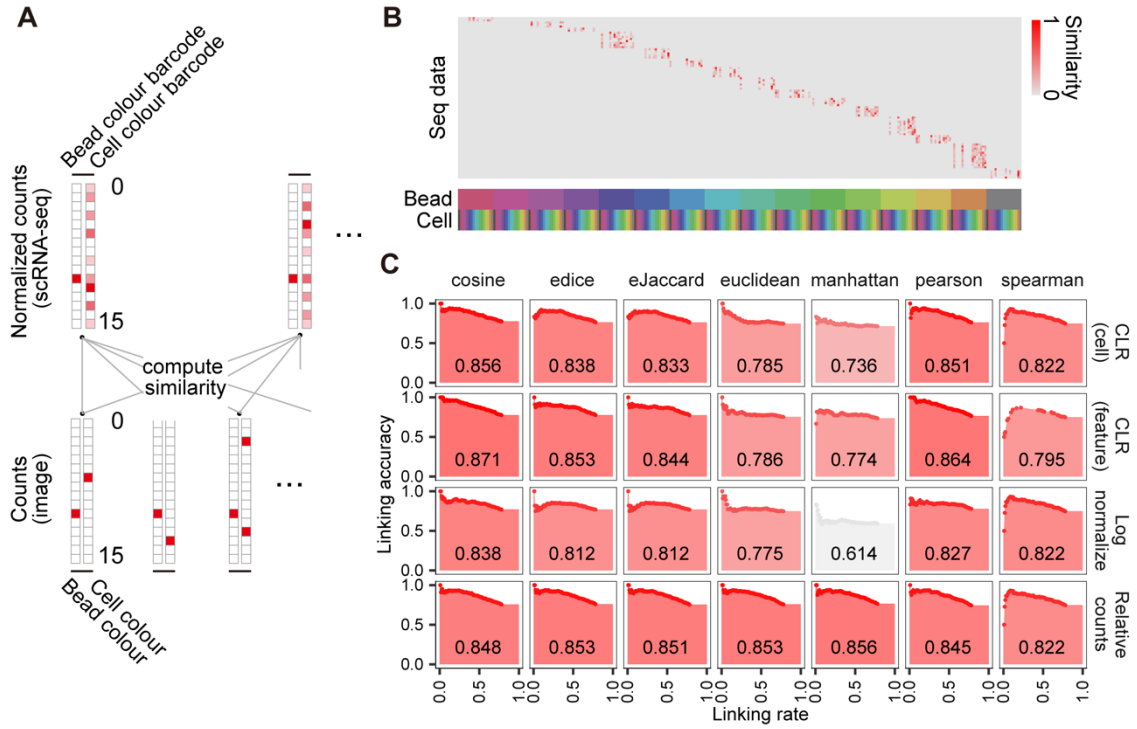


Figure 2.6 A computational framework to link the single-cell image and gene expression via joint colour codes

A. Schematic image of computation for the cosine similarity. B. A look-up table that shows the matching of sequencing data (columns) and colour codes decoded from image data (rows). C. The area under the curve of linking rates (the number of linked joint colour codes over all the number of joint colour codes detected in the image) versus linking accuracies (the number of linked data with consistent species over all the number of linked data) using different similarities and normalizations of DNA tag counts. We identified the species of cells by the abundance of the transcripts when one species exceeds 70% of the total unique molecular identifier (UMI) counts.

2.3.3 Assessment of the linkage between transcriptomic data and imaging data

The sequence reads with the same cell barcode were dominantly mapped either to the homosapiens genome (GRCh38.p12) or Mus musculus genome (GRCm38.p6), supporting the successful RNA-seq at single-cell resolution (Fig. 2.7A). The scRNA-seq detected approximately 1296 ± 586 genes per cell (i.e., approximately 2839 ± 1829

unique molecular identifiers (UMIs) per cell) and approximately 1203 ± 541 genes per cell (i.e., approximately 2633 ± 1716 UMIs per cell), respectively for HeLa and NIH/3T3 cells (sequence read per cell was 30,865 on average). Of the 360 unique joint colour codes identified by fluorescence microscopy in six experimental runs, 137 were also identified in the DNA tag library and successfully linked to the scRNA-seq data. Of those, 122 cells were consistent for the species (Fig. 2.7B, C).

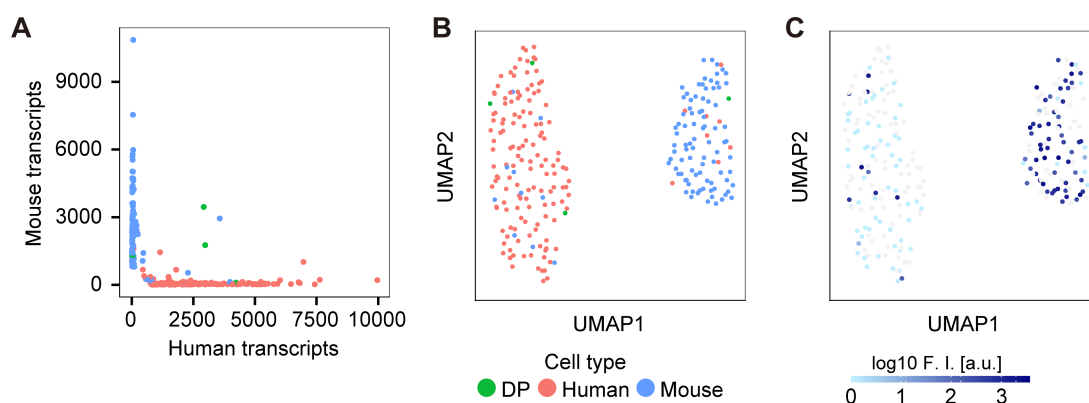


Figure 2.7 Mixed species experiment (mouse and human) validates the linkage between transcriptomic data and imaging data.

A. The number of detected transcripts associated with individual cell barcodes. B-C. Uniform manifold approximation and projection (UMAP) of the cells. The colour represents cell type identified from linked cell image (B, DP is linked to cells co-captured with cells from different species.) and logarithmic fluorescence intensities of cells unique to the mouse cells (C).

2.3.4 Evaluation of DNA tag introduction

We benchmarked two different approaches, electroporation and lipofection for labelling cells with DNA tags using fluorescently labelled DNA tags and flow cytometry (Fig. 2.8A, B). The data revealed that lipofection outperformed in delivering more DNA tags to cells than electroporation, while the amount of DNA tags resulted in a relatively large cell-to-cell variation. Furthermore, lipofection was less efficient for NIH/3T3 cells than for HeLa cells. To gain a similar sensitivity in detecting the DNA tags from HeLa

and NIH/3T3 cells, we employed electroporation in the experiments with mixed species. Alternatively, modulating the concentration of DNA tags for lipofection could tune the sensitivity (Fig. 2.8A, B). We employed lipofection in the other experiments with a cell line.

We also assessed the durability of the DNA tags within the cells by quantifying their presence over time (Fig. 2.8C). Remarkably, even after 48 h of labelling, the DNA tags remained detectable, indicating the potential for combining our approach with longitudinal live-cell imaging. Notably, the linking rate, a metric representing the fraction of cell images linked to scRNA-seq among those identified from the cell images, exhibited no degradation over time (Fig. 2.8D). Interestingly, the linking rate associated with the electroporation labelling increased over time. We hypothesise that this trend may be attributed to a selection bias in favour of healthy cells within the electroporated cell population. Furthermore, we conducted additional analyses to confirm the integration of labelled cells with the unlabelled ones in the transcriptomic space (Fig. 2.8E, F). These data serve as a clear benchmark for cell tagging achieved through DNA delivery via electroporation and lipofection.

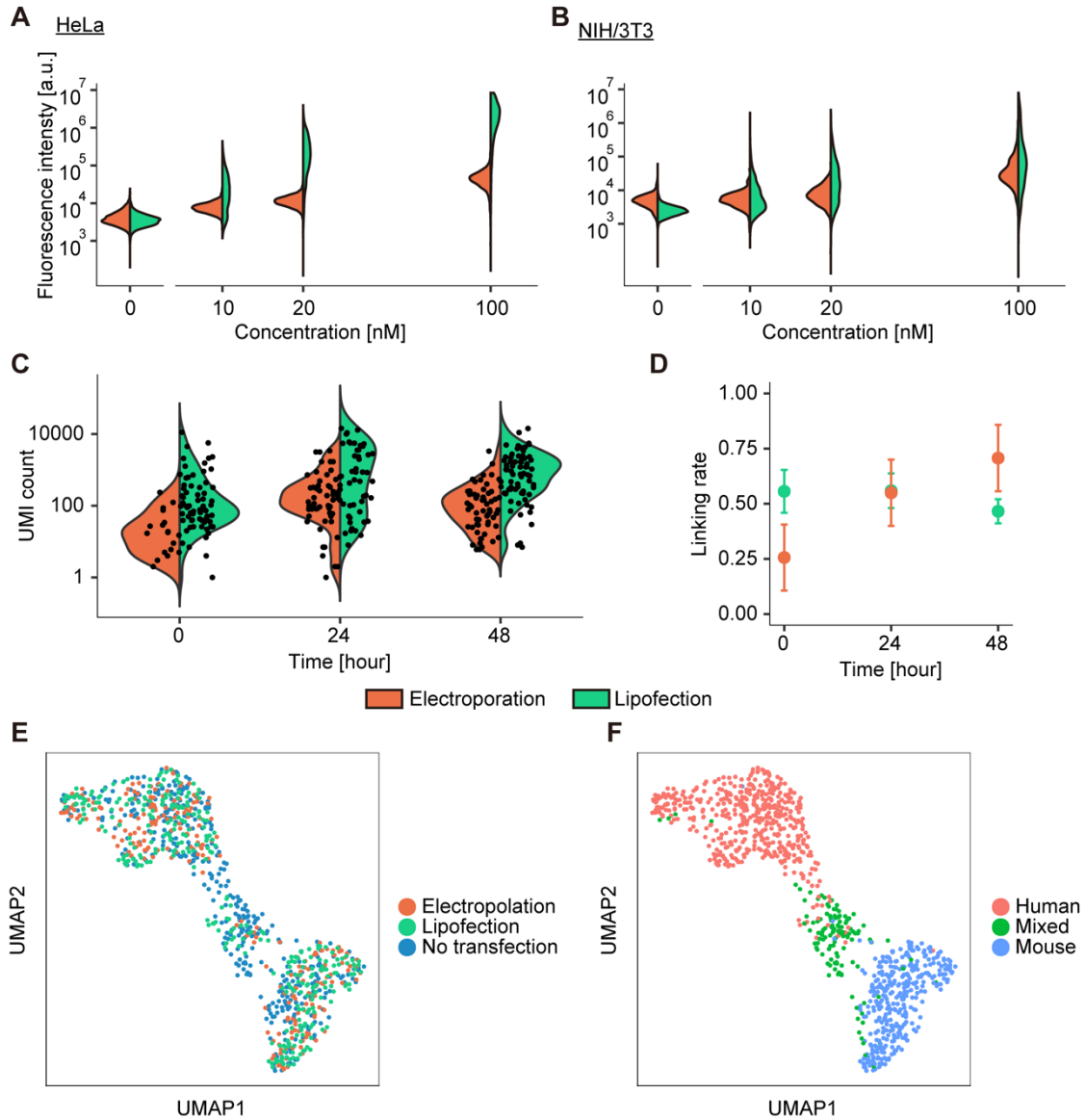


Figure 2.8 Comparison of electroporation and lipofection for DNA-tag labelling.

A-B. Quantities of FAM-labelled DNA tags at various DNA-tag concentrations for (A) HeLa cells and (B) NIH/3T3 cells. C. Unique molecular identifier (UMI) counts of DNA tag against the different durations of incubation at 20 nM for lipofection, and 100 nM for electroporation. D. Linking rates against the different durations of incubation. E-F. Uniform manifold approximation and projection (UMAP) of single cells labelled by electroporation and lipofection (E) and species determined by the ratio of UMI counts (F).

2.3.5 Integrated perturbation analysis of the phenotypic and transcriptomic response to paclitaxel

Next, we sought to determine if our approach could enhance the insights gained from chemical screening. Specifically, we investigated the cell-to-cell heterogeneity in the response of HeLa cells to the chemical impact of paclitaxel, which is a chemotherapy drug used in the clinical treatment of lung, ovarian, and breast cancer; it inhibits the growth of cancer cells by blocking cell division (Fig 2.9). Traditionally, it was believed to induce cell death through mitotic arrest. However, recent studies have suggested that tumour regression is not solely dependent on the mitotic arrest, but is influenced by multipolar spindle formation (Zasadil, L. M. *et al.*, *Sci. Transl. Med.*, 2014), leading to cell death (Scribano, C. M. *et al.*, *Sci. Transl. Med.*, 2021). In our study, we aimed to dissect the nuclear phenotype associated with multipolar spindles induced by paclitaxel and its underlying transcriptomic basis.

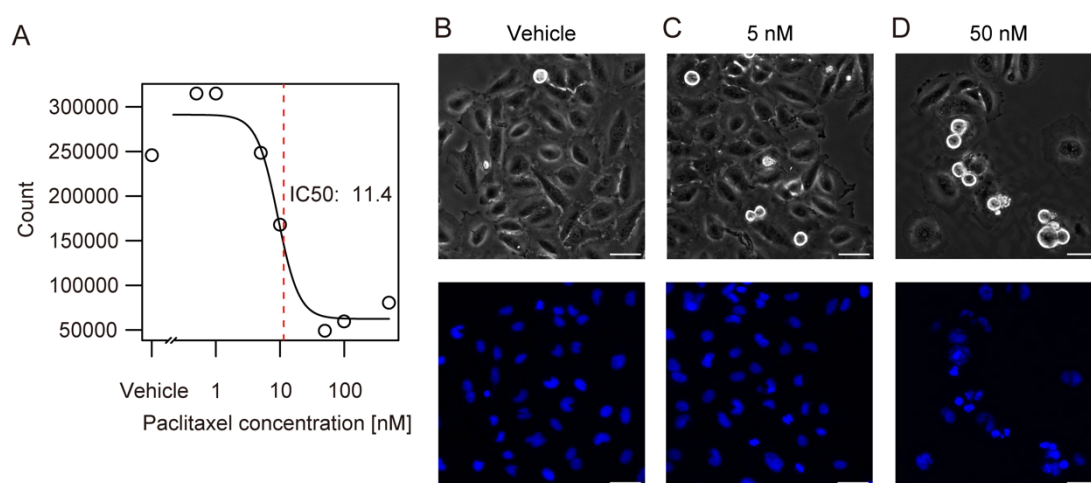


Figure 2.9 HeLa cell response to paclitaxel on dish

A. The drug response curve of HeLa cells treated with paclitaxel for 24 h. The red broken line indicates the half-maximal inhibitory concentration (IC50). B-D. Images of cells in bright fields and nuclei stained with Hoechst 33342 of HeLa cells treated with paclitaxel (B. vehicle, C. 5 nM, and D. 50 nM) for 24 h in standard culture dishes. The scale bars on the bottom right are 50 μ m.

To understand the intricate relationship between the phenotypic and transcriptomic responses at the single-cell level, we subjected the DNA-tagged and colour-coded HeLa cells to paclitaxel treatment at eight distinct concentrations, ranging from 0.5 to 500 nM, over a 24-h period. Subsequently, we analysed the combined samples using our established approach. Specifically, we utilised a single fluorescence channel to monitor the emergence of multipolar spindles as a phenotypic response to paclitaxel by staining the DNA with Hoechst 33342. The colour-decoded images of individual cells revealed that the occurrence of multipolar spindles became more prevalent at higher concentrations of paclitaxel. Notably, even at identical concentrations of paclitaxel, the number of spindles exhibited considerable heterogeneity across the cells (Fig. 2.10A, B). These observations aligned with the findings from non-pooled assays conducted in separate dishes (Fig. 2.10C, D).

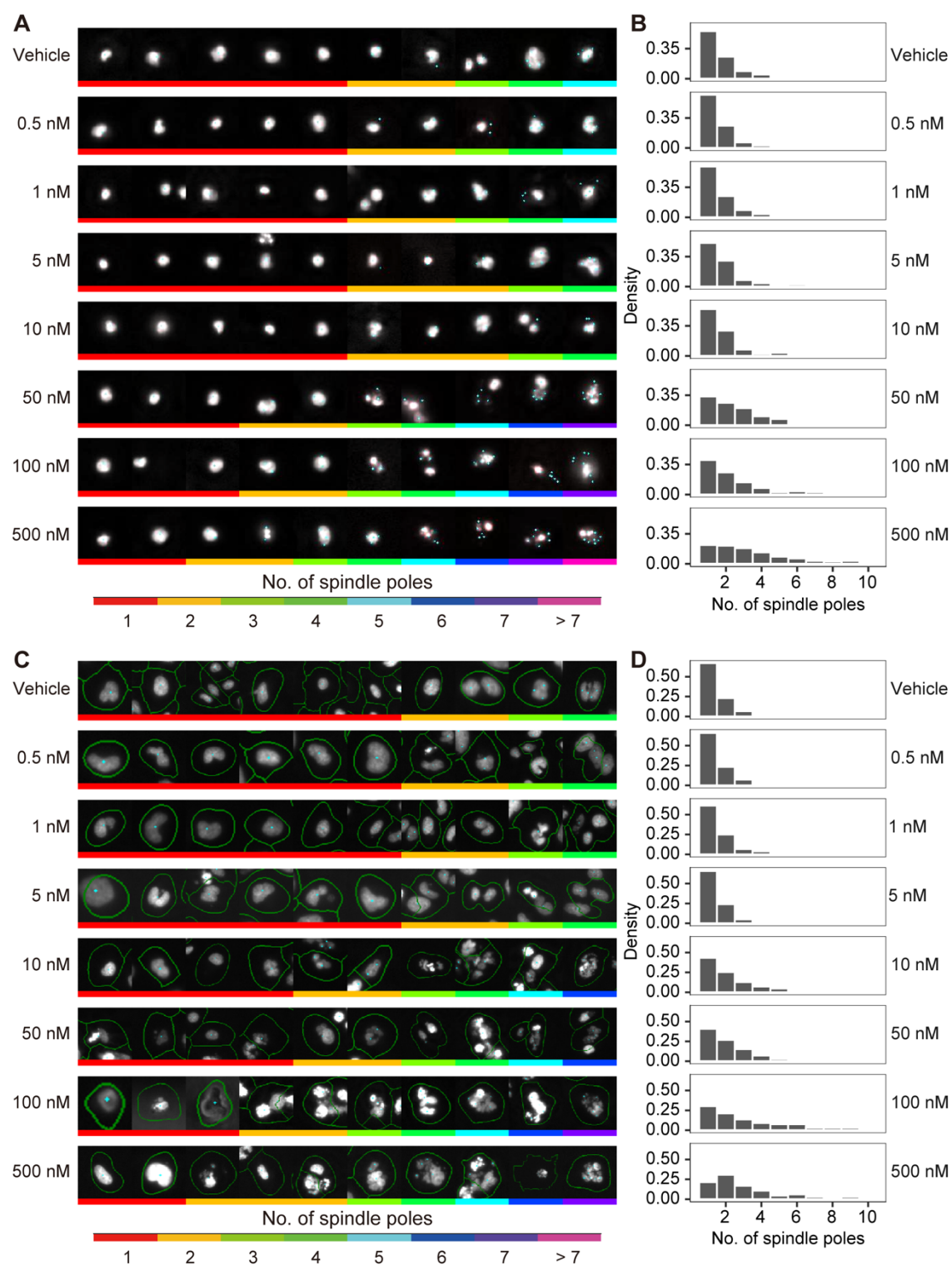


Figure 2.10 Nuclear morphological response of colour-coded HeLa cell to paclitaxel

A. On-chip images of nuclei in HeLa treated with different concentrations of paclitaxel.

Cian points are the positions of spindle poles. The colour labels on the bottom of the images indicate the number of spindle poles. The images are stratified according to the number of spindle poles. B. Distributions of the number of spindle poles in HeLa cells imaged on chip. C. On dish images of nuclei in individual HeLa cells treated with different concentrations of paclitaxel. Green line represents outline of each cell. D. Distributions of the number of spindle poles in HeLa cells imaged on dish.

To uncover the mechanism underlying the heterogeneous cellular response, we leveraged the transcriptomic data linked to the phenotypic responses. The transcriptomic data showed approximately 1364 ± 350 genes per cell (i.e., approximately 4357 ± 2366 UMIs per cell, with 106,119 sequence read per cell on average). The integrated multimodal data by weighted nearest neighbour analysis enabled inferring two distinct trajectories, related to the formation of multipolar spindles, or lack thereof, in response to paclitaxel burden within the transcriptomic data (Fig. 2.11 A-C). Gene set enrichment analysis (GSEA) revealed that the genes associated with the mitosis (*mitotic cell cycle*, *mitotic cell cycle process*, *cell cycle process*, *cell cycle G2/M phase transition*, *cell cycle phase transition*, *G2/M transition of mitotic cell cycle*, and *regulation of cell cycle*) were down-regulated with increasing paclitaxel concentration, irrespective of the presence of multipolar spindle formation (Fig. 2.11D); the genes included in the GO terms consistently exhibited the down-regulation (Fig. 2.11E). Subsequently, in trajectory 1 (no multipolar spindle formation), GSEA highlighted the induction of endoplasmic reticulum (ER) stress (Fig. 2.11G), which, if sustained or is severe, can potentially trigger apoptosis (Liao, P. C. *et al.*, *J. Cell Biochem.*, 2008). Conversely, in trajectory 2, characterised by multipolar spindle formation, the *RFC4* gene, known for its role in DNA replication and repair (Li, Y. *et al.*, *Am. J. Cancer. Res.*, 2018), consistently exhibited up-regulation in response to increasing paclitaxel exposure. These findings underscore the remarkable power of integrated multimodal data analysis, effectively distinguishing the gene regulations between the two trajectories associated with distinct phenotypic outcomes.

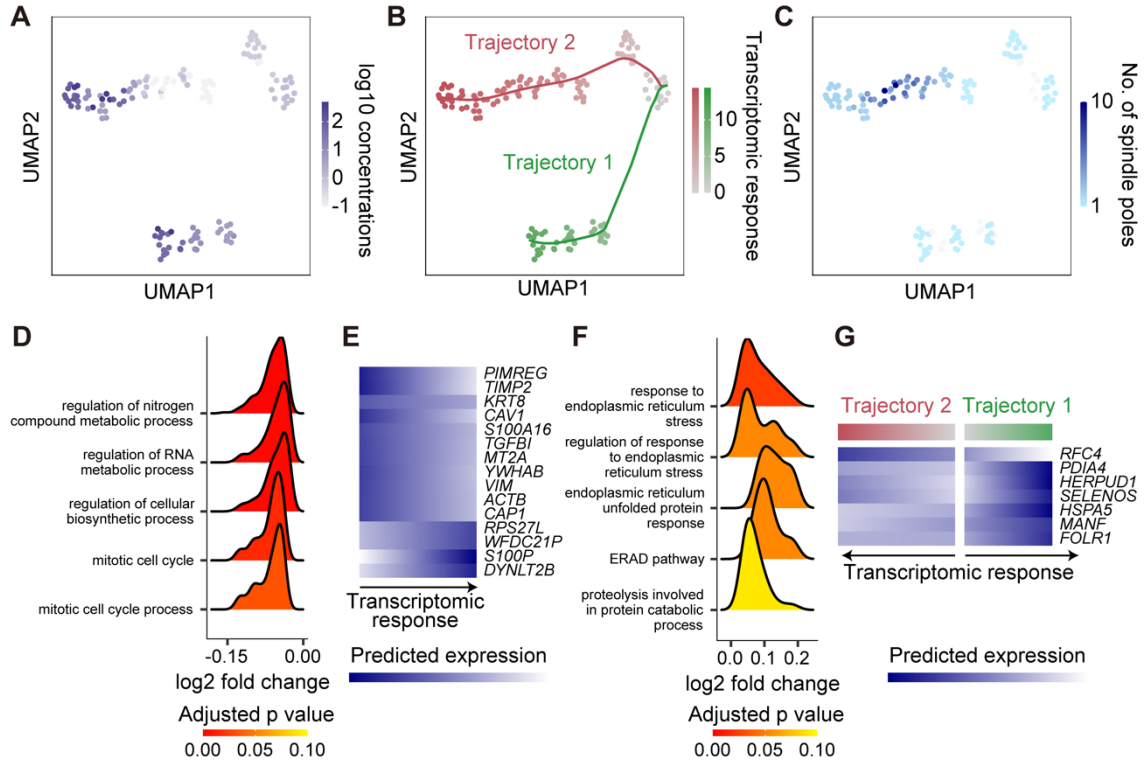


Figure 2.11 Transcriptomic response of HeLa cells to paclitaxel burden

A-C. Uniform manifold approximation and projection (UMAP) of the integrated dataset by weighted nearest neighbour analysis. The colour intensity represents the concentrations of paclitaxel, (A) the magnitudes of transcriptomic responses in the two distinct trajectories (B), and the numbers of spindle poles (C) D-E. Gene set enrichment analysis (GSEA) of differentially expressed genes associated with the increase of transcriptomic response shared among the two trajectories (D) and its predicted expression of individual genes. (E) F-G. GSEA comparing the two distinct trajectories with and without multipolar spindle formation (F) and predicted expression of individual genes (G).

2.4 Summary

In this chapter, we propose an opto-combinatorial indexing method that integrates single-cell image analysis with gene expression analysis. This method applies dual labeling, referred to as a "colour code," consisting of optical indexing using fluorescent

dyes and DNA molecular barcodes, to both cells and hydrogel beads with dT probes, respectively, in order to combine single-cell image data with sequencing data. The combination of the colour codes from cells and beads (joint colour code) is read using both imaging and next-generation sequencing, enabling the cross-referencing of single-cell data. A species-mixing experiment demonstrated a 91% cell types concordance rate between images and gene expression achieved through self-developed optimized computation. We also assessed and optimized the transient transfection of DNA molecular barcodes. Properly designed transfection showed that labeling could be sustained for up to 48 hours using both lipofection and electroporation. Furthermore, we applied this method to chemical perturbation analysis, investigating the formation of multipolar spindles in HeLa cells under paclitaxel treatments. Imaging of the spindle poles revealed an overall increase in the number of spindle poles with increasing paclitaxel concentration at the ensemble level; however, there was heterogeneity at the individual cell level. Integrated analysis with gene expression data showed a distinct separation based on the presence or absence of multipolar spindle formation. Differential gene expression analysis suggested the involvement of ER stress response and the DNA replication-related gene *RFC4* in the process.

3 Scaling up opto-combinatorial indexing for high-content imaging and cellular multiplexing

3.1 Introduction

In single-cell high-content imaging, the profiling of multiple targets and readouts within multiplexed images makes it feasible to measure multiple phenotypes (Carpenter, A. E. *et al.*, *Genome Biol.*, 2006). When applied to chemical perturbation screening, this capability allows to profile multiple drugs simultaneously and predict their MOAs (Perlman, Z. E. *et al.*, *Science*, 2004; Loo, L. H. *et al.*, *Nat. Methods*, 2007; Young, D. W. *et al.*, *Nat. Chem. Biol.*, 2008). This single-cell high-content imaging approach also enables the assessment of highly heterogeneous populations of cells (Feldman, D. *et al.*, *Cell*, 2019; Funk, L. *et al.*, *Cell*, 2022). Notably, high-content imaging analysis provides distinct and complementary information compared to that obtained from RNA-seq (Haghighi, M. *et al.*, *Nat. Methods*, 2022; Way, G. P. *et al.*, *Cell Syst.*, 2022; Moshkov, N. *et al.*, *Nat. Commun.*, 2023), providing insights into protein localization, cellular morphology, enzymatic activity, and other cytologic characteristics.

We have demonstrated the utility of integrated single-cell imaging and RNA-seq analysis for chemical perturbation to investigate the molecular systems driving cytological phenotype heterogeneity in response to paclitaxel treatment (Tsuchida, A. *et al.*, *Lab Chip*, 2024). This method, termed opto-combinatorial indexing, utilises colour code combining the bright or dim of fluorescent probes with different wavelengths, along with dual DNA molecular barcode labelling, to link cell images and sequencing data. However, despite its utility, this approach has been limited to single-readout cytological phenotyping and has not achieved high-content imaging.

A key challenge in achieving this is fluorescence spillover, caused by interference between fluorophores used for phenotype readouts and those used for colour coding. This issue also reduces the throughput of single-cell analyses, limiting the resolution in assessing sample heterogeneity in an assay.

To significantly expand the multiplexing capacity of conventional opto-combinatorial indexing, we propose a strategy to overcome fluorescence spillover. For cell, we used signal unmixing by minimizing mutual information between images. For bead, we accurately designed DNA molecular barcode on hydrogel beads with interfering fluorescence and decoded them with machine learning-based prediction of colour codes.

3.2 Method

3.2.1 Strategies to overcome the limitations of fluorescence multiplexing due to spectral overlap

We have overcome the limitation on the number of measurable molecular features due to the overlap of fluorescence wavelengths through the following two strategies: (1) We separated the fluorescence signals of molecular features from the colour codes for cells by an unsupervised unmixing algorithm. (2) We increased the number of colour codes for beads with interfering fluorescence probes and thus increased the number of multiplexed cells by developing an algorithm to generate orthogonal nucleotide sequences that do not cross-react (Fig. 3.1). In the former strategy, we achieved an increase in the number of measurable cytologic features while maintaining the number of cellular colour codes by eliminating fluorescence spillover between cytologic features and cellular colour codes through the unmixing algorithm. We confirmed that this strategy uncompromised the signals from the cytologic features and the accuracy of cell colour code decoding. In the latter strategy, by increasing the number of bead colour codes, we provide a method to scale multiplexity of cells. Our precisely designed molecular system for colour coding enables accurate decoding of the colour code by machine learning approach.

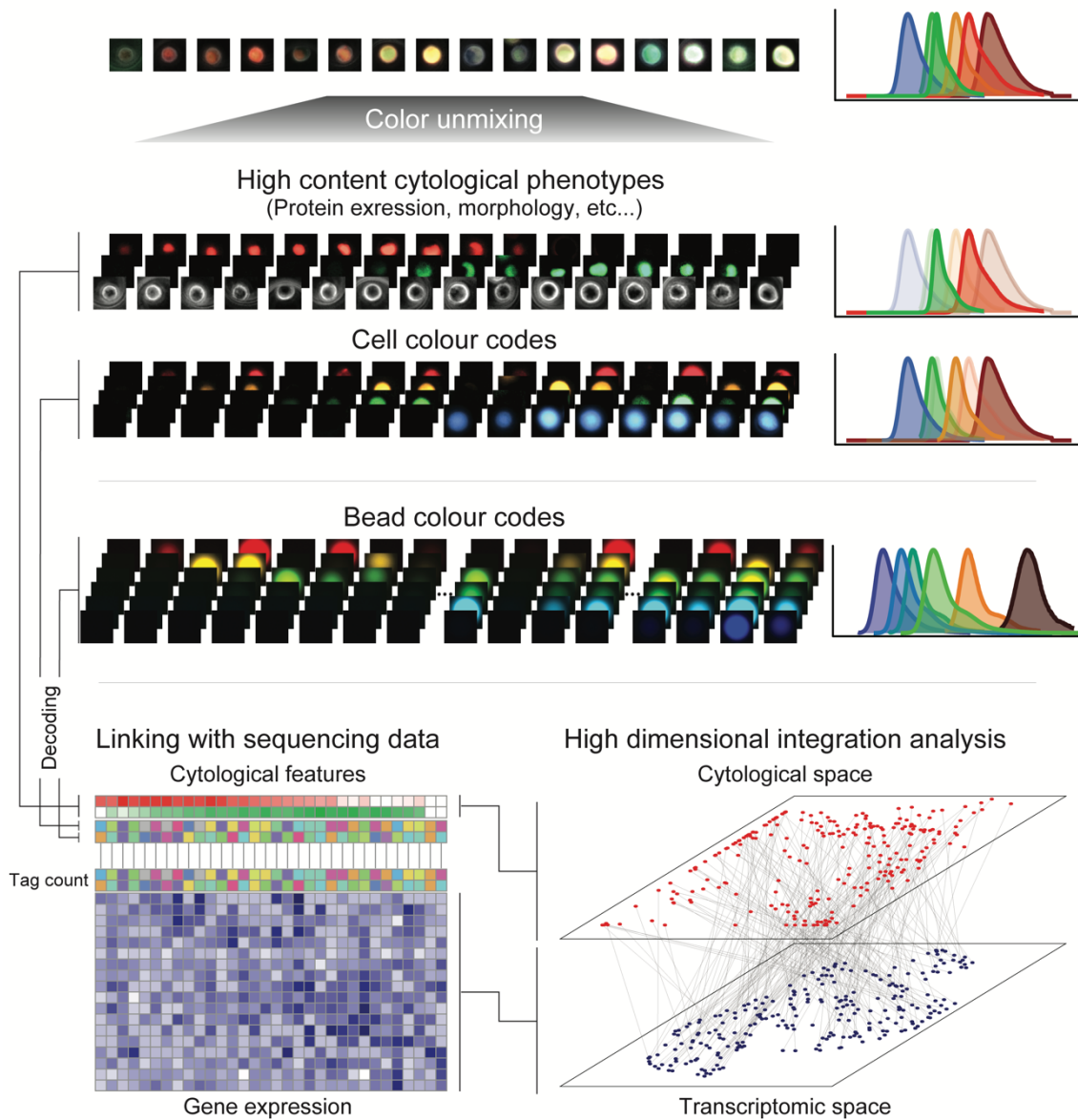


Figure 3.1 Conceptual figure to expand the multiplexity of phenotyping and the scalability of single-cell integrated analysis.

Fluorescence signals are demultiplexed according to spectral. The signals are proceeded for colour code demultiplexing and phenotypic analysis. Demultiplexed colour codes are linked to the colour codes in scRNA-seq data pool.

3.2.2 Probe design of 6-bit colour code for hydrogel bead

Our colour code beads utilise two types of anchoring probes, Branch 1 and Branch

2. Branch 1 probes contain binding sites for the colour-coded sequences on the beads and two binding sites for the Branch 2 probes. Branch 2 probes are further categorized into Branch 2-1 and Branch 2-2; Alexa488, Alexa546, and Alexa647 fluorescent probes can bind to Branch 2-1, while ATTO532, Rho101, and Alexa750 can bind to Branch 2-2. There are 64 types of bead colour-coded sequences, each corresponding to a unique Branch 1 probe. Additionally, Branch 2-1 and Branch 2-2 each have eight types of binding sequences, with unique combinations of fluorescent probes bound to each.

To design oligos for these beads, first, we selected candidate sequences for bead colour codes and binding sequences between Branch 1 and Branch 2, consisting of 20-nt sequences with reduced cross-reactivity. We generated 1,000,000 random 20-nt sequences and selected those with GC content between 40% and 60%. Next, we selected sequences with a Levenshtein distance of at least five between sequences, and a distance of at least six from reserved sequences (Readout probes, PCR handles, and bridge sequences), as well as from the binding sequences of Branch 2-1 and 2-2 Readout probes. Subsequently, we calculated binding affinities between candidate sequences using nupack-4 to consider the binding energy models and selected non-cross-reactive sequences (Fornace, M. E. *et al.*, *ChemRxiv*, 2022). We listed each candidate sequence and its reverse complement, extracted them in order, and performed binding calculations. Calculation conditions were as follows: initial strand concentration of 20 nM, temperature of 25°C, [Na⁺] concentration of 1.0 M, and a maximum complex size of 2. The cross-reactivity was evaluated by the residual strand concentration at equilibrium ($r = [\text{strand}]_{\text{eq}}/[\text{strand}]_{\text{init}}$). To optimize computational time, candidate sequences were divided into subgroups of 100, and cross-reactivity calculations and selection of non-cross-reactive sequences were performed within each subgroup iteratively to obtain the final set of candidate sequences. For removing cross-reactive sequences, we first calculated the median of residual strand concentration r across columns, excluding sequences with $r \leq 0.99$. Next, we computed the average r value across rows, excluding sequences with $r \leq 0.95$. We further removed sequences with $r \leq 0.9$ with any other sequence. For the remaining candidate sequences, binding affinities with reserved sequences were calculated, and sequences with an $r \leq 0.9$ to any reserved sequence were excluded. From the final sequence list, we randomly

selected 64 sequences for bead colour code and 16 for binding sequences between Branch 1 and Branch 2. The design steps outlined above are summarized in Table 1.

Next, we designed the cell barcodes to identify each bead. We designed the cell barcode with the combination of Barcode 1 and Barcode 2 sequences, both 10-nt in length, ligating via a bridge sequence in the split and pool synthesis. First, we generated all possible 10-nt sequences ($4^{10} = 1,048,576$) composed of A, T, G, and C, and selected those with GC content between 40% and 60%. We then filtered sequences with a Levenshtein distance of at least three between sequences and with their reverse complements. We calculated binding affinities with Readout, Branch 1, Branch 2, colour-code sequences, PCR handles, and bridge sequences, and selected sequences with $r > 0.9$. In the subsequent step, we virtually synthesized the sequence of bead primer by concatenating the sequence of PCR handle, colour code, Barcode 1, Bridge, Barcode 2, UMI, and polyT sequence, and calculated the cross-reactivity. To reduce calculation costs, we divided the sequence of bead primer into two parts and calculated the cross-reactivity; the first part consisted of PCR handle, colour code, Barcode 1, and the first half of the Bridge sequence. The second part consists of the second half of the Bridge sequence, Barcode 2, UMI, and polyT sequence. We calculated cross-reactivity between the candidates and Branch 1 and selected the candidates with minimum r values > 0.8 . Additionally, cross-reactivity between the candidates and Branch 2 and Readout was calculated, selecting the candidates with $r > 0.8$. Of the remaining candidates containing barcode 1 sequences, the top 30 sequences with the least cross-reactivity for each colour code were selected. For the candidate containing barcode 2, the top 100 sequences with minimal cross-reactivity were selected. All combinations of these barcode 1 and barcode 2 sequences were used to generate full- sequence of bead primer, and cross-reactivity with Branch 1, Branch 2, and Readout was calculated. Based on these calculations, sequences with r median values ≤ 0.8 for barcode 1 and barcode 2 directions were excluded. Furthermore, sequences with minimum r values of ≤ 0.2 for the barcode 2 direction were eliminated. Barcode 2 sequences common to all colour codes were extracted, and self-annealing values were calculated to exclude dimer-forming sequences. Finally, for each stem sequence, binding calculations were performed under conditions of an initial

concentration of 20 nM, temperature of 25°C, [Na⁺] = 1.0 M, and maximum complex size of 2. Dimer concentrations were obtained, and the dimer formation rate was calculated as $1 - [\text{dimer}]_{\text{eq}} / [\text{strand}]_{\text{init}}$, excluding barcode 1 sequences with a median dimer formation rate of ≥ 0.2 . From the final candidate sequences, the top 15 sequences with the least cross-reactivity were selected for barcode 1 for each colour code, while all remaining candidate sequences were adopted for barcode 2.

Table 1 Procedure to generate 20-nt orthogonal barcodes with minimized cross-reactivity

Step	Process	The number of candidate barcodes
1	Generate random 20-nt sequence	1,000,000
2	Select barcodes with GC content between 40% and 60%	303,666
3	Select barcodes with a Levenshtein distance of at least 5 between barcodes	73,125
4	Select barcodes with a Levenshtein distance of at least 6 from the reserved sequences	19,374
5	Remove cross-reactive barcodes based on calculation of binding affinities with unpack	398

3.2.3 Synthesis of DNA barcoded hydrogel beads

We synthesized the polyacrylamide hydrogel beads with poly(dT) sequences through two rounds of split-pool ligation as previously described in Chapter 2. We generated droplets of acrylamide premix with 50 μM acrydited primer (Table S3, TypeA/B base PCR, 5'-Acryd/AAGCAGTGGTATCAACGCAGAGTACGACGCTCTT-3') using a simple coflow microfluidic device. The final bead size was approximately 40 μm . We then ligated the first barcode fragments containing the bead colour code to the first part of the cell barcode (Table S3, Stem_6bit_CC00_Bcd1-00–Stem_6bit_CC63_Bcd1-09). In the second round of ligation, we added fragments with the second part of the cell barcode, UMI and poly(dT)

(NNNNNNNNTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTVN) (Table S3, Stem_6bit_Bcd2-00–Stem_6bit_Bcd2-11). The combination of the first and second parts of the cell barcode created 7,680 unique barcodes.

3.2.4 Staining of beads with colour codes

To stain beads with colour codes, we pooled 5×10^3 beads containing cell barcodes into a single tube. The beads were washed three times with 2X hybridization buffer (4X SSC, 40% formamide, and 0.2% v/v Tween-20). For each wash, 140 μ L of 2X hybridization buffer was added to the bead solution, briefly vortexed, and then centrifuged at 1,000 g for 3 minutes to pellet the beads. After centrifugation, 140 μ L of the supernatant was removed. The bead solution was adjusted to a final volume of 25 μ L by adding 2X hybridization buffer.

Next, 25 μ L of the bead solution was combined with 25 μ L of 2X Branch 1 probe mixture, which consisted of 0.4 μ M Branch 1 oligonucleotides (Table S3, Branch1-00_000000–Branch1-63_111111) and 0.4 mg/mL salmon sperm DNA. The beads were incubated in the Branch 1 probe mixture for 1 hour at room temperature, followed by two washes with 140 μ L of washing buffer (2X SSC and 0.1% Tween-20). The beads were then washed three times with 2X hybridization buffer and adjusted to a final volume of 25 μ L.

Subsequently, 25 μ L of the bead solution was combined with 25 μ L of 2X Branch 2 probe mixture, consisting of 0.4 μ M Branch 2-1 and Branch 2-2 oligos (Table S3, Branch2-1-00_0X0X0X–Branch2-2-15_X1X1X1) and 0.4 mg/mL salmon sperm DNA. The beads were incubated in the Branch 2 probe mixture for 1 hour at room temperature, followed by two washes with 140 μ L of washing buffer. The beads were washed three additional times with 2X hybridization buffer and adjusted to a final volume of 25 μ L.

Finally, 25 μ L of the bead solution was combined with 25 μ L of 2X Readout probe mixture, containing 0.4 μ M Readout oligonucleotides (Table S3, Alexa647, Alexa488, Alexa546, Alexa750, ATTO532, Rho101, antiAlexa647, antiAlexa488, antiAlexa546, antiAlexa750, antiATTO532, antiRho101) and 0.4 mg/mL salmon sperm DNA. The

beads were incubated in the Readout probe mixture for 1 hour at room temperature, followed by three washes with 140 μ L of washing buffer.

3.2.5 Cell colour coding and drug treatment

HeLa/Fucci2 (RCB2867, RIKEN BRC), HeLa (RCB0007, RIKEN BRC), and NIH/3T3 (RCB2767, RIKEN BRC), cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, 08456-65). The culture medium was supplemented with 10% fetal bovine serum (FBS, 26140-079, gibco) and 1% penicillin-streptomycin (P/S, P4333-100ML, Sigma-Aldrich).

For experiment in section 3.3.1, we seeded the HeLa/Fucci2 cells in 16 wells of a 24-well plate and cultured them for one day. We individually transfected cells with different types of DNA tags using Lipofectamine® 3000 reagent (Invitrogen™) according to the manufacturer's protocol (incubation for 4 h at 20 nM DNA tag concentration). After lipofection, we washed the cells three times with culture medium. We individually stained the cells in each well with 16 different combinations of four CellTrace dyes (5 μ M Violet, 250 nM CFSE, 250 nM Yellow, and 50 nM Far Red). We washed the cells three times with $1\times$ PBS. After trypsinisation, the 16 sub-pools of cells at equal concentration were pooled equally into one tube. We then suspended the 16 sub-pools of cells in a tube of imaging medium (DMEM, 08490-05, Nacalai Tesque, without FBS and P/S) at equal concentration.

For experiment in section 3.3.3, we stained the HeLa and NIH/3T3 cells individually in each well with 16 different combinations of four CellTrace (2.5 μ M Violet, 2.5 μ M CFSE, 2.5 μ M Yellow, and 0.5 μ M Far Red) according to the manufacturer's protocol. The cells were suspended in 200 μ L Gene Pulser® Electroporation Buffer with 100 nM DNA tags and performed electroporation using Gene Pulser Mxcell™ Electroporation System (Bio-Rad) with the following parameters: 250 V, 2000 μ F, ∞ Ohm, 20 ms for HeLa, 400 V, 950 μ F, ∞ Ohm, 20 ms for NIH/3T3, and voltage of 200 V. Subsequently, we added 1 mL medium immediately and incubated 1 h. After wash with $1\times$ phosphate-buffered saline (PBS, 14249-24, Nacalai Tesque), We then suspended the

16 sub-pools of cells in a tube of imaging medium at equal concentration.

3.2.6 On-chip experiment procedure

The workflow employed in this study was adapted from protocols reported in Chapter 2. In brief, we prepared functionalised arrays and placed one on a glass-based dish (3961-035, IWAKI) and seeded pools of colour-coded cells by dropping them onto the microwell array. After washing the array surface, we imaged the cells in the imaging medium using the setup described in Table S4. Next, we seeded 2.0×10^4 of colour-coded beads suspended in bead loading buffer (1% polyvinylpyrrolidone in $1\times$ PBS) and dropped them onto the microwell array for 10 min. We then tapped the array and allowed it to rest for 1 min at 37°C, repeating this process five times. To seal the microwells, we pre-treated a track-etched membrane with 10 nm diameter nanopores (Sterlitech) using a UV ozone cleaner (ASM401N, Asumi Giken, Limited) for 5 min and then hydrated it in PBS. We pressed the membrane and the PDMS slab together by placing an 8 mm square glass slide (S7214, MATSUNAMI) and a 100 g weight on top at 37°C for 30 min. We lysed the cells with 3 mL of cell lysis buffer (5 M guanidine thiocyanate, 1 mM EDTA, 0.50% sarkosyl (N-laurylsarcosine), and 1.0% 2-mercaptoethanol) for 20 min, allowing the mRNA and DNA tags to hybridize with dT primers on the hydrogel beads for 40 min while agitating the mixture at 5–60 rpm. After removing the membrane from the microwell array, we captured images of the microwell array containing hydrogel beads using the setup described in Table S5. All hydrogel beads from the microwells were collected into a tube by flushing them with a collection buffer composed of 2 M NaCl, 3 mM MgCl₂, 20 mM Tris-HCl (pH 8.0), 0.64% PEG8000, and 0.1% Tween 20. The beads were then suspended in a total volume of 200 µL. Following this, the hydrogel beads were washed twice using a washing buffer, with buffer exchange performed through centrifugation at 300 g for 5 min at 4°C. The composition of the washing buffer was adjusted according to the type of reverse transcriptase being used. For SMARTScribe Reverse Transcriptase, the washing buffer contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM MgCl₂, 0.4 U/µL Recombinant RNase inhibitor (2313A, Takara), and 0.1%

Tween 20. In contrast, for Maxima H Minus Reverse Transcriptase, the buffer contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 0.2 U/μL Recombinant RNase inhibitor (2313A, Takara), 0.1% Tween 20, and 1 mM Dithiothreitol (DTT, 14128-46, Nacalai Tesque). Finally, the total volume of the bead suspension was reduced to 10 μL after centrifugation at 1000 g for 5 min at 4°C.

3.2.7 Library preparation

We prepared sequencing libraries using two types of reverse transcriptase. The protocol for SMARTScribe Reverse Transcriptase is the same as in Chapter 2. Here, we describe the protocol for Maxima H Minus Reverse Transcriptase (EP0751, Thermo Scientific).

For bulk experiments, we extracted purified total RNA from HeLa cells using the NucleoSpin® RNA XS kit. A 100 pg of total RNA was suspended in 5 μL of buffer containing 0.5 μL of reaction buffer (9.5X Lysis buffer, 635013, Takara, and 4 U/μL Recombinant RNase inhibitor) and hydrogel beads carrying dT probes at an equivalent concentration of 1.2 -12 μM dT primer. Total RNA and dT primers were heat denatured at 72°C for 3 min followed by rapid cooling on ice for 2 min. We subsequently added 5 μL of reverse transcription (RT) enzyme mix. The RT enzyme mix was adjusted to yield a final concentration of 1X RT buffer, 0.5 mM dNTP mix, 1 U/μL recombinant RNase inhibitor, 2 μM biotinylated template switching oligonucleotide (TSO, Qiagen), and 2 U/μL Maxima H Minus Reverse Transcriptase.

For the on-chip experiment, we added 2X RT buffer, 1 mM dNTP mix, 2 U/μL recombinant RNase inhibitor, 4 μM biotinylated TSO, and 4 U/μL Maxima H Minus Reverse Transcriptase to the hydrogel-beads suspensions.

We synthesized the first strand cDNA in a thermal cycler at 42°C for 90 min, followed by 10 cycles of 50°C for 2 min and 42°C for 2 min, and a final step at 85°C for 5 min (Hagemann-Jensen, M. *et al.*, *Nat. Biotechnol.*, 2020). To remove excess dT primer, we added Exonuclease I (2650A, Takara) at a working concentration of 0.24 U/μL.

Samples were incubated at 37°C for 30 minutes followed by 80°C for 20 minutes to inactivate the enzyme. We added 60 U/μL ribonuclease H (2150A, Takara) at a working concentration of 2.73 U/μL and incubated for 30 min at 37°C followed by 20 min at 65°C. Then, we synthesized the second strand cDNA by adding 10 mM dNTP, 100 μM dN-SMART primer, and 5,000 U/mL DNA Polymerase I, Large (Klenow) Fragment (M0210S, New England Biolabs), which were adjusted at a working concentration of 0.947 mM, 9.82 μM, and 175 U/mL, respectively, and incubated at 37°C for 60 min followed by heating at 75°C for 20 min. We washed the samples three times with 200 μL of washing buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 1 mM DTT, and 0.657X Lysis buffer). After each wash, we centrifuged the samples at 1000 g for 5 min at 4°C, reducing the supernatant volume to 40 μL. After the final wash, we concentrated the suspension to 11 μL. The samples were then amplified by PCR in a 25 μL reaction containing 0.24 μM primer2, 9 nM additive primer, 1x SeqAmp PCR Buffer and 0.025 U/μL SeqAmp DNA Polymerase (Takara) using the following program: 95°C for 1 min; 16-18 cycles of 98°C for 10 s and 65°C for 30 s; 68°C for 4 min; and 72°C for 10 min. Following the PCR, we fractionated the long cDNA derived from cellular mRNA (retained on beads) against the short cDNA tag fraction. The long cDNA fraction was processed according to the protocol to generate the transcriptome library. To capture short cDNA tag in the supernatant, 1.4x volume of SPRI beads was added to the supernatant and processed. The Purified DNA was eluted in the 13 μL or 11 μL of elution buffer (10 mM Tris-HCl, pH 8.5), respectively. We examined the long cDNA yield and quality using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and a quantitative real-time PCR (qPCR) targeting human *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Hs02758991_g1, Thermo Fisher Scientific).

The following procedures are the same as Chapter 2. In brief, long cDNA were fragmented and PCR with Nextera XT (Illumina) and custom primers, followed by further purification by SPRIselect bead. The DNA tag library was amplified using KAPA Hifi Hotstart (Roche) and purified by SPRIselect beads. The library was sequenced on a MiSeq (Illumina) instrument with 2 x 150 bp paired-end reads.

3.2.8 Imaging analysis procedures

We acquired images of cells and hydrogel beads scanning the entire regions of each microwell array while controlling the sCMOS camera (SONA-4BV6U, Andor), auto XY stage, LED light source (Xcite® Novem XT910, Excelitas Technologies Corp.), filter wheels (FW102C, Thorlabs), and microscope via MicroManager (Edelstein, A. D. *et al.*, *J. Biol. Methods*, 2014). Uneven illuminations are corrected by dividing estimated flat-field image before stitching them together.

For cell images, we performed spectral unmixing using the PICASSO algorithm (Seo, J. *et al.*, *Nat. Commun.*, 2022). During each iteration of the unmixing process, we applied a mask to the images using a gridded region with a Structural Similarity Index (SSIM) value of 0.5 or higher to minimize overcorrections (Cang, H. *et al.*, *Bioinformatics*, 2024). This was followed by cross-channel registration, resizing the images to 40% of their original scale to account for chromatic aberration, and background subtraction using a top-hat filter provided as a built-in MATLAB function.

3.2.9 Colour code prediction model

We created a model for the bead colour code prediction by random forest. For reference data, we synthesised 64 different colours of beads separately and imaged the beads respectively. We extracted the mean intensities of the beads from all the fluorescence channels as described in the Table S4 for both reference data and test data. The intensities of beads are transformed to a centred log ratio across beads in the same chip or reference data. To remove batch effects in the test data, we integrate data from different batches to reference data using the functions of ‘*SelectIntegrationFeatures*’, ‘*FindIntegrationAnchors*’, and ‘*IntegrationData*’ from Seurat (version 5.1) package. We trained the prediction model using the mean intensities from reference (n = 2880) data and predicted the colour of test data using caret (method "rf") R package.

3.3 Results

3.3.1 Compatible implementation for scalable colour coding and multiplexed phenotypic imaging

We demonstrated the compatibility of the signals of molecular features and cell colour codes by unmixing the signals of a fluorescent ubiquitination-based cell cycle indicator (Fucci) in HeLa/Fucci(SA)2 cells labelled with 16 colour codes. HeLa/Fucci(SA)2 cells show the expressions of green (mVenus) and red fluorescent proteins (mCherry), which maximised the fluorescence in the G2 and the late G1 phases, respectively. We labelled the cells with a combination of four different dyes (CellTrace Violet, CFSE, Yellow, and Far Red from Thermo Fisher Scientific), with emission spectra overlapping between CFSE and mVenus, and between Yellow and mCherry. We designed the combination of excitation and emission filters to ensure that the intended fluorescence signal was prominent from other spillovers in each channel (Table S3). Our unmixing technique based on the minimization of mutual information between mixed images successfully resolved these overlapping signals from the individual dyes/proteins (Fig. 3.2A). To confirm that the cellular states defined by Fucci signal intensity were not perturbed by the unmixing process, we analysed the cell cycle distribution with and without unmixing. In cells stained with colour codes, the expression patterns of mVenus and mCherry, as well as the proportions of cell cycle phases defined by these signals, differed significantly from those in cells without colour code staining due to the impact of fluorescence spillover (Fig. 3.2B, C). In contrast, in the data processed with unmixing, the effects of fluorescence spillover were removed, and the expression patterns of mVenus and mCherry, as well as the proportions of cell cycle phases, showed no differences between cells with and without colour code staining (Fig. 3.2B, C). Next, we demonstrated the unmixing process rescues the accuracy of decoding of cell colour codes. Without unmixing, the data points of different colour codes were intermixed on the UMAP (Fig. 3.2D). In contrast, after unmixing, the data points of each cell were clearly separated into 16 distinct regions according to their colour codes. The precision-recall curves of the colour code separation show that, with unmixing, the accuracy of the colour code decoding is compatible between with and without Fucci cells (0.873 and 0.855),

while the decoding accuracy is low in the case without unmixing (0.722) (Fig. 3.2E). Taken together, these results demonstrated unmixing allows for the measurement of cellular states defined by the two fluorescent proteins of Fucci while maintaining the number of cell colour codes.

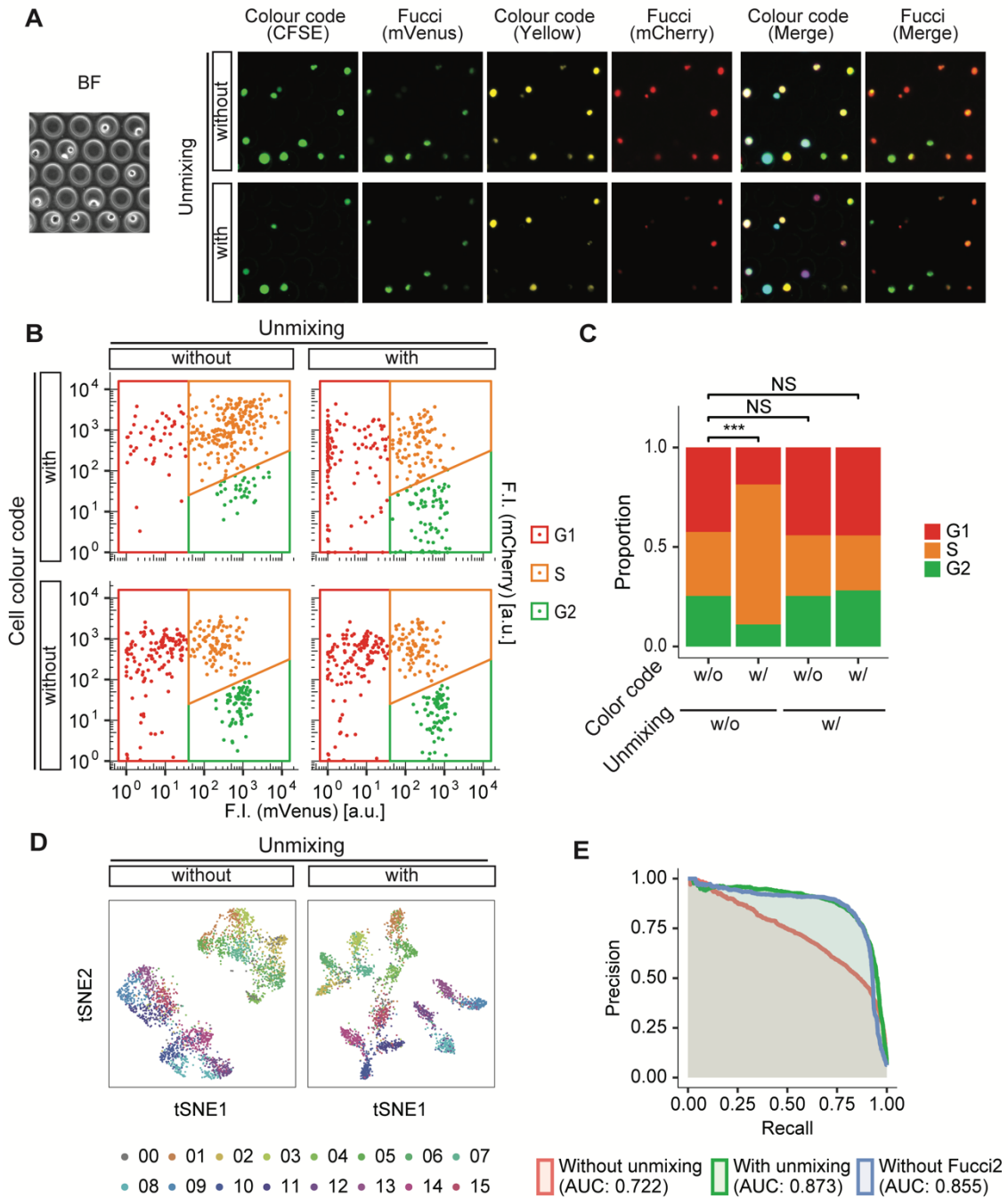


Figure 3.2 Benchmarking the unmixing of fluorescence for the multiplexed phenotypic imaging

A. Representative fluorescence images of colour-coded HeLa/Fucci(SA)2 cells before and after unmixing. B. Scatter plot of Fucci(SA)2 signals before and after unmixing. The colours represent the cell cycle stage decided with the corresponding gates. C. Proportions of the cell cycle stages in (B). P values are derived from Pearson's chi-square test. D. Uniform manifold approximation and projection (UMAP) shows cell colour code before and after unmixing. E. Precision-Recall curves of the colour-code segregation. The values are micro averages of all 16 IDs. We modelled the classification score using logistic regression to estimate the positive and negative cells in channels for colour codes.

Further, the opto-combinatorial indexing leveraging the unmixing showcases the multimodal analysis on the dynamics of cell cycle progression by combining the protein and mRNA expression of single cells (Fig. 3.3). The S score and G2M score, which characterize transcriptomic state for cell cycle phases of single cells, show on average the expected trend along the progression of cell cycle and further interesting cell-to-cell heterogeneity.

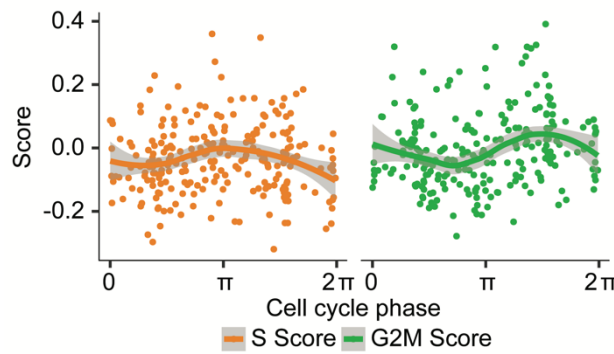


Figure 3.3 Cell cycle scores of gene expression against cell cycle phase defined by unmixed Fucci2 signals

Lines represents LOESS curves and areas represents their 95% confidence intervals.

3.3.2 Improvement of library qualities

In this study, we optimized conditions to enhance sequencing library quality for capturing cell-to-cell heterogeneity in RNA expression across cytological phenotypes. A summary of all examined experimental conditions is provided in Appendix Table S6. Here, we focus on the key experimental methods pertinent to this study.

First, we evaluated reverse transcription conditions using bulk purified RNA. Our previous protocol used SmartScribe reverse transcriptase. Prior studies have shown that using Maxima H Minus improves cDNA yield (Bagnoli, J. W. *et al.*, *Nat. Commun.*, 2018), and that second-strand synthesis using random primers can recover cDNA affected by template switching, enhancing gene detection (Hughes, T. K. *et al.*, *Immunity*, 2020). Our experiments confirmed that combining Maxima H Minus and second-strand synthesis increased cDNA yield (Fig. 3.4A).

We further assessed the number of detected transcripts and detected genes per sequencing read (Fig. 3.4B, C). We observed the number of detected transcripts improved when the reverse transcriptase switched from SmartScribe to Maxima H Minus, and the number of detected genes improved with the introduction of second-strand synthesis. In on-chip experiments, similar comparisons revealed that using Maxima H Minus and second-strand synthesis enhanced cDNA yield and library complexity (Fig. 3.4D-F). Specifically, gene detection in the on-chip experiment was 531 ± 234 per 828 ± 460 UMIs per cell with Maxima H Minus, compared to 419 ± 244 genes per 601 ± 437 UMIs per cell with SmartScribe RT, using 5000 reads per cell.

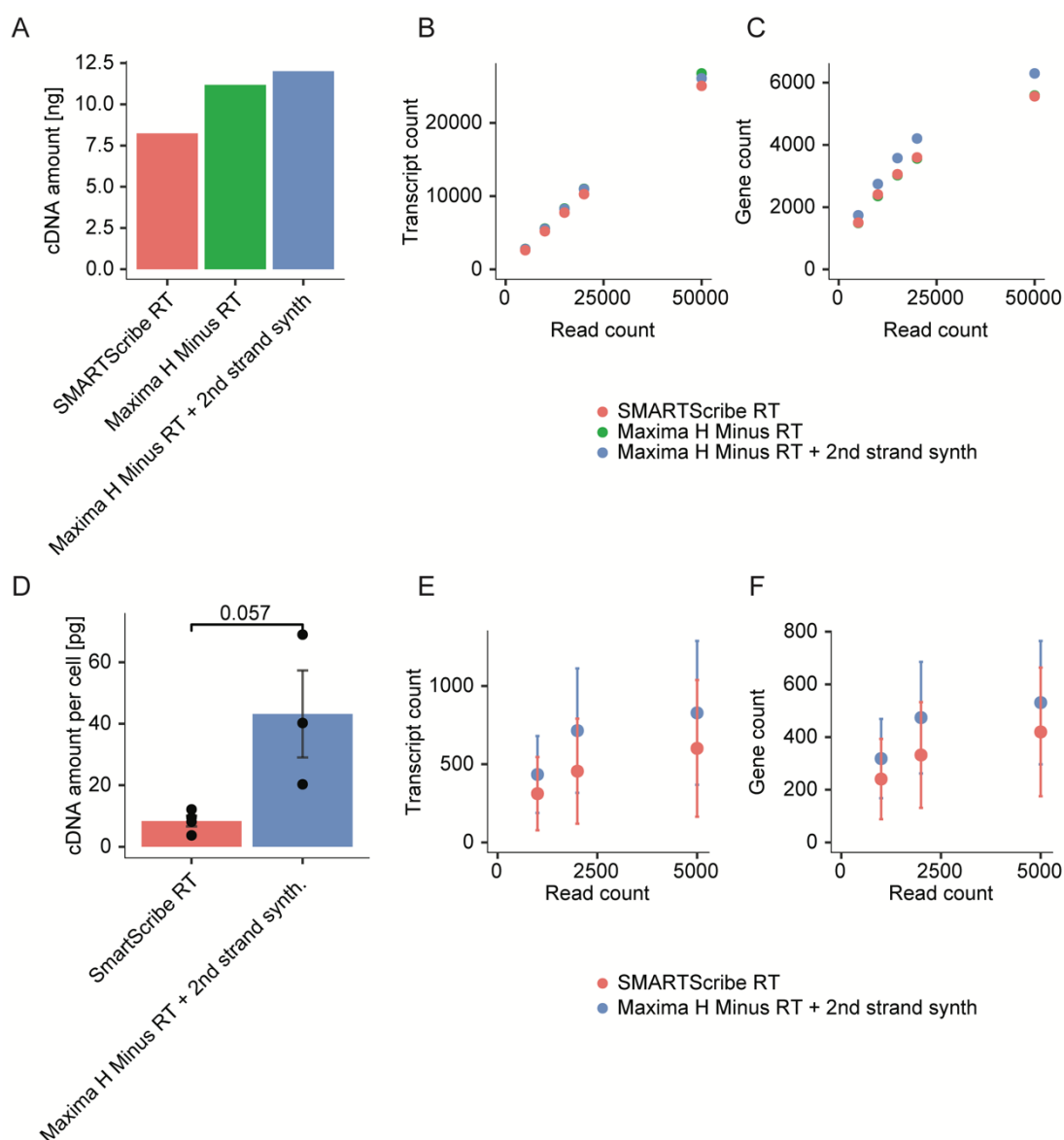


Figure 3.4 Improvement of cDNA library preparations.

A. cDNA yields synthesised from purified RNA after reverse transcription and PCR amplification, comparing different reverse transcription enzymes and second-strand synthesis probes. B. C. Scatter plot showing transcript counts (B) and gene counts (C) against read count, comparing different library preparation protocols synthesized from purified RNA. D. cDNA yields synthesized from on-chip experiment after reverse transcription and PCR amplification, comparing different library preparation protocols. The p value was calculated using the Wilcoxon rank sum tests. E. F. transcript counts (E) and detected gene count (F) of on-chip experiment comparing different library

preparation protocols.

3.3.3 Scaling the capability of multiplexing by increasing the number of colour codes for beads

Increasing the number of colour codes for beads allows for an increase in the multiplex number of cells. To achieve this, we established a pipeline for designing probe sequences for colour-coded beads by generating orthogonal barcode sequences, based on binding energy calculations and minimized cross-reactivity among different IDs (see methods).

As a proof of concept, we designed colour-coded beads with 64 colour codes, using six fluorescent dyes (Fig. 3.5A). To decode the colour code sequences by the combinations of six fluorescent dyes (Alexa488, ATTO532, Alexa546, Rho101, Alexa647, and Alexa750), we developed a molecular system of sequentially hybridized probes consist of Branch 1, Branch 2, fluorescent dyes conjugated DNA probes. From the 1,000,000 candidate sequences, we selected 398 orthogonal barcode sequences which have minimized cross-reactivity (Table 1). Using these orthogonal barcodes, we designed 64 colour-coded sequences, 64 Branch 1 sequences, 8 Branch 2-1 sequences, and 8 Branch 2-2 sequences (Fig. 3.5B). Additionally, we designed 7,680 unique cell barcode 640 cell barcode1 sequences and 12 cell barcode2 sequences that do not cross-react with probe sequences, creating a set of colour-coded beads with 64 colours and a total of 7,680 unique cell barcodes (Table S4). Visualizing the high-dimensional data defined by the fluorescence intensities of the six dyes using UMAP, we observed 64 distinct clusters, confirming that the colour-coded beads were successfully fabricated with 64 unique colours as designed (Fig. 3.5C). Furthermore, using data from individually stained samples for each of the 64 colour codes as training data, we employed a random forest classifier to identify the bead colour codes. The predictions on reference data achieved an accuracy of 98.4% for decoding the colour codes (Fig. 3.5D),

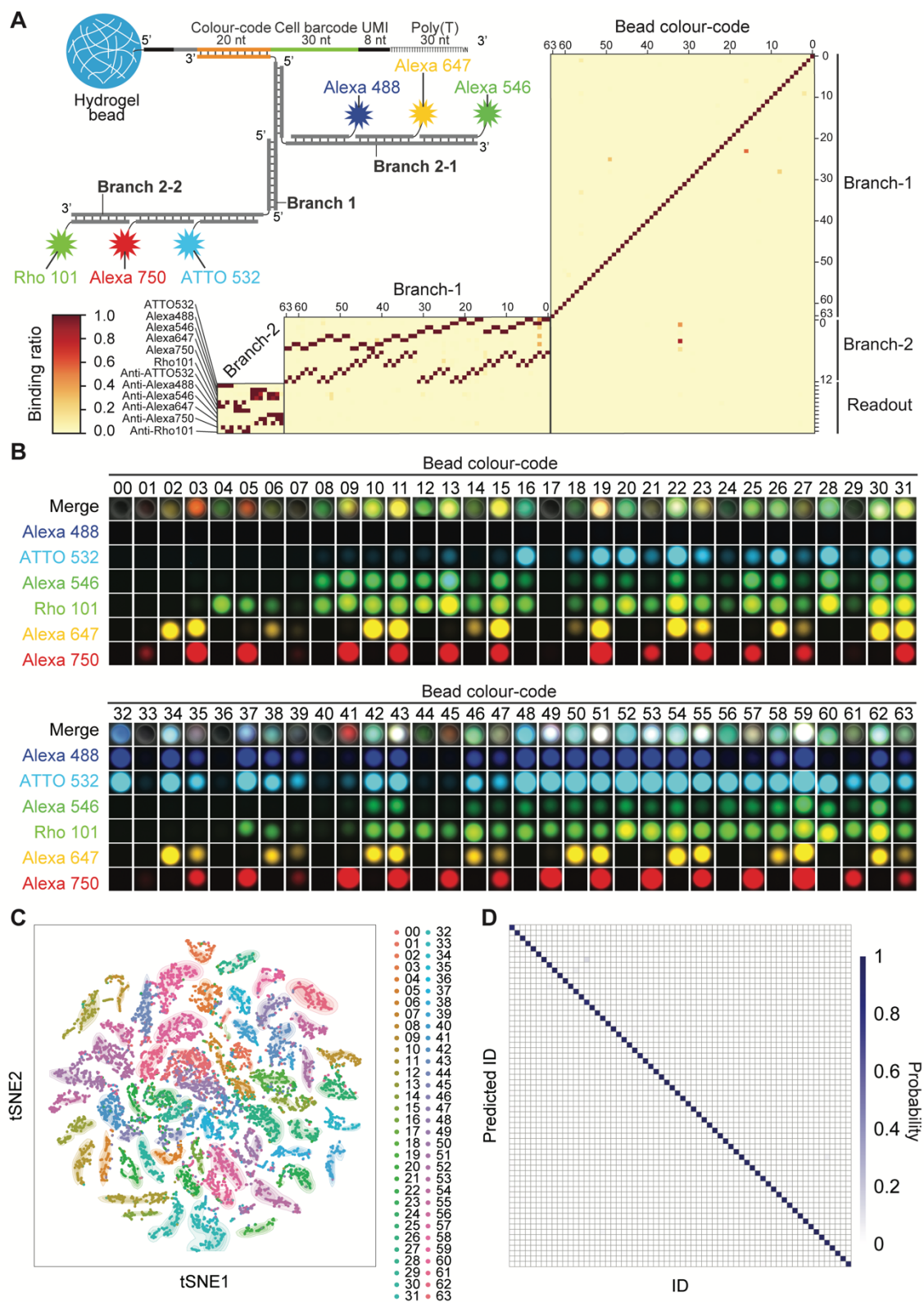


Figure 3.5 Preparation of 6-bit colour coded beads.

A. Design of 6-bit colour-coded beads. (Left) Molecular configuration. Branch 1 and Branch 2 probes convert the colour-coded sequence into a combination of six fluorescent dyes. (Right) Cross-reactivity of probes for colour codes. B. Fluorescence images of 6-bit colour-code beads. C. Uniform manifold approximation and projection (UMAP) showing the fluorescence of 6-bit colour-coded beads. Points represent the beads imaged on the chip, and contours represent the probability distributions of the reference data, with each contour binned at intervals of 0.2. The colors represent the IDs predicted by our random forest model or the reference data IDs. D. Confusion matrix showing bead colour prediction accuracy with our random forest model.

3.4 Summary

In this chapter, we propose a strategy to scale opto-combinatorial indexing to achieve more multiplexed imaging and higher throughput of single cell analysis. This strategy unmix the fluorescence signals used for cytological phenotyping from those used for colour coding by minimizing mutual information between images. In experiments using HeLa Fucci(SA)2 cells, we successfully separated the cell cycle indicator signals mVenus and mCherry of Fucci2 from CellTrace Violet, CFSE, Yellow, and Far Red fluorescence signals without disrupting cell cycle distribution, while maintaining the separation required for optical indexing. Furthermore, through the implementation of opto-combinatorial indexing, we observed heterogeneity in cell cycle scores of gene expression along Fucci2 signals.

To capture this gene expression heterogeneity in detail, we also worked on improving library preparation. By switching the reverse transcriptase to Maxima H Minus and incorporating second-strand synthesis, we confirmed an increase in cDNA yield and the number of detected genes in both bulk and on-chip single-cell experiments.

Additionally, to enhance the number of cells that can be analysed using this method, we designed a DNA molecular barcoding system. This system accurately separates hydrogel beads stained with overlapping fluorescent DNA probes within a multidimensional space and uses a random forest algorithm to decode 64 different bead

colours with an accuracy of 0.984.

4 Conclusion

Multiplex chemical transcriptomics provides mechanistic insights into the cellular responses to the chemical perturbations at the molecular level and offers a comprehensive understanding across pooled conditions, suppressing the batch effect (McFarland, J. M. *et al.*, *Nat. Commun.*, 2020; Srivatsan, S. R. *et al.*, *Science*, 2020). Cellular tagging is a key to demultiplex genetically identical cells, extending its applicability to study chemical-dependent or dose-dependent responses. However, transcriptomics-based screening still faces difficulty in linking molecular responses to key phenotypic expression, such as cell proliferation and morphological change, which are typically profiled by quantitative optical microscopy. The integration of microscopical phenotyping and molecular profiling provides a unique opportunity to dissect the molecular cascades that cause the specific phenotypic expression (Chen, T. N. *et al.*, *Lab Chip*, 2020; Srivatsan, S. R. *et al.*, *Science*, 2020).

There are several strategies for the integrated phenotypic and transcriptomics analysis. The first is the optical decoding of the barcode sequence by sequential fluorescence in situ hybridisation, and the second is physical isolation of the interested cell and indexing by known barcode tags. SCOPE-seq2 employs the former strategy, decoding the cell barcode of the hydrogel beads by performing cyclic hybridisation and readout with automated microfluidic control and microscopic imaging (Liu, Z. *et al.*, *Sci. Rep.*, 2020). As an example of the latter strategy, an automated cell picking system was employed to isolate single cells into 96 well plates and then process them for scRNA-seq (Jin, J. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2023). In contrast to these methods, to link the cellular phenotype and transcriptomics, our approach uses a combination of colour codes of cells and hydrogel beads to optically index pairs of single cells and hydrogel beads. Our approach is free of automated microfluidic controls and robotic systems, has fewer on-chip steps, and is compatible with standard epi-fluorescence microscopy, which are advantageous features to be implemented in a standard laboratory.

Our approach can be readily integrated with the profiling of surface protein via

CITE-seq (Stoeckius, M. *et al.*, *Nat. Methods*, 2017) thereby enabling the analysis to couple with another omics layer. The proposed opto-combinatorial indexing is also compatible with cell-hashing using DNA-tagged antibodies (Stoeckius, M. *et al.*, *Genome Biol.*, 2018) or lipids (McGinnis, C. S. *et al.*, *Nat. Methods*, 2019). The transfection-based approaches (electroporation or lipofection) used in our demonstration are robust and cost-effective, for instance, when assaying cells from non-model organisms.

As demonstrated in our experiments for the paclitaxel burden on HeLa cells, the cell colour code also works as cell hashing for multiplex chemical screening. Our analysis revealed two distinct trajectories in transcriptomic response by paclitaxel treatment, which correspond to distinct phenotypic reactions. The trajectory that involved no multipolar spindle formation showed up-regulation of ER stress response. Prolonged and severe ER stress may induce apoptosis, otherwise leading to the acquisition of drug resistance (Cubillos-Ruiz, J. R. *et al.*, *Cell*, 2017) through the activation of unfolded protein response (UPR), a signalling pathway involved in both adaptive and apoptotic response (Shore, G. C. *et al.*, *Curr. Opin. Cell Biol.*, 2011). The second trajectory exhibited generations of multipolar spindles, leading to chromosome missegregation and cell death (Scribano, C. M. *et al.*, *Sci. Transl. Med.*, 2021).

In conclusion, opto-combinatorial indexing provides a simplified strategy to analyse the image and gene expressions simultaneously from single cells, and effectively dissect the molecular background of distinct phenotypic behaviours by integrating cytologic phenotype and transcriptomics data.

4.1 Recommendations for future studies

Our approach has the potential to be extended to the integrated analysis of dynamic phenotyping and transcriptomics using longitudinal live-cell imaging. The DNA tags were retained within the cells even after 48 h of labelling. For instance, the integration of our approach with longitudinal imaging of leukocytes at the sites of active inflammation can potentially classify leukocytes by spatio-temporal behaviours (Crainiciuc, G. *et al.*, *Nature*, 2022) and uncover the molecular background.

In this study, we demonstrated the unmixing of cell cycle indicators using HeLa Fucci(SA)2 cells. The cell cycle is a highly valuable target for cancer therapy; for example, Palbociclib, a cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) inhibitor, is used in the treatment of ER+ HER2- breast cancer. Previous studies have suggested that cells can adapt to Palbociclib treatment in the early stages (Herrera-Abreu, M. T. *et al.*, *Cancer Res.*, 2016), with cellular heterogeneity playing a significant role in this fractional resistance (Asghar, U. S. *et al.*, *Clin. Cancer Res.*, 2017; Zikry, T. M. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2024). Using our technique, we aim to integratively examine the cellular heterogeneity in Fucci-indicated cell cycle phases and the molecular background associated with cancer drug-induced cell cycle arrest.

We expect that by advancing this approach to incorporate more multiplexed perturbations and accumulating the resulting data, it will be possible to create a comprehensive atlas that describes the overall relationship between cellular phenotypes and gene expression. This method is applicable not only to chemical perturbations but also to genetic and environmental perturbations. By examining changes in cellular phenotypes and gene expression across these diverse perturbations, it becomes possible to identify shared patterns and uncover previously unrecognized relationships. This effort will allow us to map how changes in gene expression, in alignment with the flow of the central dogma, drive changes in cellular phenotypes and their regulation. Ultimately, this approach will provide the holistic perspective on the flow of biological information, and practical guidance on how to design perturbations for cellular manipulation to achieve desired cellular phenotypes.

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List of publications

Journal papers included in this thesis

Chapter 2

Arata Tsuchida, Taikopaul Kaneko, Kaori Nishikawa, Mayu Kawasaki, Ryuji Yokokawa, and Hirofumi Shintaku Opto-combinatorial indexing enables high-content transcriptomics by linking cell images and transcriptomes. *Lab on a Chip* **24**(8): 2287-2297 (2024).

Other journal papers

1. Akifumi Shiomi, Taikopaul Kaneko, Kaori Nishikawa, Arata Tsuchida, Takashi Isoshima, Mayuko Sato, Kiminori Toyooka, Kentaro Doi, Hidekazu Nishikii, and Hirofumi Shintaku High-throughput mechanical phenotyping and transcriptomics of single cells. *Nature Communications* **15**(1): 3812 (2024).
2. Jianming Chen, Arata Tsuchida, Ali D. Malay, Kousuke Tsuchiya, Hiroyasu Masunaga, Yui Tsuji, Mako Kuzumoto, Kenji Urayama, Hirofumi Shintaku, and Keiji Numata Replicating shear-mediated self-assembly of spider silk through microfluidics. *Nature Communications* **15**(1): 527 (2024).
3. Sangamithirai Subramanian Parimalam, Mahmoud N. Abdelmoez, Arata Tsuchida, Naoyuki Sotta, Mayuki Tanaka, Takashi Kuromori, Toru Fujiwara, Masami Yokota Hirai, Ryuji Yokokawa, Yusuke Oguchi, and Hirofumi Shintaku Targeted permeabilization of the cell wall and extraction of charged molecules from single cells in intact plant clusters using a focused electric field. *Analyst* **146**(5): 1604-1611 (2021).
4. Sangamithirai Subramanian Parimalam, Yusuke Oguchi, Mahmoud N. Abdelmoez, Arata Tsuchida, Yuka Ozaki, Ryuji Yokokawa, Hidetoshi Kotera, and Hirofumi Shintaku Electrical Lysis and RNA Extraction from Single Cells Fixed by Dithiobis(succinimidyl propionate). *Analytical Chemistry* **90**(21): 12512-12518 (2018).

Conference presentations and posters

1. Arata Tsuchida, Taikopaul Kaneko, Kaori Nishikawa, Mayu Kawasaki, and Hirofumi Shintaku, Expanding the capacity of opto-combinatorial indexing for multimodal single-cell analysis, *MicroTAS 2024*, Montreal, 14th October (2024).
2. Arata Tsuchida, Taikopaul Kaneko, Kaori Nishikawa, Mayu Kawasaki, and Hirofumi Shintaku, Profiling transcriptomic responses to chemical perturbation by opto-

combinatorial indexing, *Serendipity Symposium 2024*, Ishigaki city, 12th March (2024).

3. 土田 新, 金子 泰洸ポール, 川崎 真由, 横川 隆司, 新宅 博文, DNA バーコード技術を用いた 1 細胞画像・遺伝子発現量解析の統合 1 細胞多階層相関解析, *日本機械学会 2022 年度年次大会*, J224-03, 富山市, 2022 年 9 月 13 日.
4. Arata Tsuchida, Mahmoud N. Abdelmoez, Taikopaul Kaneko, Ryuji Yokokawa, Hirofumi Shintaku, Multimodal profiling of single cells via on-chip electrophoresis and RNA sequencing, *The 2021 International Chemical Congress of Pacific Basin Societies*, Virtual, 20th December (2021).
5. 土田 新, マハムド N. アブデイルモエズ, 金子 泰洸ポール, 横川 隆司, 新宅 博文, 1 細胞電気泳動および遺伝子発現の統合解析に向けた並列マイクロ流体システムの開発, 第 12 回マイクロ・ナノ工学シンポジウム, オンライン, 2021 年 11 月 9 日.
6. 土田 新, マハムド N. アブデイルモエズ, 横川 隆司, 新宅 博文, オンチップ電気泳動を用いた 1 細胞多階層相関解析, *日本機械学会 2020 年度年次大会*, 名古屋市, 2020 年 9 月 16 日.
7. 土田 新, 横川 隆司, 新宅 博文, 1 細胞解析のためのオンチップ電気泳動システムの開発, 第 10 回マイクロ・ナノ工学シンポジウム, 浜松市, 2019 年 11 月 20 日.
8. 土田 新, 横川 隆司, 新宅 博文, オンチップ電気泳動を用いた 1 細胞解析システムの開発, *シングルセルゲノミクス研究会 2019*, 柏市, 2019 年 8 月 29 日.
9. Arata Tsuchida, Ryuji Yokokawa and Hirofumi Shintaku, Integration of on-chip electrophoretic analysis on cytoplasmic RNA of single cells and high-throughput RNA-sequencing, *ASME - JSME - KSME Joint Fluids Engineering Conference 2019*, San Francisco, USA, 31st July (2019).
10. 土田 新, 畑 翔太, 横川 隆司, 小寺 秀俊, 新宅 博文, 一細胞 RNA 網羅解析に向けた ribosomal RNA 除去法の開発, *日本機械学会 2017 年度年次大会 流体工学部門優秀講演セッション*, さいたま市, 2017 年 9 月 4 日.
11. 土田 新, 畑 翔太, 横川 隆司, 小寺 秀俊, 新宅 博文, 一細胞 RNA 網羅解析に向けた ribosomal RNA 除去法の開発, *日本機械学会 2017 年度年次大会*,

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Appendix

Table S1. Sequences and modifications of the oligonucleotides for 4 bit beads

Oligonucleotide Name	Oligonucleotide Sequence and Modification
TypeA/B base PCR	/5Acrd/AAGCAGTGGTATCAACGCAGAGTACGA CGCTCTT
Bridge A	AGATCGG AAGAGCG
Bridge C	GACTTGT CTAGAGC
Readout Alexa647	/Alexa647/GAGGGCGTTCAATCACTC
Readout Alexa488	/Alexa488/GGAATAACGAAGCACCCA
Readout Alexa555	/Alexa555/CTGTGTAGAGGGCGAGAC
Readout Alexa750	/Alexa750/GGTGTGTGTGCTTCAAAC
Readout R0	TGATGTTGTACCTGCGAG
Readout G0	GAACCAGGGACATTCCAG
Readout P0	AGCATTTACGGGATCAGC
Readout B0	GATTGGGAATGTCTGCCA
Branch 00 NNNN	CTGGAATGTCCTGGTTC CTCGAGGTACAACATCA TGGCAGACATTCCCAATC CGTGATCCCGTAAATGCT GTCTAATAGGTAGGCCTAT
Branch 01 NNNR	CTGGAATGTCCTGGTTC GAGTGATTGAACGCCCTC TGGCAGACATTCCCAATC CGTGATCCCGTAAATGCT TTCTGAGGACATACCTCGAC
Branch 02 NNPN	CTGGAATGTCCTGGTTC CTCGAGGTACAACATCA TGGCAGACATTCCCAATC GTCTCGCCCTCTACACAG AGCATACGGCGTTCGGTATC
Branch 03 NNPR	CTGGAATGTCCTGGTTC GAGTGATTGAACGCCCTC TGGCAGACATTCCCAATC GTCTCGCCCTCTACACAG TAGCTGCTGTAGTAACTT
Branch 04 NGNN	TGGGTGCTTCGTTATTCC CTCGAGGTACAACATCA TGGCAGACATTCCCAATC CGTGATCCCGTAAATGCT AAGCGTATATTTCGTCGAC
Branch 05 NGNR	TGGGTGCTTCGTTATTCC GAGTGATTGAACGCCCTC TGGCAGACATTCCCAATC CGTGATCCCGTAAATGCT AATCTCGCGTTTGAATCGCT
Branch 06 NGPN	TGGGTGCTTCGTTATTCC CTCGAGGTACAACATCA TGGCAGACATTCCCAATC GTCTCGCCCTCTACACAG GCCTTACCGATAAGGCTGGT
Branch 07 NGPR	TGGGTGCTTCGTTATTCC GAGTGATTGAACGCCCTC TGGCAGACATTCCCAATC GTCTCGCCCTCTACACAG CCGCAATCCGTTGGTAATA
Branch 08 BNNN	CTGGAATGTCCTGGTTC CTCGAGGTACAACATCA GTTTGAAGCACACACACC CGTGATCCCGTAAATGCT TCCGAGACCATAGAGTCTCA
Branch 09 BNNR	CTGGAATGTCCTGGTTC GAGTGATTGAACGCCCTC GTTTGAAGCACACACACC CGTGATCCCGTAAATGCT GAGAGGAATTTGCTCTCAGG
Branch 10 BNPN	CTGGAATGTCCTGGTTC CTCGAGGTACAACATCA GTTTGAAGCACACACACC GTCTCGCCCTCTACACAG AAGGCCATCATTTGCCAGGT
Branch 11 BNPR	CTGGAATGTCCTGGTTC GAGTGATTGAACGCCCTC GTTTGAAGCACACACACC GTCTCGCCCTCTACACAG AGCTGCCTCAAGGCATTCTAT
Branch 12 BGNN	TGGGTGCTTCGTTATTCC CTCGAGGTACAACATCA GTTTGAAGCACACACACC CGTGATCCCGTAAATGCT TCAATATCGACGAGTTCGCC
Branch 13 BGNR	TGGGTGCTTCGTTATTCC GAGTGATTGAACGCCCTC GTTTGAAGCACACACACC CGTGATCCCGTAAATGCT TTATGACCAACCGGTCTCTAA
Branch 14 BGNP	TGGGTGCTTCGTTATTCC CTCGAGGTACAACATCA GTTTGAAGCACACACACC GTCTCGCCCTCTACACAG GTAGGAACCAATCCAACCTAC
Branch 15 BGPR	TGGGTGCTTCGTTATTCC GAGTGATTGAACGCCCTC GTTTGAAGCACACACACC GTCTCGCCCTCTACACAG GTTCTCTCCTTCGGGAGGAA
Stem CC00 ID01	CCGATCT ATAGGCCTCACCTATTAGAC CATCCGTA GCTCTAG
Stem CC00 ID02	CCGATCT ATAGGCCTCACCTATTAGAC GTCTAGTG GCTCTAG
Stem CC00 ID03	CCGATCT ATAGGCCTCACCTATTAGAC CGGAAGAA GCTCTAG
Stem CC00 ID04	CCGATCT ATAGGCCTCACCTATTAGAC GCCAAGAA GCTCTAG
Stem CC00 ID05	CCGATCT ATAGGCCTCACCTATTAGAC GGAGAGAA GCTCTAG
Stem CC00 ID06	CCGATCT ATAGGCCTCACCTATTAGAC ACGGAGAA GCTCTAG
Stem CC00 ID07	CCGATCT ATAGGCCTCACCTATTAGAC CACGAGAA GCTCTAG
Stem CC00 ID08	CCGATCT ATAGGCCTCACCTATTAGAC CCACAGAA GCTCTAG
Stem CC00 ID09	CCGATCT ATAGGCCTCACCTATTAGAC GAGCAGAA GCTCTAG
Stem CC00 ID10	CCGATCT ATAGGCCTCACCTATTAGAC AGCCAGAA GCTCTAG
Stem CC00 ID11	CCGATCT ATAGGCCTCACCTATTAGAC TCGAGGAA GCTCTAG
Stem CC00 ID12	CCGATCT ATAGGCCTCACCTATTAGAC CTCAGGAA GCTCTAG
Stem CC00 ID13	CCGATCT ATAGGCCTCACCTATTAGAC GGTAGGAA GCTCTAG
Stem CC00 ID14	CCGATCT ATAGGCCTCACCTATTAGAC TGACGGAA GCTCTAG
Stem CC00 ID15	CCGATCT ATAGGCCTCACCTATTAGAC ATGCGGAA GCTCTAG
Stem CC00 ID16	CCGATCT ATAGGCCTCACCTATTAGAC GCATGGAA GCTCTAG
Stem CC00 ID17	CCGATCT ATAGGCCTCACCTATTAGAC CAGTGGAA GCTCTAG
Stem CC00 ID21	CCGATCT ATAGGCCTCACCTATTAGAC TCAGCGAA GCTCTAG

Stem CC00 ID22	CCGATCT ATAGGCCTCACCTATTAGAC ATCGCGAA GCTCTAG
Stem CC00 ID24	CCGATCT ATAGGCCTCACCTATTAGAC CGATCGAA GCTCTAG
Stem CC01 ID01	CCGATCT GTCGAGGTATGTCCTCAGAA CATCOGTA GCTCTAG
Stem CC01 ID02	CCGATCT GTCGAGGTATGTCCTCAGAA GTCTAGTG GCTCTAG
Stem CC01 ID03	CCGATCT GTCGAGGTATGTCCTCAGAA CGGAAGAA GCTCTAG
Stem CC01 ID04	CCGATCT GTCGAGGTATGTCCTCAGAA GCCAAGAA GCTCTAG
Stem CC01 ID06	CCGATCT GTCGAGGTATGTCCTCAGAA ACGGAGAA GCTCTAG
Stem CC01 ID08	CCGATCT GTCGAGGTATGTCCTCAGAA CCACAGAA GCTCTAG
Stem CC01 ID09	CCGATCT GTCGAGGTATGTCCTCAGAA GAGCAGAA GCTCTAG
Stem CC01 ID10	CCGATCT GTCGAGGTATGTCCTCAGAA AGCCAGAA GCTCTAG
Stem CC01 ID11	CCGATCT GTCGAGGTATGTCCTCAGAA TCGAGGAA GCTCTAG
Stem CC01 ID12	CCGATCT GTCGAGGTATGTCCTCAGAA CTCAGGAA GCTCTAG
Stem CC01 ID13	CCGATCT GTCGAGGTATGTCCTCAGAA GGTAGGAA GCTCTAG
Stem CC01 ID14	CCGATCT GTCGAGGTATGTCCTCAGAA TGACGGAA GCTCTAG
Stem CC01 ID15	CCGATCT GTCGAGGTATGTCCTCAGAA ATGCGGAA GCTCTAG
Stem CC01 ID16	CCGATCT GTCGAGGTATGTCCTCAGAA GCATGGAA GCTCTAG
Stem CC01 ID17	CCGATCT GTCGAGGTATGTCCTCAGAA CAGTGGAA GCTCTAG
Stem CC01 ID21	CCGATCT GTCGAGGTATGTCCTCAGAA TCAGCGAA GCTCTAG
Stem CC01 ID22	CCGATCT GTCGAGGTATGTCCTCAGAA ATCGCGAA GCTCTAG
Stem CC01 ID23	CCGATCT GTCGAGGTATGTCCTCAGAA GATGCGAA GCTCTAG
Stem CC01 ID24	CCGATCT GTCGAGGTATGTCCTCAGAA CGATCGAA GCTCTAG
Stem CC01 ID25	CCGATCT GTCGAGGTATGTCCTCAGAA CTGGTGAA GCTCTAG
Stem CC02 ID02	CCGATCT GATACGGAACGCCGTATGCT GTCTAGTG GCTCTAG
Stem CC02 ID03	CCGATCT GATACGGAACGCCGTATGCT CGGAAGAA GCTCTAG
Stem CC02 ID05	CCGATCT GATACGGAACGCCGTATGCT GGAGAGAA GCTCTAG
Stem CC02 ID06	CCGATCT GATACGGAACGCCGTATGCT ACGGAGAA GCTCTAG
Stem CC02 ID07	CCGATCT GATACGGAACGCCGTATGCT CACGAGAA GCTCTAG
Stem CC02 ID08	CCGATCT GATACGGAACGCCGTATGCT CCACAGAA GCTCTAG
Stem CC02 ID09	CCGATCT GATACGGAACGCCGTATGCT GAGCAGAA GCTCTAG
Stem CC02 ID10	CCGATCT GATACGGAACGCCGTATGCT AGCCAGAA GCTCTAG
Stem CC02 ID12	CCGATCT GATACGGAACGCCGTATGCT CTCAGGAA GCTCTAG
Stem CC02 ID14	CCGATCT GATACGGAACGCCGTATGCT TGACGGAA GCTCTAG
Stem CC02 ID15	CCGATCT GATACGGAACGCCGTATGCT ATGCGGAA GCTCTAG
Stem CC02 ID16	CCGATCT GATACGGAACGCCGTATGCT GCATGGAA GCTCTAG
Stem CC02 ID17	CCGATCT GATACGGAACGCCGTATGCT CAGTGGAA GCTCTAG
Stem CC02 ID22	CCGATCT GATACGGAACGCCGTATGCT ATCGCGAA GCTCTAG
Stem CC02 ID24	CCGATCT GATACGGAACGCCGTATGCT CGATCGAA GCTCTAG
Stem CC02 ID25	CCGATCT GATACGGAACGCCGTATGCT CTGGTGAA GCTCTAG
Stem CC02 ID26	CCGATCT GATACGGAACGCCGTATGCT GTCCTGAA GCTCTAG
Stem CC02 ID32	CCGATCT GATACGGAACGCCGTATGCT CTCACAA GCTCTAG
Stem CC02 ID33	CCGATCT GATACGGAACGCCGTATGCT GGTCAAA GCTCTAG
Stem CC02 ID35	CCGATCT GATACGGAACGCCGTATGCT GAGAGAA GCTCTAG
Stem CC03 ID02	CCGATCT AAGTTACCTAGCAGCAGCTA GTCTAGTG GCTCTAG
Stem CC03 ID03	CCGATCT AAGTTACCTAGCAGCAGCTA CGGAAGAA GCTCTAG
Stem CC03 ID04	CCGATCT AAGTTACCTAGCAGCAGCTA GCCAAGAA GCTCTAG
Stem CC03 ID05	CCGATCT AAGTTACCTAGCAGCAGCTA GGAGAGAA GCTCTAG
Stem CC03 ID06	CCGATCT AAGTTACCTAGCAGCAGCTA ACGGAGAA GCTCTAG
Stem CC03 ID08	CCGATCT AAGTTACCTAGCAGCAGCTA CCACAGAA GCTCTAG
Stem CC03 ID10	CCGATCT AAGTTACCTAGCAGCAGCTA AGCCAGAA GCTCTAG
Stem CC03 ID12	CCGATCT AAGTTACCTAGCAGCAGCTA CTCAGGAA GCTCTAG
Stem CC03 ID13	CCGATCT AAGTTACCTAGCAGCAGCTA GGTAGGAA GCTCTAG
Stem CC03 ID14	CCGATCT AAGTTACCTAGCAGCAGCTA TGACGGAA GCTCTAG

Stem CC03 ID15	CCGATCT AAGTTACCTAGCAGCAGCTA ATGCGGAA GCTCTAG
Stem CC03 ID16	CCGATCT AAGTTACCTAGCAGCAGCTA GCATGGAA GCTCTAG
Stem CC03 ID17	CCGATCT AAGTTACCTAGCAGCAGCTA CAGTGGAA GCTCTAG
Stem CC03 ID21	CCGATCT AAGTTACCTAGCAGCAGCTA TCAGCGAA GCTCTAG
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Stem CC03 ID24	CCGATCT AAGTTACCTAGCAGCAGCTA CGATCGAA GCTCTAG
Stem CC03 ID25	CCGATCT AAGTTACCTAGCAGCAGCTA CTGGTGAA GCTCTAG
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Stem CC03 ID27	CCGATCT AAGTTACCTAGCAGCAGCTA CGTCTGAA GCTCTAG
Stem CC04 ID00	CCGATCT GTCGACGGAAATATACGCTT TGGCATGA GCTCTAG
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Stem CC04 ID02	CCGATCT GTCGACGGAAATATACGCTT GTCTAGTG GCTCTAG
Stem CC04 ID03	CCGATCT GTCGACGGAAATATACGCTT CGGAAGAA GCTCTAG
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Stem CC04 ID09	CCGATCT GTCGACGGAAATATACGCTT GAGCAGAA GCTCTAG
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Stem CC04 ID14	CCGATCT GTCGACGGAAATATACGCTT TGACGGAA GCTCTAG
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Stem CC04 ID20	CCGATCT GTCGACGGAAATATACGCTT CCTACGAA GCTCTAG
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Stem CC04 ID28	CCGATCT GTCGACGGAAATATACGCTT GTGGACAA GCTCTAG
Stem CC05 ID00	CCGATCT AGCGATTCAAACGCGAGATT TGGCATGA GCTCTAG
Stem CC05 ID01	CCGATCT AGCGATTCAAACGCGAGATT CATCCGTA GCTCTAG
Stem CC05 ID02	CCGATCT AGCGATTCAAACGCGAGATT GTCTAGTG GCTCTAG
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Stem CC05 ID28	CCGATCT AGCGATTCAAACGCGAGATT GTGGACAA GCTCTAG
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Stem CC06 ID02	CCGATCT ACCAGCCTTATCGGTAAGGC GTCTAGTG GCTCTAG
Stem CC06 ID03	CCGATCT ACCAGCCTTATCGGTAAGGC CGGAAGAA GCTCTAG
Stem CC06 ID04	CCGATCT ACCAGCCTTATCGGTAAGGC GCCAAGAA GCTCTAG
Stem CC06 ID05	CCGATCT ACCAGCCTTATCGGTAAGGC GGAGAGAA GCTCTAG
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Stem CC07 ID02	CCGATCT TATTACCAACGGATTGCCGG GTCTAGTG GCTCTAG
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Stem CC13 ID17	CCGATCT TTAGGACCGGTTGGTCATAA CAGTGGAA GCTCTAG
Stem CC13 ID21	CCGATCT TTAGGACCGGTTGGTCATAA TCAGCGAA GCTCTAG
Stem CC13 ID22	CCGATCT TTAGGACCGGTTGGTCATAA ATCGCGAA GCTCTAG

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Stem CC14 ID02	CCGATCT GTAGTTGGATTGGTTCCTAC GTCTAGTG GCTCTAG
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Stem CC15 ID05	CCGATCT TTCCTCCGCAAGGAGAGAAAC GGAGAGAA GCTCTAG
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Stem CC15 ID08	CCGATCT TTCCTCCGCAAGGAGAGAAAC CCACAGAA GCTCTAG
Stem CC15 ID09	CCGATCT TTCCTCCGCAAGGAGAGAAAC GAGCAGAA GCTCTAG
Stem CC15 ID10	CCGATCT TTCCTCCGCAAGGAGAGAAAC AGCCAGAA GCTCTAG
Stem CC15 ID11	CCGATCT TTCCTCCGCAAGGAGAGAAAC TCGAGGAA GCTCTAG
Stem CC15 ID12	CCGATCT TTCCTCCGCAAGGAGAGAAAC CTCAGGAA GCTCTAG
Stem CC15 ID13	CCGATCT TTCCTCCGCAAGGAGAGAAAC GGTAGGAA GCTCTAG
Stem CC15 ID14	CCGATCT TTCCTCCGCAAGGAGAGAAAC TGACGGAA GCTCTAG
Stem CC15 ID15	CCGATCT TTCCTCCGCAAGGAGAGAAAC ATGCGGAA GCTCTAG
Stem CC15 ID16	CCGATCT TTCCTCCGCAAGGAGAGAAAC GCATGGAA GCTCTAG
Stem CC15 ID17	CCGATCT TTCCTCCGCAAGGAGAGAAAC CAGTGGAA GCTCTAG
Stem CC15 ID21	CCGATCT TTCCTCCGCAAGGAGAGAAAC TCAGCGAA GCTCTAG
Stem CC15 ID22	CCGATCT TTCCTCCGCAAGGAGAGAAAC ATCGCGAA GCTCTAG
Stem CC15 ID23	CCGATCT TTCCTCCGCAAGGAGAGAAAC GATGCGAA GCTCTAG
Stem CC15 ID24	CCGATCT TTCCTCCGCAAGGAGAGAAAC CGATCGAA GCTCTAG
Stem CC15 ID25	CCGATCT TTCCTCCGCAAGGAGAGAAAC CTGGTGAA GCTCTAG
ID00 dT	ACAAGTC AACGCATG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID01 dT	ACAAGTC GTTGCATG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID03 dT	ACAAGTC CCTTCATG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID05 dT	ACAAGTC GCTCTATG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID06 dT	ACAAGTC CTGAAGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID07 dT	ACAAGTC TGCAAGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID08 dT	ACAAGTC GCTAAGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID09 dT	ACAAGTC TCAGAGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID10 dT	ACAAGTC CGATAGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
ID15 dT	ACAAGTC TATCGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN

ID16 dT	ACAAGTC ACATCGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID21 dT	ACAAGTC CAGTTGTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID23 dT	ACAAGTC ACTCACTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID24 dT	ACAAGTC GAGTACTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID25 dT	ACAAGTC TCCTACTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID26 dT	ACAAGTC TCAAGCTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID28 dT	ACAAGTC ATTGGCTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID30 dT	ACAAGTC GTATCCTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID31 dT	ACAAGTC CTCATCTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN
ID32 dT	ACAAGTC TTGGTCTG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTVN

Table S2. Primer and oligonucleotides sequences for library preparation.

barcoded oligo-dT primers & TSO

barcode	sequence
ATCCTGTA	AGACGTGTGCTCTTCCGATCTACTCTGTABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
ATTGAGGA	AGACGTGTGCTCTTCCGATCTATTGAGGABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CAACCACA	AGACGTGTGCTCTTCCGATCTCAACCACABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
GACTAGTA	AGACGTGTGCTCTTCCGATCTGACTAGTABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CAATGGAA	AGACGTGTGCTCTTCCGATCTCAATGGAABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CACTTCGA	AGACGTGTGCTCTTCCGATCTCACTTCGABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CAGCGTIA	AGACGTGTGCTCTTCCGATCTCAGCGTTABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CATACCAA	AGACGTGTGCTCTTCCGATCTCATACCAABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CCAGTICA	AGACGTGTGCTCTTCCGATCTCCAGTTCABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CCGAAGTA	AGACGTGTGCTCTTCCGATCTCCGAAGTABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CCGTGAGA	AGACGTGTGCTCTTCCGATCTCCGTGAGABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CCTCCTGA	AGACGTGTGCTCTTCCGATCTCCTCTGABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CGAACTIA	AGACGTGTGCTCTTCCGATCTCGAACTTABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CGACTGGA	AGACGTGTGCTCTTCCGATCTCGACTGGABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CGCATACA	AGACGTGTGCTCTTCCGATCTCGCATACABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CTCAATGA	AGACGTGTGCTCTTCCGATCTCTCAATGABAAAAAAAAAAAAAAAAAAAAAAAAAAAA
biotinylated template switching oligonucleotide (TSO)	Bio-AAGCAGTGGTATCAACGCAGGTACrGrG+G

SeqAmp PCR primers

name	sequence
primer2	AAGCAGTGGTATCAACGCAGAGT
additive primer	AGACGTGTGCTCTTCCGATCT

Indexing primers (TruSeq P5)

name	sequence
501	AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTCCCTACACGACGCTCTTCCGATCT
502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTCCCTACACGACGCTCTTCCGATCT
503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTCCCTACACGACGCTCTTCCGATCT
504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGAACACTCTTCCCTACACGACGCTCTTCCGATCT
505	AATGATACGGCGACCACCGAGATCTACAGTAAGGAGACACTCTTCCCTACACGACGCTCTTCCGATCT

506	AATGATACGGCGACCACCGAGATCTACACTGCATAAAGCTCTTTCCCTACACGACGCTCTTCCGATCT
507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTAAGCTCTTTCCCTACACGACGCTCTTCCGATCT
508	AATGATACGGCGACCACCGAGATCTACACCTAAGCTTACACTCTTTCCCTACACGACGCTCTTCCGATCT

Indexing primers (TruSeq DNA Methylation P7)

name	sequence
TruSeq DNA Methy index1	CAAGCAGAAGACGGCATACGAGATATCACGGTGAAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index2	CAAGCAGAAGACGGCATACGAGATCGATGTGTGACTGGAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index3	CAAGCAGAAGACGGCATACGAGATTTAGGCGTGAAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index4	CAAGCAGAAGACGGCATACGAGATTGACCAAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index5	CAAGCAGAAGACGGCATACGAGATACAGTGGTGAAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index6	CAAGCAGAAGACGGCATACGAGATGCAATGTGAAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index7	CAAGCAGAAGACGGCATACGAGATCAGTCGTGAAGTTTCAGACGTGTGCTCTTCCGATCT
TruSeq DNA Methy index8	CAAGCAGAAGACGGCATACGAGATACTGAGTGAAGTTTCAGACGTGTGCTCTTCCGATCT

Table S3. Sequences and modifications of the oligonucleotides for 6 bit beads

Oligonucleotide Name	Oligonucleotide Sequence and Modification
TypeA/B base PCR	/5Acrd/AAGCAGTGGTATCAACGACAGTACGA CGCTCTT
Bridge A	AGATCGG AAGAGCG
Bridge C	GACTTGT CTAGAGC
Readout Alexa647	/Alexa647/GAGGGCGTTCAATCACTC
Readout Alexa488	/Alexa488/GGAATAACGAAGCACCCA
Readout Alexa546	/Alexa546/CTGTGTAGAGGGCGAGAC
Readout Alexa750	/Alexa750/GGTGTGTGTGCTTCAAAC
Readout ATTO532	/ATTO532/TAGCTTACACGTGCACGC
Readout Rho101	/Rho101/GCTGTAGTGAAGACACC
Readout antiAlexa647	TGATGTTGTACCTGCGAG
Readout antiAlexa488	GAACGAGGACATTCCAG
Readout antiAlexa546	AGCATTACGGGATCAG
Readout antiAlexa750	GATTGGGAATGTCTGCCA
Readout antiATTO532	ACGTGGCTTATGCACGAG
Readout antiRho101	AAGAGTTGCACTAATGCC
Branch1-00 000000	TTCTAGTCAACATTGACCGG GGTACACCGTATAAGTACCA CCGATGAGTTCGTCTCATAG
Branch1-01 000001	TTCTAGTCAACATTGACCGG CATGCGCATAACTGTGCATT ATTACGACGAACCGCGTAA
Branch1-02 000010	ATGGCATCAACGACTGCCAC GGTACACCGTATAAGTACCA GCGAGGTATTCATGCCTCTG
Branch1-03 000011	ATGGCATCAACGACTGCCAC CATGCGCATAACTGTGCATT GCGATTACTTCTGTGTAATG
Branch1-04 000100	TTCTAGTCAACATTGACCGG TGACAACCTGGATGGTTGAT GGAGAACAAGACTCCTAATC
Branch1-05 000101	TTCTAGTCAACATTGACCGG TAGGTCTCACACGACCTGTT TGTGCAGGATAATCGCACTA
Branch1-06 000110	ATGGCATCAACGACTGCCAC TGACAACCTGGATGGTTGAT AGCCTTACATTCTGTACGGC
Branch1-07 000111	ATGGCATCAACGACTGCCAC TAGGTCTCACACGACCTGTT AACTACAAGCCACTTGTAG
Branch1-08 001000	CTGCGACACCGCATTGTATT GGTACACCGTATAAGTACCA CGGCTACATAATGATGCAGC
Branch1-09 001001	CTGCGACACCGCATTGTATT CATGCGCATAACTGTGCATT AACACAAGTGGCTTGTGGAA
Branch1-10 001010	CAGCCTTAGCGAAGAAGGCC GGTACACCGTATAAGTACCA TGATAGTAGTCATCTTGACC
Branch1-11 001011	CAGCCTTAGCGAAGAAGGCC CATGCGCATAACTGTGCATT TCGACAGCTCCTGTATTATC
Branch1-12 001100	CTGCGACACCGCATTGTATT TGACAACCTGGATGGTTGAT CGCAACCATTTAATTGGTG
Branch1-13 001101	CTGCGACACCGCATTGTATT TAGGTCTCACACGACCTGTT ACGACCATGTGCAAGATGGA
Branch1-14 001110	CAGCCTTAGCGAAGAAGGCC TGACAACCTGGATGGTTGAT CCGGTACTACTTGTAAACGGT
Branch1-15 001111	CAGCCTTAGCGAAGAAGGCC TAGGTCTCACACGACCTGTT GCACATAGAGTTCTATAGGA
Branch1-16 010000	TTCTAGTCAACATTGACCGG TGGACGAGGTATATCTCGTA TCTCAACGACTTATGGTTGA

Branch1-17 010001	TTCTAGTCAACATTGACCGG GGACGAGCTAATGGCTCACG CATCGCCGAGAACGGCGAAT
Branch1-18 010010	ATGGCATCAACGACTGCCAC TGGACGAGGTATATCTCGTA TCGACCAATCATGGTCTCT
Branch1-19 010011	ATGGCATCAACGACTGCCAC GGACGAGCTAATGGCTCACG CGCCAGATGAGTATCTGACA
Branch1-20 010100	TTCTAGTCAACATTGACCGG CAGGTTGAGTAACCTCTACG CTGCGGTCTATAACCGCGTC
Branch1-21 010101	TTCTAGTCAACATTGACCGG GTAATGCTGCGCAATTACCT GATGATTGTCCGCAATCACC
Branch1-22 010110	ATGGCATCAACGACTGCCAC CAGGTTGAGTAACCTCTACG AGTACAGGAACGTGTGTGCC
Branch1-23 010111	ATGGCATCAACGACTGCCAC GTAATGCTGCGCAATTACCT GTTATGGTTGATCTCCATAC
Branch1-24 011000	CTGCGACACCGCATTGTATT TGGACGAGGTATATCTCGTA GGTATCGATATGAATCGATC
Branch1-25 011001	CTGCGACACCGCATTGTATT GGACGAGCTAATGGCTCACG AGTACTTAGCACGGCTAAGG
Branch1-26 011010	CAGCCTTAGCGAAGAAGGCC TGGACGAGGTATATCTCGTA AAGATTCATTCCGCGAATCT
Branch1-27 011011	CAGCCTTAGCGAAGAAGGCC GGACGAGCTAATGGCTCACG AAGACAATTACTCGTCCAC
Branch1-28 011100	CTGCGACACCGCATTGTATT CAGGTTGAGTAACCTCTACG GCTATGCATAGATTATGCCA
Branch1-29 011101	CTGCGACACCGCATTGTATT GTAATGCTGCGCAATTACCT TTGGCAGCGTGAGCTGGCAA
Branch1-30 011110	CAGCCTTAGCGAAGAAGGCC CAGGTTGAGTAACCTCTACG CGGCCTTAAGCCTATTACTA
Branch1-31 011111	CAGCCTTAGCGAAGAAGGCC GTAATGCTGCGCAATTACCT TTCTGAGAGCTCTTGCTCGG
Branch1-32 100000	GGCCATTGATAATATGGTGC GGTACACCGTATAAGTACCA AAGCCGAGTGATCCGGAA
Branch1-33 100001	GGCCATTGATAATATGGTGC CATGCGCATAACTGTGCATT AAGTTGGCCAGATGGCCACT
Branch1-34 100010	CGACATTCGATATACGAATC GGTACACCGTATAAGTACCA AGCCGCTATCGGCATTGAAT
Branch1-35 100011	CGACATTCGATATACGAATC CATGCGCATAACTGTGCATT TTAGCAGGCATATACTGCCA
Branch1-36 100100	GGCCATTGATAATATGGTGC TGACAACCTGGATGGTTGAT CCAGCTTCCCTCAGGAGCTG
Branch1-37 100101	GGCCATTGATAATATGGTGC TAGGTCTCACACGACCTGTT CTTGGCTTAAGATGCCACTC
Branch1-38 100110	CGACATTCGATATACGAATC TGACAACCTGGATGGTTGAT GCTATATCTCGCATAGCTT
Branch1-39 100111	CGACATTCGATATACGAATC TAGGTCTCACACGACCTGTT GGTAAAGGTTCATATTGAACC
Branch1-40 101000	AGAGACGTTGAGCAACGTCT GGTACACCGTATAAGTACCA GTTCTCAAGTCATTGAATCC
Branch1-41 101001	AGAGACGTTGAGCAACGTCT CATGCGCATAACTGTGCATT TTACCGAAGACTCGGAATCT
Branch1-42 101010	AACCACGAACAAGCGTGGAG GGTACACCGTATAAGTACCA CCTCCAAGGAATACTACTTG
Branch1-43 101011	AACCACGAACAAGCGTGGAG CATGCGCATAACTGTGCATT TTGCGGAATCAATCCGAGAG
Branch1-44 101100	AGAGACGTTGAGCAACGTCT TGACAACCTGGATGGTTGAT AAGGACACGATAGTCTCCA
Branch1-45 101101	AGAGACGTTGAGCAACGTCT TAGGTCTCACACGACCTGTT CCTCAACAAGATGAGAAGAG
Branch1-46 101110	AACCACGAACAAGCGTGGAG TGACAACCTGGATGGTTGAT TATGAGTCCAGGTGACTTAA
Branch1-47 101111	AACCACGAACAAGCGTGGAG TAGGTCTCACACGACCTGTT TCAGGACTCAATGTGAGTAC
Branch1-48 110000	GGCCATTGATAATATGGTGC TGGACGAGGTATATCTCGTA GTCCGAATGGCTACAATCGG
Branch1-49 110001	GGCCATTGATAATATGGTGC GGACGAGCTAATGGCTCACG CACCGCATATCTATTGGCGG
Branch1-50 110010	CGACATTCGATATACGAATC TGGACGAGGTATATCTCGTA AATTGGCAGTGTTCGCCGCTT
Branch1-51 110011	CGACATTCGATATACGAATC GGACGAGCTAATGGCTCACG CTAGTTGGTGTCAACTATAC
Branch1-52 110100	GGCCATTGATAATATGGTGC CAGGTTGAGTAACCTCTACG CCATGGACGAAGTCCGTGA
Branch1-53 110101	GGCCATTGATAATATGGTGC GTAATGCTGCGCAATTACCT CTGCAGTCCATGAGCTATA
Branch1-54 110110	CGACATTCGATATACGAATC CAGGTTGAGTAACCTCTACG GGCTTCAAGACCTTGAATAT
Branch1-55 110111	CGACATTCGATATACGAATC GTAATGCTGCGCAATTACCT CGTTGGATATTACCAACACC
Branch1-56 111000	AGAGACGTTGAGCAACGTCT TGGACGAGGTATATCTCGTA GGACAGAGACCTGTGCATCC
Branch1-57 111001	AGAGACGTTGAGCAACGTCT GGACGAGCTAATGGCTCACG GGCCTAGTTGTGCGCTACT
Branch1-58 111010	AACCACGAACAAGCGTGGAG TGGACGAGGTATATCTCGTA TGTTGCCATCCATAGCAACC
Branch1-59 111011	AACCACGAACAAGCGTGGAG GGACGAGCTAATGGCTCACG TGGTAGAGATTCTCTGCCG
Branch1-60 111100	AGAGACGTTGAGCAACGTCT CAGGTTGAGTAACCTCTACG AGACGCCACACTTCTGGCTC
Branch1-61 111101	AGAGACGTTGAGCAACGTCT GTAATGCTGCGCAATTACCT ATCTGGCCATGTGTGGCGT
Branch1-62 111110	AACCACGAACAAGCGTGGAG CAGGTTGAGTAACCTCTACG CTAATCGGACCTATGTCCAA
Branch1-63 111111	AACCACGAACAAGCGTGGAG GTAATGCTGCGCAATTACCT CACCTCCTTAATAGGTATGT
Branch2-1-00 0X0X0X	CCGGTCAATGTTAGTAGAA CTGGAATGTCCCTGGTTC CTCGCAGGTACAACATCA CGTGATCCCGTAAATGCT
Branch2-1-01 0X0X1X	GTGGCAGTCGTTGATGCCAT CTGGAATGTCCCTGGTTC GAGTGATTGAACGCCCTC CGTGATCCCGTAAATGCT
Branch2-1-02 0X1X0X	AATACAATGCGGTGTGCGAG CTGGAATGTCCCTGGTTC CTCGCAGGTACAACATCA GTCTCGCCCTCTACACAG
Branch2-1-03 0X1X1X	GGCCTTCTCGCTAAGGCTG CTGGAATGTCCCTGGTTC GAGTGATTGAACGCCCTC GTCTCGCCCTCTACACAG

Branch2-1-04 1X0X0X	GCACCATATTATCAATGGCC TGGGTGCTTCGTTATTCC CTCGCAGGTACAACATCA CGTGATCCCGTAAATGCT
Branch2-1-05 1X0X1X	GATTTCGTATATCGAATGTCG TGGGTGCTTCGTTATTCC GAGTGATTGAACGCCCTC CGTGATCCCGTAAATGCT
Branch2-1-06 1X1X0X	AGACGTTGCTCAACGCTCTCT TGGGTGCTTCGTTATTCC CTCGCAGGTACAACATCA GTCTCGCCCTCTACACAG
Branch2-1-07 1X1X1X	CTCCACGCTTGTTCGTGGTT TGGGTGCTTCGTTATTCC GAGTGATTGAACGCCCTC GTCTCGCCCTCTACACAG
Branch2-2-08 X0X0X0	TGGTACTTATACGGTGTACC CTCGTGCATAAGCCACGT TGGCAGACATTCCCAATC GGCATTAGTGCAACTCTT
Branch2-2-09 X0X0X1	AATGCACAGTTATGCGCATG CTCGTGCATAAGCCACGT GTTTGAAGCACACACACC GGCATTAGTGCAACTCTT
Branch2-2-10 X0X1X0	ATCAACCATCCAGGTTGTCA CTCGTGCATAAGCCACGT TGGCAGACATTCCCAATC GGTGTCTTACACTACAGC
Branch2-2-11 X0X1X1	AACAGGTCGTGTGAGACCTA CTCGTGCATAAGCCACGT GTTTGAAGCACACACACC GGTGTCTTACACTACAGC
Branch2-2-12 X1X0X0	TACGAGATATACCTCGTCCA GCGTGCACGTGTAAGCTA TGGCAGACATTCCCAATC GGCATTAGTGCAACTCTT
Branch2-2-13 X1X0X1	CGTGAGCATTAGTCTCGCC GCGTGCACGTGTAAGCTA GTTTGAAGCACACACACC GGCATTAGTGCAACTCTT
Branch2-2-14 X1X1X0	CGTAGAGGTTACTCAACCTG GCGTGCACGTGTAAGCTA TGGCAGACATTCCCAATC GGTGTCTTACACTACAGC
Branch2-2-15 X1X1X1	AGGTAATTGCGCAGCATTAC GCGTGCACGTGTAAGCTA GTTTGAAGCACACACACC GGTGTCTTACACTACAGC
Stem 6bit CC00 Bcd1-00	CCGATCT CTATGAGACGAACTCATCGG TGCTACCCAC GCTCTAG
Stem 6bit CC00 Bcd1-01	CCGATCT CTATGAGACGAACTCATCGG TGCTACCTTC GCTCTAG
Stem 6bit CC00 Bcd1-02	CCGATCT CTATGAGACGAACTCATCGG CAACCACTAC GCTCTAG
Stem 6bit CC00 Bcd1-03	CCGATCT CTATGAGACGAACTCATCGG GGACCACTTC GCTCTAG
Stem 6bit CC00 Bcd1-04	CCGATCT CTATGAGACGAACTCATCGG TACAACGTCT GCTCTAG
Stem 6bit CC00 Bcd1-05	CCGATCT CTATGAGACGAACTCATCGG ATACAGTGCA GCTCTAG
Stem 6bit CC00 Bcd1-06	CCGATCT CTATGAGACGAACTCATCGG TTGACTGCAT GCTCTAG
Stem 6bit CC00 Bcd1-07	CCGATCT CTATGAGACGAACTCATCGG TGATAGCTGA GCTCTAG
Stem 6bit CC00 Bcd1-08	CCGATCT CTATGAGACGAACTCATCGG CTGCTAGTT GCTCTAG
Stem 6bit CC00 Bcd1-09	CCGATCT CTATGAGACGAACTCATCGG ACGCCTGCAA GCTCTAG
Stem 6bit CC01 Bcd1-00	CCGATCT TTACGCCGGTTCGTCGTAAT GTGCTACCAC GCTCTAG
Stem 6bit CC01 Bcd1-01	CCGATCT TTACGCCGGTTCGTCGTAAT TCGAGACCAC GCTCTAG
Stem 6bit CC01 Bcd1-02	CCGATCT TTACGCCGGTTCGTCGTAAT GGACCACTTC GCTCTAG
Stem 6bit CC01 Bcd1-03	CCGATCT TTACGCCGGTTCGTCGTAAT TTGCTGATAC GCTCTAG
Stem 6bit CC01 Bcd1-04	CCGATCT TTACGCCGGTTCGTCGTAAT CAACCACTAC GCTCTAG
Stem 6bit CC01 Bcd1-05	CCGATCT TTACGCCGGTTCGTCGTAAT CCGACCACTT GCTCTAG
Stem 6bit CC01 Bcd1-06	CCGATCT TTACGCCGGTTCGTCGTAAT TACAACGTCT GCTCTAG
Stem 6bit CC01 Bcd1-07	CCGATCT TTACGCCGGTTCGTCGTAAT GCGTCACTGA GCTCTAG
Stem 6bit CC01 Bcd1-08	CCGATCT TTACGCCGGTTCGTCGTAAT ACCGTGCATA GCTCTAG
Stem 6bit CC01 Bcd1-09	CCGATCT TTACGCCGGTTCGTCGTAAT TACCACAAGC GCTCTAG
Stem 6bit CC02 Bcd1-00	CCGATCT CAGAGGCATGAATACCTCGC GGTGAACCAA GCTCTAG
Stem 6bit CC02 Bcd1-01	CCGATCT CAGAGGCATGAATACCTCGC GCGATACCGT GCTCTAG
Stem 6bit CC02 Bcd1-02	CCGATCT CAGAGGCATGAATACCTCGC GTGCTACCAC GCTCTAG
Stem 6bit CC02 Bcd1-03	CCGATCT CAGAGGCATGAATACCTCGC CAACCACTAC GCTCTAG
Stem 6bit CC02 Bcd1-04	CCGATCT CAGAGGCATGAATACCTCGC TGCTCTATTG GCTCTAG
Stem 6bit CC02 Bcd1-05	CCGATCT CAGAGGCATGAATACCTCGC TACAACGTCT GCTCTAG
Stem 6bit CC02 Bcd1-06	CCGATCT CAGAGGCATGAATACCTCGC GTTACTGCAC GCTCTAG
Stem 6bit CC02 Bcd1-07	CCGATCT CAGAGGCATGAATACCTCGC GGACACTGGT GCTCTAG
Stem 6bit CC02 Bcd1-08	CCGATCT CAGAGGCATGAATACCTCGC TGCTTACGAT GCTCTAG
Stem 6bit CC02 Bcd1-09	CCGATCT CAGAGGCATGAATACCTCGC TTCTCTTGA GCTCTAG
Stem 6bit CC03 Bcd1-00	CCGATCT CATTACACAGAAGTAATCGC GGTGAACCAA GCTCTAG
Stem 6bit CC03 Bcd1-01	CCGATCT CATTACACAGAAGTAATCGC GCGATACCGT GCTCTAG
Stem 6bit CC03 Bcd1-02	CCGATCT CATTACACAGAAGTAATCGC GTGCTACCAC GCTCTAG
Stem 6bit CC03 Bcd1-03	CCGATCT CATTACACAGAAGTAATCGC TGCTCTATTG GCTCTAG
Stem 6bit CC03 Bcd1-04	CCGATCT CATTACACAGAAGTAATCGC CAACCACTAC GCTCTAG
Stem 6bit CC03 Bcd1-05	CCGATCT CATTACACAGAAGTAATCGC TACAACGTCT GCTCTAG
Stem 6bit CC03 Bcd1-06	CCGATCT CATTACACAGAAGTAATCGC GTTACTGCAC GCTCTAG
Stem 6bit CC03 Bcd1-07	CCGATCT CATTACACAGAAGTAATCGC TGCTTACGAT GCTCTAG
Stem 6bit CC03 Bcd1-08	CCGATCT CATTACACAGAAGTAATCGC GCCTGCCTAA GCTCTAG

Stem 6bit CC03 Bcd1-09	CCGATCT CATTACACAGAAGTAATCGC GGACACTGGT GCTCTAG
Stem 6bit CC04 Bcd1-00	CCGATCT GATTAGGAGTCTTGTCTCC CAACCACTAC GCTCTAG
Stem 6bit CC04 Bcd1-01	CCGATCT GATTAGGAGTCTTGTCTCC GGACCACTTC GCTCTAG
Stem 6bit CC04 Bcd1-02	CCGATCT GATTAGGAGTCTTGTCTCC TTCAACACTG GCTCTAG
Stem 6bit CC04 Bcd1-03	CCGATCT GATTAGGAGTCTTGTCTCC TTCCACTCAC GCTCTAG
Stem 6bit CC04 Bcd1-04	CCGATCT GATTAGGAGTCTTGTCTCC TCACTATTCC GCTCTAG
Stem 6bit CC04 Bcd1-05	CCGATCT GATTAGGAGTCTTGTCTCC TAGACACTGG GCTCTAG
Stem 6bit CC04 Bcd1-06	CCGATCT GATTAGGAGTCTTGTCTCC TACAACGTCT GCTCTAG
Stem 6bit CC04 Bcd1-07	CCGATCT GATTAGGAGTCTTGTCTCC CCGACCACTT GCTCTAG
Stem 6bit CC04 Bcd1-08	CCGATCT GATTAGGAGTCTTGTCTCC AACCAGTGAT GCTCTAG
Stem 6bit CC04 Bcd1-09	CCGATCT GATTAGGAGTCTTGTCTCC CACTTAGGCT GCTCTAG
Stem 6bit CC05 Bcd1-00	CCGATCT TAGTGCATTATCCTGCACA AAGCCTGCCT GCTCTAG
Stem 6bit CC05 Bcd1-01	CCGATCT TAGTGCATTATCCTGCACA CAACCACTAC GCTCTAG
Stem 6bit CC05 Bcd1-02	CCGATCT TAGTGCATTATCCTGCACA TACAACGTCT GCTCTAG
Stem 6bit CC05 Bcd1-03	CCGATCT TAGTGCATTATCCTGCACA GTGCTACCAC GCTCTAG
Stem 6bit CC05 Bcd1-04	CCGATCT TAGTGCATTATCCTGCACA CCGACCACTT GCTCTAG
Stem 6bit CC05 Bcd1-05	CCGATCT TAGTGCATTATCCTGCACA CTGCTTAGTT GCTCTAG
Stem 6bit CC05 Bcd1-06	CCGATCT TAGTGCATTATCCTGCACA TACCACAAGC GCTCTAG
Stem 6bit CC05 Bcd1-07	CCGATCT TAGTGCATTATCCTGCACA AAGTACCGCT GCTCTAG
Stem 6bit CC05 Bcd1-08	CCGATCT TAGTGCATTATCCTGCACA AGTACCACCA GCTCTAG
Stem 6bit CC05 Bcd1-09	CCGATCT TAGTGCATTATCCTGCACA CGTACTACT GCTCTAG
Stem 6bit CC06 Bcd1-00	CCGATCT GCCGTACAGAATGTAAGGCT GTGCTACCAC GCTCTAG
Stem 6bit CC06 Bcd1-01	CCGATCT GCCGTACAGAATGTAAGGCT TCGAGACCAC GCTCTAG
Stem 6bit CC06 Bcd1-02	CCGATCT GCCGTACAGAATGTAAGGCT GGACCACTTC GCTCTAG
Stem 6bit CC06 Bcd1-03	CCGATCT GCCGTACAGAATGTAAGGCT GCGTATACCA GCTCTAG
Stem 6bit CC06 Bcd1-04	CCGATCT GCCGTACAGAATGTAAGGCT CAACCACTAC GCTCTAG
Stem 6bit CC06 Bcd1-05	CCGATCT GCCGTACAGAATGTAAGGCT CCGACCACTT GCTCTAG
Stem 6bit CC06 Bcd1-06	CCGATCT GCCGTACAGAATGTAAGGCT GGACACTGGT GCTCTAG
Stem 6bit CC06 Bcd1-07	CCGATCT GCCGTACAGAATGTAAGGCT CTGCTTAGTT GCTCTAG
Stem 6bit CC06 Bcd1-08	CCGATCT GCCGTACAGAATGTAAGGCT TACAACGTCT GCTCTAG
Stem 6bit CC06 Bcd1-09	CCGATCT GCCGTACAGAATGTAAGGCT TACCACAAGC GCTCTAG
Stem 6bit CC07 Bcd1-00	CCGATCT CTACAAGTGGCTTGTAGTGT GATACATGGT GCTCTAG
Stem 6bit CC07 Bcd1-01	CCGATCT CTACAAGTGGCTTGTAGTGT GATAGCATCG GCTCTAG
Stem 6bit CC07 Bcd1-02	CCGATCT CTACAAGTGGCTTGTAGTGT CAACCACTAC GCTCTAG
Stem 6bit CC07 Bcd1-03	CCGATCT CTACAAGTGGCTTGTAGTGT GATAGCCAAG GCTCTAG
Stem 6bit CC07 Bcd1-04	CCGATCT CTACAAGTGGCTTGTAGTGT AACCAGTGAT GCTCTAG
Stem 6bit CC07 Bcd1-05	CCGATCT CTACAAGTGGCTTGTAGTGT TGATAGCTGA GCTCTAG
Stem 6bit CC07 Bcd1-06	CCGATCT CTACAAGTGGCTTGTAGTGT CTGCTTAGTT GCTCTAG
Stem 6bit CC07 Bcd1-07	CCGATCT CTACAAGTGGCTTGTAGTGT ACCGTGCATA GCTCTAG
Stem 6bit CC07 Bcd1-08	CCGATCT CTACAAGTGGCTTGTAGTGT GATCTTCCAT GCTCTAG
Stem 6bit CC07 Bcd1-09	CCGATCT CTACAAGTGGCTTGTAGTGT GATAACGACG GCTCTAG
Stem 6bit CC08 Bcd1-00	CCGATCT GCTGCATCATTATGTAGCCG GGTAATCGCG GCTCTAG
Stem 6bit CC08 Bcd1-01	CCGATCT GCTGCATCATTATGTAGCCG GAGTATTCCG GCTCTAG
Stem 6bit CC08 Bcd1-02	CCGATCT GCTGCATCATTATGTAGCCG CATCAATCGC GCTCTAG
Stem 6bit CC08 Bcd1-03	CCGATCT GCTGCATCATTATGTAGCCG AAGAGGCTCG GCTCTAG
Stem 6bit CC08 Bcd1-04	CCGATCT GCTGCATCATTATGTAGCCG GGAAGTCCGA GCTCTAG
Stem 6bit CC08 Bcd1-05	CCGATCT GCTGCATCATTATGTAGCCG TCTTATACGG GCTCTAG
Stem 6bit CC08 Bcd1-06	CCGATCT GCTGCATCATTATGTAGCCG GCTTAGGCTT GCTCTAG
Stem 6bit CC08 Bcd1-07	CCGATCT GCTGCATCATTATGTAGCCG CTTAACCGGC GCTCTAG
Stem 6bit CC08 Bcd1-08	CCGATCT GCTGCATCATTATGTAGCCG GTGCTACCAC GCTCTAG
Stem 6bit CC08 Bcd1-09	CCGATCT GCTGCATCATTATGTAGCCG ATATTTCGGC GCTCTAG

Stem 6bit CC09 Bcd1-00	CCGATCT TTCCACAAGCCACTTGTGTT TCGAGACCAC GCTCTAG
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Stem 6bit CC09 Bcd1-02	CCGATCT TTCCACAAGCCACTTGTGTT GATACATGGT GCTCTAG
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Stem 6bit CC10 Bcd1-02	CCGATCT GGTC AAGATGACTACTATCA TACAAC TGCT GCTCTAG
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Stem 6bit CC10 Bcd1-07	CCGATCT GGTC AAGATGACTACTATCA CTATTACCAC GCTCTAG
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Stem 6bit CC10 Bcd1-09	CCGATCT GGTC AAGATGACTACTATCA TCAGTAGGCT GCTCTAG
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Stem 6bit CC11 Bcd1-04	CCGATCT GATAATACAGGAGCTGTGCGA CCGACCACTT GCTCTAG
Stem 6bit CC11 Bcd1-05	CCGATCT GATAATACAGGAGCTGTGCGA CTGCTTAGTT GCTCTAG
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Stem 6bit CC11 Bcd1-08	CCGATCT GATAATACAGGAGCTGTGCGA TGTTCGGGTT GCTCTAG
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Stem 6bit CC13 Bcd1-03	CCGATCT TCCATCTTGACATGGTCGT GCGTATACCA GCTCTAG
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Stem 6bit CC13 Bcd1-06	CCGATCT TCCATCTTGACATGGTCGT TACCACAAG GCTCTAG
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Stem 6bit CC13 Bcd1-09	CCGATCT TCCATCTTGACATGGTCGT TCATGTTCCCT GCTCTAG
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Stem 6bit CC29 Bcd1-06	CCGATCT TTGCCAGCTCACGCTGCCAA TACAACGTCT GCTCTAG
Stem 6bit CC29 Bcd1-07	CCGATCT TTGCCAGCTCACGCTGCCAA TGCCAATTCC GCTCTAG
Stem 6bit CC29 Bcd1-08	CCGATCT TTGCCAGCTCACGCTGCCAA TGCTTACGAT GCTCTAG
Stem 6bit CC29 Bcd1-09	CCGATCT TTGCCAGCTCACGCTGCCAA CTGCTTAGTT GCTCTAG
Stem 6bit CC30 Bcd1-00	CCGATCT TAGTAATAGGCTTAAGGCCG CAACCACTAC GCTCTAG
Stem 6bit CC30 Bcd1-01	CCGATCT TAGTAATAGGCTTAAGGCCG ATACAGTGCA GCTCTAG
Stem 6bit CC30 Bcd1-02	CCGATCT TAGTAATAGGCTTAAGGCCG CTGCTTAGTT GCTCTAG
Stem 6bit CC30 Bcd1-03	CCGATCT TAGTAATAGGCTTAAGGCCG GGACCACTTC GCTCTAG
Stem 6bit CC30 Bcd1-04	CCGATCT TAGTAATAGGCTTAAGGCCG GTGCTACCAC GCTCTAG
Stem 6bit CC30 Bcd1-05	CCGATCT TAGTAATAGGCTTAAGGCCG TTGACTGCAT GCTCTAG
Stem 6bit CC30 Bcd1-06	CCGATCT TAGTAATAGGCTTAAGGCCG AGATCTGACT GCTCTAG
Stem 6bit CC30 Bcd1-07	CCGATCT TAGTAATAGGCTTAAGGCCG AGTACCACCA GCTCTAG
Stem 6bit CC30 Bcd1-08	CCGATCT TAGTAATAGGCTTAAGGCCG CATAAGCCTT GCTCTAG
Stem 6bit CC30 Bcd1-09	CCGATCT TAGTAATAGGCTTAAGGCCG CGAAGCTCCT GCTCTAG
Stem 6bit CC31 Bcd1-00	CCGATCT CCGAGCAAGAGCTCTCAGAA CAACCACTAC GCTCTAG
Stem 6bit CC31 Bcd1-01	CCGATCT CCGAGCAAGAGCTCTCAGAA TACAACGTCT GCTCTAG
Stem 6bit CC31 Bcd1-02	CCGATCT CCGAGCAAGAGCTCTCAGAA TACCACAAGC GCTCTAG
Stem 6bit CC31 Bcd1-03	CCGATCT CCGAGCAAGAGCTCTCAGAA AGTACCACCA GCTCTAG
Stem 6bit CC31 Bcd1-04	CCGATCT CCGAGCAAGAGCTCTCAGAA GGAAGACACT GCTCTAG
Stem 6bit CC31 Bcd1-05	CCGATCT CCGAGCAAGAGCTCTCAGAA CTATTACCAC GCTCTAG
Stem 6bit CC31 Bcd1-06	CCGATCT CCGAGCAAGAGCTCTCAGAA CAATACCATC GCTCTAG
Stem 6bit CC31 Bcd1-07	CCGATCT CCGAGCAAGAGCTCTCAGAA TGTGATACCA GCTCTAG
Stem 6bit CC31 Bcd1-08	CCGATCT CCGAGCAAGAGCTCTCAGAA TCGTCGCACT GCTCTAG
Stem 6bit CC31 Bcd1-09	CCGATCT CCGAGCAAGAGCTCTCAGAA GTGATCAACT GCTCTAG
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Stem 6bit CC33 Bcd1-02	CCGATCT AGTGGCCATCTGGCCAATT CAACCACTAC GCTCTAG
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Stem 6bit CC33 Bcd1-05	CCGATCT AGTGGCCATCTGGCCAATT CTGCTTAGTT GCTCTAG
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Stem 6bit CC33 Bcd1-08	CCGATCT AGTGGCCATCTGGCCAATT GTGATCAACT GCTCTAG
Stem 6bit CC33 Bcd1-09	CCGATCT AGTGGCCATCTGGCCAATT GATGTAGCTT GCTCTAG
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Stem 6bit CC34 Bcd1-03	CCGATCT ATTCAATGCCGATAGCGGCT CAACCACTAC GCTCTAG
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Stem 6bit CC35 Bcd1-07	CCGATCT TGGCAGTATATGCCTGCTAA TGTGATACCA GCTCTAG
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Stem 6bit CC35 Bcd1-09	CCGATCT TGGCAGTATATGCCTGCTAA GGAAGACACT GCTCTAG
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Stem 6bit CC36 Bcd1-01	CCGATCT CAGCTCCTGAAGGAAGCTGG TACAAC T GCTCTAG
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Stem 6bit CC37 Bcd1-00	CCGATCT GAGTGGCATCTTAAGCCAAG ATACCTCCAT GCTCTAG
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Stem 6bit CC37 Bcd1-02	CCGATCT GAGTGGCATCTTAAGCCAAG ATACCTACGA GCTCTAG
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Stem 6bit CC37 Bcd1-04	CCGATCT GAGTGGCATCTTAAGCCAAG ATACAGTGCA GCTCTAG
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Stem 6bit CC38 Bcd1-00	CCGATCT AAGCTATCGCGAGATATAGC ACTGAGGAAT GCTCTAG
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Stem 6bit CC51 Bcd1-08	CCGATCT GTATAGTTGACACCAACTAG TTGACTGCAT GCTCTAG
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Stem 6bit CC52 Bcd1-03	CCGATCT TCACGGACCTTCGTCCATGG GTGGATTGCT GCTCTAG
Stem 6bit CC52 Bcd1-04	CCGATCT TCACGGACCTTCGTCCATGG GGACTAGGCT GCTCTAG
Stem 6bit CC52 Bcd1-05	CCGATCT TCACGGACCTTCGTCCATGG TGTGGCTTCT GCTCTAG
Stem 6bit CC52 Bcd1-06	CCGATCT TCACGGACCTTCGTCCATGG CTTATTGGCT GCTCTAG
Stem 6bit CC52 Bcd1-07	CCGATCT TCACGGACCTTCGTCCATGG TCGTCGCACT GCTCTAG
Stem 6bit CC52 Bcd1-08	CCGATCT TCACGGACCTTCGTCCATGG ATGGTCCACG GCTCTAG
Stem 6bit CC52 Bcd1-09	CCGATCT TCACGGACCTTCGTCCATGG TCATGTTCCCT GCTCTAG
Stem 6bit CC53 Bcd1-00	CCGATCT TATAGCTCATGGAGCTGCAG AAGCCTGCCT GCTCTAG
Stem 6bit CC53 Bcd1-01	CCGATCT TATAGCTCATGGAGCTGCAG TCGAGACCAC GCTCTAG
Stem 6bit CC53 Bcd1-02	CCGATCT TATAGCTCATGGAGCTGCAG ATGTGACCAT GCTCTAG
Stem 6bit CC53 Bcd1-03	CCGATCT TATAGCTCATGGAGCTGCAG GTGCTACCAC GCTCTAG
Stem 6bit CC53 Bcd1-04	CCGATCT TATAGCTCATGGAGCTGCAG ATACCTACGA GCTCTAG
Stem 6bit CC53 Bcd1-05	CCGATCT TATAGCTCATGGAGCTGCAG TAGTAGCAGT GCTCTAG
Stem 6bit CC53 Bcd1-06	CCGATCT TATAGCTCATGGAGCTGCAG AAGGATGCGT GCTCTAG
Stem 6bit CC53 Bcd1-07	CCGATCT TATAGCTCATGGAGCTGCAG TTGTGCCGCT GCTCTAG
Stem 6bit CC53 Bcd1-08	CCGATCT TATAGCTCATGGAGCTGCAG ATACCTCCAT GCTCTAG
Stem 6bit CC53 Bcd1-09	CCGATCT TATAGCTCATGGAGCTGCAG TTGCTGATAC GCTCTAG
Stem 6bit CC54 Bcd1-00	CCGATCT ATATTCAAGGTCTTGAAGCC CAACCACTAC GCTCTAG
Stem 6bit CC54 Bcd1-01	CCGATCT ATATTCAAGGTCTTGAAGCC ACTGAGGAAT GCTCTAG
Stem 6bit CC54 Bcd1-02	CCGATCT ATATTCAAGGTCTTGAAGCC TACAACGCT GCTCTAG
Stem 6bit CC54 Bcd1-03	CCGATCT ATATTCAAGGTCTTGAAGCC ACTGAAGACA GCTCTAG
Stem 6bit CC54 Bcd1-04	CCGATCT ATATTCAAGGTCTTGAAGCC ACTGGATAAC GCTCTAG
Stem 6bit CC54 Bcd1-05	CCGATCT ATATTCAAGGTCTTGAAGCC GGACACTGGT GCTCTAG
Stem 6bit CC54 Bcd1-06	CCGATCT ATATTCAAGGTCTTGAAGCC ACTCTCTTCA GCTCTAG
Stem 6bit CC54 Bcd1-07	CCGATCT ATATTCAAGGTCTTGAAGCC ACTGACTGAA GCTCTAG
Stem 6bit CC54 Bcd1-08	CCGATCT ATATTCAAGGTCTTGAAGCC TGCTTACGAT GCTCTAG

Stem 6bit CC54 Bcd1-09	CCGATCT ATATTCAAGGTCTTGAAGCC GGACCACTTC GCTCTAG
Stem 6bit CC55 Bcd1-00	CCGATCT GGTGTTGGTAATATCCAACG ATACCTCCAT GCTCTAG
Stem 6bit CC55 Bcd1-01	CCGATCT GGTGTTGGTAATATCCAACG GTGCTACCAC GCTCTAG
Stem 6bit CC55 Bcd1-02	CCGATCT GGTGTTGGTAATATCCAACG TACAACGTCT GCTCTAG
Stem 6bit CC55 Bcd1-03	CCGATCT GGTGTTGGTAATATCCAACG CAACCACTAC GCTCTAG
Stem 6bit CC55 Bcd1-04	CCGATCT GGTGTTGGTAATATCCAACG CCGACCACTT GCTCTAG
Stem 6bit CC55 Bcd1-05	CCGATCT GGTGTTGGTAATATCCAACG ATACAGTGCA GCTCTAG
Stem 6bit CC55 Bcd1-06	CCGATCT GGTGTTGGTAATATCCAACG TTGACTGCAT GCTCTAG
Stem 6bit CC55 Bcd1-07	CCGATCT GGTGTTGGTAATATCCAACG CTGCTTAGTT GCTCTAG
Stem 6bit CC55 Bcd1-08	CCGATCT GGTGTTGGTAATATCCAACG ACGCCTGCAA GCTCTAG
Stem 6bit CC55 Bcd1-09	CCGATCT GGTGTTGGTAATATCCAACG ACAGAATCCT GCTCTAG
Stem 6bit CC56 Bcd1-00	CCGATCT GGATGCACAGGTCTCTGTCC TGCTCTATTG GCTCTAG
Stem 6bit CC56 Bcd1-01	CCGATCT GGATGCACAGGTCTCTGTCC TGCTACCAC GCTCTAG
Stem 6bit CC56 Bcd1-02	CCGATCT GGATGCACAGGTCTCTGTCC GCGTATACCA GCTCTAG
Stem 6bit CC56 Bcd1-03	CCGATCT GGATGCACAGGTCTCTGTCC CAACCACTAC GCTCTAG
Stem 6bit CC56 Bcd1-04	CCGATCT GGATGCACAGGTCTCTGTCC TACAACGTCT GCTCTAG
Stem 6bit CC56 Bcd1-05	CCGATCT GGATGCACAGGTCTCTGTCC ACTGAGGAAT GCTCTAG
Stem 6bit CC56 Bcd1-06	CCGATCT GGATGCACAGGTCTCTGTCC GTTACTGCAC GCTCTAG
Stem 6bit CC56 Bcd1-07	CCGATCT GGATGCACAGGTCTCTGTCC ACTGAAGACA GCTCTAG
Stem 6bit CC56 Bcd1-08	CCGATCT GGATGCACAGGTCTCTGTCC ACTGGATAAC GCTCTAG
Stem 6bit CC56 Bcd1-09	CCGATCT GGATGCACAGGTCTCTGTCC ACTCTCTTCA GCTCTAG
Stem 6bit CC57 Bcd1-00	CCGATCT AGTAGCGCACAAC TAGCGCC TGCTCTATTG GCTCTAG
Stem 6bit CC57 Bcd1-01	CCGATCT AGTAGCGCACAAC TAGCGCC TACAACGTCT GCTCTAG
Stem 6bit CC57 Bcd1-02	CCGATCT AGTAGCGCACAAC TAGCGCC CAACCACTAC GCTCTAG
Stem 6bit CC57 Bcd1-03	CCGATCT AGTAGCGCACAAC TAGCGCC TGCTTACGAT GCTCTAG
Stem 6bit CC57 Bcd1-04	CCGATCT AGTAGCGCACAAC TAGCGCC CCGACCACTT GCTCTAG
Stem 6bit CC57 Bcd1-05	CCGATCT AGTAGCGCACAAC TAGCGCC ACTGACTGAA GCTCTAG
Stem 6bit CC57 Bcd1-06	CCGATCT AGTAGCGCACAAC TAGCGCC GGACACTGGT GCTCTAG
Stem 6bit CC57 Bcd1-07	CCGATCT AGTAGCGCACAAC TAGCGCC GGACCACTTC GCTCTAG
Stem 6bit CC57 Bcd1-08	CCGATCT AGTAGCGCACAAC TAGCGCC TCGAGACCAC GCTCTAG
Stem 6bit CC57 Bcd1-09	CCGATCT AGTAGCGCACAAC TAGCGCC ACGGCTTACT GCTCTAG
Stem 6bit CC58 Bcd1-00	CCGATCT GGTGCTATGGATGGCAACA GTGCTACCAC GCTCTAG
Stem 6bit CC58 Bcd1-01	CCGATCT GGTGCTATGGATGGCAACA GGACCACTTC GCTCTAG
Stem 6bit CC58 Bcd1-02	CCGATCT GGTGCTATGGATGGCAACA TCGAGACCAC GCTCTAG
Stem 6bit CC58 Bcd1-03	CCGATCT GGTGCTATGGATGGCAACA TATCTCCACT GCTCTAG
Stem 6bit CC58 Bcd1-04	CCGATCT GGTGCTATGGATGGCAACA TCCGCCACAT GCTCTAG
Stem 6bit CC58 Bcd1-05	CCGATCT GGTGCTATGGATGGCAACA TTCCACTCAC GCTCTAG
Stem 6bit CC58 Bcd1-06	CCGATCT GGTGCTATGGATGGCAACA TTCCTTAGCA GCTCTAG
Stem 6bit CC58 Bcd1-07	CCGATCT GGTGCTATGGATGGCAACA CCGACCACTT GCTCTAG
Stem 6bit CC58 Bcd1-08	CCGATCT GGTGCTATGGATGGCAACA TACAACGTCT GCTCTAG
Stem 6bit CC58 Bcd1-09	CCGATCT GGTGCTATGGATGGCAACA CGTGATACTG GCTCTAG
Stem 6bit CC59 Bcd1-00	CCGATCT CGGCAGAGGAATCTCTACCA CGCTTGAGTC GCTCTAG
Stem 6bit CC59 Bcd1-01	CCGATCT CGGCAGAGGAATCTCTACCA CTCATCTAGG GCTCTAG
Stem 6bit CC59 Bcd1-02	CCGATCT CGGCAGAGGAATCTCTACCA CTGTTGAAGT GCTCTAG
Stem 6bit CC59 Bcd1-03	CCGATCT CGGCAGAGGAATCTCTACCA CTACAGAATG GCTCTAG
Stem 6bit CC59 Bcd1-04	CCGATCT CGGCAGAGGAATCTCTACCA CCACTTGACT GCTCTAG
Stem 6bit CC59 Bcd1-05	CCGATCT CGGCAGAGGAATCTCTACCA CTACTCTCTG GCTCTAG
Stem 6bit CC59 Bcd1-06	CCGATCT CGGCAGAGGAATCTCTACCA CGGTTGCCTA GCTCTAG
Stem 6bit CC59 Bcd1-07	CCGATCT CGGCAGAGGAATCTCTACCA CTCACAGAGG GCTCTAG
Stem 6bit CC59 Bcd1-08	CCGATCT CGGCAGAGGAATCTCTACCA CTACGAGTAT GCTCTAG
Stem 6bit CC59 Bcd1-09	CCGATCT CGGCAGAGGAATCTCTACCA CTGCTCTGATG GCTCTAG

Stem 6bit CC60 Bcd1-00	CCGATCT GAGCCAGAAGTGTGGCGTCT GTGCTACCAC GCTCTAG
Stem 6bit CC60 Bcd1-01	CCGATCT GAGCCAGAAGTGTGGCGTCT GGACCACTTC GCTCTAG
Stem 6bit CC60 Bcd1-02	CCGATCT GAGCCAGAAGTGTGGCGTCT GCTAAGTTCT GCTCTAG
Stem 6bit CC60 Bcd1-03	CCGATCT GAGCCAGAAGTGTGGCGTCT GCGTATACCA GCTCTAG
Stem 6bit CC60 Bcd1-04	CCGATCT GAGCCAGAAGTGTGGCGTCT GCGTCACTGA GCTCTAG
Stem 6bit CC60 Bcd1-05	CCGATCT GAGCCAGAAGTGTGGCGTCT CATGGCTCCT GCTCTAG
Stem 6bit CC60 Bcd1-06	CCGATCT GAGCCAGAAGTGTGGCGTCT TGTTCGGGT GCTCTAG
Stem 6bit CC60 Bcd1-07	CCGATCT GAGCCAGAAGTGTGGCGTCT GCTCAGCCAT GCTCTAG
Stem 6bit CC60 Bcd1-08	CCGATCT GAGCCAGAAGTGTGGCGTCT CGTACTACT GCTCTAG
Stem 6bit CC60 Bcd1-09	CCGATCT GAGCCAGAAGTGTGGCGTCT CTTCTGATCT GCTCTAG
Stem 6bit CC61 Bcd1-00	CCGATCT ACGCCATCACATGGCCAGAT TCAGACCCAC GCTCTAG
Stem 6bit CC61 Bcd1-01	CCGATCT ACGCCATCACATGGCCAGAT GTGCTACCAC GCTCTAG
Stem 6bit CC61 Bcd1-02	CCGATCT ACGCCATCACATGGCCAGAT GATACATGGT GCTCTAG
Stem 6bit CC61 Bcd1-03	CCGATCT ACGCCATCACATGGCCAGAT TGAGACCCAA GCTCTAG
Stem 6bit CC61 Bcd1-04	CCGATCT ACGCCATCACATGGCCAGAT GGACCACTTC GCTCTAG
Stem 6bit CC61 Bcd1-05	CCGATCT ACGCCATCACATGGCCAGAT CAACCACTAC GCTCTAG
Stem 6bit CC61 Bcd1-06	CCGATCT ACGCCATCACATGGCCAGAT TTCCACTCAC GCTCTAG
Stem 6bit CC61 Bcd1-07	CCGATCT ACGCCATCACATGGCCAGAT CCGACCACTT GCTCTAG
Stem 6bit CC61 Bcd1-08	CCGATCT ACGCCATCACATGGCCAGAT TACAAGTCT GCTCTAG
Stem 6bit CC61 Bcd1-09	CCGATCT ACGCCATCACATGGCCAGAT GGACACTGGT GCTCTAG
Stem 6bit CC62 Bcd1-00	CCGATCT TTGGACATAGGTCCGATTAG ATACCTCCAT GCTCTAG
Stem 6bit CC62 Bcd1-01	CCGATCT TTGGACATAGGTCCGATTAG TACAAGTCT GCTCTAG
Stem 6bit CC62 Bcd1-02	CCGATCT TTGGACATAGGTCCGATTAG ATACAGTCA GCTCTAG
Stem 6bit CC62 Bcd1-03	CCGATCT TTGGACATAGGTCCGATTAG CTGCTAGTT GCTCTAG
Stem 6bit CC62 Bcd1-04	CCGATCT TTGGACATAGGTCCGATTAG ATACCAAGAG GCTCTAG
Stem 6bit CC62 Bcd1-05	CCGATCT TTGGACATAGGTCCGATTAG TACCACAAGC GCTCTAG
Stem 6bit CC62 Bcd1-06	CCGATCT TTGGACATAGGTCCGATTAG ATGGTCCACG GCTCTAG
Stem 6bit CC62 Bcd1-07	CCGATCT TTGGACATAGGTCCGATTAG GGAAGACACT GCTCTAG
Stem 6bit CC62 Bcd1-08	CCGATCT TTGGACATAGGTCCGATTAG ATGAACCAAC GCTCTAG
Stem 6bit CC62 Bcd1-09	CCGATCT TTGGACATAGGTCCGATTAG AGACCGACCT GCTCTAG
Stem 6bit CC63 Bcd1-00	CCGATCT ACATACCTATTAAGGAGGTG ATACAGTGCA GCTCTAG
Stem 6bit CC63 Bcd1-01	CCGATCT ACATACCTATTAAGGAGGTG TTGACTGCAT GCTCTAG
Stem 6bit CC63 Bcd1-02	CCGATCT ACATACCTATTAAGGAGGTG CTGCTAGTT GCTCTAG
Stem 6bit CC63 Bcd1-03	CCGATCT ACATACCTATTAAGGAGGTG TACAAGTCT GCTCTAG
Stem 6bit CC63 Bcd1-04	CCGATCT ACATACCTATTAAGGAGGTG ATACCAAGAG GCTCTAG
Stem 6bit CC63 Bcd1-05	CCGATCT ACATACCTATTAAGGAGGTG TAGCAACACT GCTCTAG
Stem 6bit CC63 Bcd1-06	CCGATCT ACATACCTATTAAGGAGGTG GTCCTGTACT GCTCTAG
Stem 6bit CC63 Bcd1-07	CCGATCT ACATACCTATTAAGGAGGTG TCGTCGCACT GCTCTAG
Stem 6bit CC63 Bcd1-08	CCGATCT ACATACCTATTAAGGAGGTG TTCCGATTCT GCTCTAG
Stem 6bit CC63 Bcd1-09	CCGATCT ACATACCTATTAAGGAGGTG AGACCGACCT GCTCTAG
Stem 6bit Bcd2-00	ACAAGTC TACAGTCTAG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-01	ACAAGTC CAAGGTTGTC NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-02	ACAAGTC TTGACTCGTC NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-03	ACAAGTC CTGTCATAAC NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-04	ACAAGTC TATTCGTGTC NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-05	ACAAGTC GTGCTTGGT NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-06	ACAAGTC GGCTTCTGTC NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-07	ACAAGTC AGCTTGTCAA NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-08	ACAAGTC CTAACCTGTC NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-09	ACAAGTC CCTTGGCTAG NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN
Stem 6bit Bcd2-10	ACAAGTC TCCATGCTT NNNNNNNN TTTTTTTTTTTTTTTTTTTTTTVN

Stem 6bit Bcd2-11	ACAAGTC TCCTGCTGTC NNNNNNN TTTTTTTTTTTTTTTTTTTTTTTVN
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Table S4 cell imaging setup

Channel name	Target fluorophore				LED		Mirror unit		Emission filter			
	Name	Excitation	Emission	Vendor	Name	Vendor	Name	Vendor	Name	Centre	Width	Vendor
Channel 1	CellTrace™ Violet	405	450	Invitrogen™	Xcite® Novem XT910 (365)	Excelitas Technologies Corp.	89402	Chroma Technology Corp.	ET434/32m	434	32	Chroma Technology Corp.
Channel 2	CellTrace™ CFSE	495	519	Invitrogen™	Xcite® Novem XT910 (475)	Excelitas Technologies Corp.	89402	Chroma Technology Corp.	ZET514/10x	514	10	Chroma Technology Corp.
Channel3	mVenus	515	527	Invitrogen™	Xcite® Novem XT910 (475)	Excelitas Technologies Corp.	89402	Chroma Technology Corp.	ET545/40m	545	40	Chroma Technology Corp.
Channel 4	CellTrace™ Yellow	546	579	Invitrogen™	Xcite® Novem XT910 (500-600)	Excelitas Technologies Corp.	89402	Chroma Technology Corp.	ET595/33m	595	33	Chroma Technology Corp.
Channel 5	mCherry	587	610	Invitrogen™	Xcite® Novem XT910 (500-600)	Excelitas Technologies Corp.	89402	Chroma Technology Corp.	ET595/33m	595	33	Chroma Technology Corp.

Table S5 bead imaging setup

Channel name	Target fluorophore			LED		Mirror unit		Emission filter			
	Name	Excitation	Emission	Name	Vendor	Name	Vendor	Name	Centre	Width	Vendor
Channel 1	Alexa 488	490	525	Xcite® Novem XT910 (475)	Excelitas Technologies Corp.	U-FBW	Evident Corp.	ET519/26m	519	26	Chroma Technology Corp.
Channel 2	ATTO 532	534	554	Xcite® Novem XT910 (475)	Excelitas Technologies Corp.	U-FBW	Evident Corp.	ET550/20x	550	20	Chroma Technology Corp.
Channel3	Alexa 546	556	573	Xcite® Novem XT910 (500-600)	Excelitas Technologies Corp.	U-FGW	Evident Corp.	FF03-575/25	575	25	Semrock Inc.
Channel 4	ATTO Rho101	592	609	Xcite® Novem XT910 (500-600)	Excelitas Technologies Corp.	U-FGW	Evident Corp.	FF01-624/40	624	40	Semrock Inc.
Channel 5	Alexa 647	650	665	Xcite® Novem XT910 (635)	Excelitas Technologies Corp.	XF407	Omega Optical, LLC	FF01-676/29	676	29	Semrock Inc.
Channel 6	Alexa 750	749	775	Xcite® Novem XT910 (735)	Excelitas Technologies Corp.	U-DM3-CY7	Evident Corp.	-	-	-	-

Table S6 Sequenced librarys

Library No.	Type	Preparation date	Preparation method	Enzyme	Second strand synthesis	Sample type	Cell No.	Seq Amp cycles	cdNA yield (ng)	Ct value (CAPI10)	Note
1	On-chip, Human mouse	20230211	Opto-combinatorial indexing (electroporation, 100 nM input)	SmartScribe		HeLa & NIH3T3	144	16	37.4	16.7	Presented in Chapter 2.
2	On-chip, Human mouse	20230212	Opto-combinatorial indexing (electroporation, 100 nM input)	SmartScribe		HeLa & NIH3T3	177	16	18.3	17.4	Presented in Chapter 2.
3	On-chip, Human mouse	20230415	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa & NIH3T3	106	17	19.8	17.5	Presented in Chapter 2.
4	On-chip, Human mouse	20230419	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa & NIH3T3	88	17	44.5	15.8	Presented in Chapter 2.
5	On-chip, Human mouse	20230419	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa & NIH3T3	101	17	16.5	17.1	Presented in Chapter 2.
6	On-chip, Human mouse	20230419	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa & NIH3T3	48	17	16.4	17.0	Presented in Chapter 2.
7	On-chip, Human mouse	20240903	Opto-combinatorial indexing (electroporation, 100 nM input)	SmartScribe		HeLa & NIH3T3	497	15	1.8	21.3	
8	On-chip, Human mouse	20240903	Opto-combinatorial indexing (electroporation, 100 nM input)	SmartScribe		HeLa & NIH3T3	297	15	2.4	19.8	
9	On-chip, Human mouse	20240917	Opto-combinatorial indexing (electroporation, 100 nM input)	SmartScribe		HeLa & NIH3T3	644	15	7.8	20.3	
10	On-chip, Human mouse	20240917	Opto-combinatorial indexing (electroporation, 100 nM input)	SmartScribe		HeLa & NIH3T3	523	15	4.9	20.8	Presented in Chapter 3.
11	On-chip, paclitaxel	20230219	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	105	17	120.1	17.6	Presented in Chapter 2.
12	On-chip, paclitaxel	20230225	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	190	17	23.5	18.8	Presented in Chapter 2.
13	On-chip, paclitaxel	20230227	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	96	17	25.7	20.0	Presented in Chapter 2.
14	On-chip, paclitaxel	20230301	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	121	17	5.2	20.0	Presented in Chapter 2.
15	On-chip, paclitaxel	20230302	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	188	17	32.2	16.8	Presented in Chapter 2.

16	On-chip, paclitaxel	20230303	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	214	17	51.7	18.4	Presented in Chapter 2.
17	On-chip, paclitaxel	20230304	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	84	18	55.6	17.4	Presented in Chapter 2.
18	On-chip, paclitaxel	20230304	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	99	18	64.0	17.7	Presented in Chapter 2.
19	On-chip, paclitaxel	20230307	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	169	17	56.4	18.6	Presented in Chapter 2.
20	On-chip, paclitaxel	20230307	Opto-combinatorial indexing (lipofection, 20 nM input)	SmartScribe		HeLa (paclitaxel treated)	57	17	37.7	17.5	Presented in Chapter 2.
21	On-chip, HeLa Fucci2	20230713	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	175	16	21.3	17.2	Presented in Chapter 3.
22	On-chip, HeLa Fucci2	20230714	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	133	16	15.5	17.7	Presented in Chapter 3.
23	On-chip, HeLa Fucci2	20230714	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	137	16	9.1	18.8	Presented in Chapter 3.
24	On-chip, HeLa Fucci2	20230718	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	73	17	33.3	17.2	Presented in Chapter 3.
25	On-chip, HeLa Fucci2	20230718	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	134	16	12.8	17.8	Presented in Chapter 3.
26	On-chip, HeLa Fucci2	20230719	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	90	16	20.4	17.9	Presented in Chapter 3.
27	On-chip, HeLa Fucci2	20230719	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	96	16	19.1	17.4	Presented in Chapter 3.
28	On-chip, HeLa Fucci2	20230720	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	155	16	25.0	16.7	Presented in Chapter 3.
29	On-chip, HeLa Fucci2	20230725	Opto-combinatorial indexing (lipofection, 100 nM input)	SmartScribe		HeLa Fucci(SA)2	28	17	22.1	17.4	Presented in Chapter 3.
30	On-chip, No tag	20230202	Opto-combinatorial indexing (no transfection)	SmartScribe		HeLa & NIH3T3	80	16	25.4	16.5	Presented in Chapter 2.
31	On-chip, No tag	20230206	Opto-combinatorial indexing (no transfection)	SmartScribe		HeLa & NIH3T3	59	17	21.5	16.8	Presented in Chapter 2.
32	On-chip, No tag	20230223	Opto-combinatorial indexing (no transfection)	SmartScribe		HeLa & NIH3T3	228	16	35.4	16.1	Presented in Chapter 2.
33	On-chip, Maxima H Minus + 2nd strand synthesis	20240903	Opto-combinatorial indexing (electroporation, 100 nM input)	Maxima H Minus	✓	HeLa & NIH3T3	379	15	7.7	23.1	
34	On-chip, Maxima H Minus + 2nd strand synthesis	20240917	Opto-combinatorial indexing (electroporation, 100 nM input)	Maxima H Minus	✓	HeLa & NIH3T3	686	15	47.3	18.9	
35	On-chip, Maxima H Minus + 2nd strand synthesis	20240917	Opto-combinatorial indexing (electroporation, 100 nM input)	Maxima H Minus	✓	HeLa & NIH3T3	769	15	30.9	18.9	Presented in Chapter 3.
36	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	14.6	13.9	
37	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	14.8	13.8	
38	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	13.9	13.6	
39	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	12.0	13.6	
40	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	12.5	13.4	
41	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	21.6	12.9	
42	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	17.8	13.2	
43	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	18.6	13.2	
44	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	16.3	13.2	
45	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	23.3	12.5	
46	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	17.9	13.2	
47	Index sorting, HeLa Fucci2	20220711	Index sorting	SmartScribe		HeLa Fucci(SA)2	16	15	21.6	12.9	
48	Single cell, K562	20190904	Reaction in tube from single cell	SmartScribe		K562	1	18	13.0	20.6	
49	Single cell, K562	20190904	Reaction in tube from single cell	SmartScribe		K562	1	18	17.3	19.9	
50	Single cell, K562	20190904	Reaction in tube from single cell	SmartScribe		K562	1	18	9.1	20.8	
51	Bulk, SmartScribe	20240522	Reaction in tube from 100 pg purified RNA	SmartScribe		HeLa	-	15	4.1	16.6	Presented in Chapter 3. See table S5, Sample ID 137.
52	Bulk, Maxima H Minus	20240529	Reaction in tube from 100 pg purified RNA	Maxima H Minus		HeLa	-	15	5.6	16.0	Presented in Chapter 3. See table S5, Sample ID 151.
53	Bulk, Maxima H Minus + 2nd strand synthesis	20240529	Reaction in tube from 100 pg purified RNA	Maxima H Minus	✓	HeLa	-	15	6.0	16.8	Presented in Chapter 3. See table S5, Sample ID 153.

Table S7 Number of detected transcripts and genes from different downsampled reads and libraries

Type	No. of reads	No. of experiment	No of cell	Transcript count_mean	Transcript count_std	Gene count_mean	Gene count_std
On-chip, Human mouse	1000	7	725	239	197	192	139

On-chip, Human mouse	2000	7	721	287	266	223	173
On-chip, Human mouse	5000	7	719	337	344	253	212
On-chip, Human mouse	10000	7	474	200	162	166	124
On-chip, Human mouse	20000	7	474	200	162	166	124
On-chip, paclitaxel	1000	10	293	139	153	116	119
On-chip, paclitaxel	2000	10	293	139	153	116	119
On-chip, paclitaxel	5000	10	293	139	153	116	119
On-chip, paclitaxel	10000	10	293	139	153	116	119
On-chip, paclitaxel	20000	10	293	139	153	116	119
On-chip, HeLa Fucci2	1000	9	746	235	186	195	134
On-chip, HeLa Fucci2	2000	9	746	235	186	195	134
On-chip, HeLa Fucci2	5000	9	746	235	186	195	134
On-chip, HeLa Fucci2	10000	9	746	235	186	195	134
On-chip, HeLa Fucci2	20000	9	746	235	186	195	134
On-chip, No tag	1000	3	292	234	161	195	121
On-chip, No tag	2000	3	292	234	161	195	121
On-chip, No tag	5000	3	292	234	161	195	121
On-chip, No tag	10000	3	292	234	161	195	121
On-chip, No tag	20000	3	292	234	161	195	121
On-chip, Maxima H Minus + 2nd strand sythesis	1000	1	519	434	246	318	150
On-chip, Maxima H Minus + 2nd strand sythesis	2000	1	514	715	398	474	212
On-chip, Maxima H Minus + 2nd strand sythesis	5000	1	515	828	460	531	234
Index sorting, HeLa Fucci2	1000	12	159	278	142	242	112
Index sorting, HeLa Fucci2	2000	12	160	555	284	445	199
Index sorting, HeLa Fucci2	5000	12	159	1411	712	954	381
Index sorting, HeLa Fucci2	10000	12	159	2792	1400	1568	567
Index sorting, HeLa Fucci2	20000	12	158	5534	2734	2435	780
Single cell, K562	1000	3	3	486	75	381	38
Single cell, K562	2000	3	3	957	136	662	60
Single cell, K562	5000	3	3	2259	287	1241	70
Single cell, K562	10000	3	3	4658	648	1936	98
Single cell, K562	20000	3	3	8623	1194	2785	158
Bulk, SmartScribe	1000	1	1	500	-	413	-
Bulk, SmartScribe	2000	1	1	1042	-	761	-
Bulk, SmartScribe	5000	1	1	2625	-	1504	-
Bulk, SmartScribe	10000	1	1	5209	-	2406	-
Bulk, SmartScribe	20000	1	1	10274	-	3597	-
Bulk, Maxima H Minus	1000	1	1	576	-	443	-
Bulk, Maxima H Minus	2000	1	1	1145	-	766	-
Bulk, Maxima H Minus	5000	1	1	2807	-	1490	-
Bulk, Maxima H Minus	10000	1	1	5584	-	2356	-
Bulk, Maxima H Minus	20000	1	1	11004	-	3561	-
Bulk, Maxima H Minus + 2nd strand sythesis	1000	1	1	547	-	468	-
Bulk, Maxima H Minus + 2nd strand sythesis	2000	1	1	1121	-	861	-
Bulk, Maxima H Minus + 2nd strand sythesis	5000	1	1	2785	-	1739	-
Bulk, Maxima H Minus + 2nd strand sythesis	10000	1	1	5529	-	2745	-
Bulk, Maxima H Minus + 2nd strand sythesis	20000	1	1	10955	-	4204	-

Table S7 List of libraries used to examine the preparation process.

Sample ID	RNA input (ng)	RT primer (bead/free)	RT primer final conc. (μM)	TSO (μM) final conc.	GC% of barcode	PEG8000 (%), final	GTP (mM), final	RT enzyme	RT treated amounts (units/μL)	RT buffer condition	DTT (mM) final conc.	Reaction vol. @RT (μL)	Heat-deactivated RNA+RT	RT cycling	End-treated (units/μL)	Exo incubation time (min)	Reacted substrate (Estimation)	2nd strand synthesis primer	2nd primer final conc.	Add dNTP final conc.	buffer exchange	Sequencing PCR cycles	Amplify vol.	10x analysis buffer @Amplify reaction	elution vol. (μL)	cDNA conc. (ng/μL)	cDNA amounts (ng)	ap-ct (1x GAPDH)
1	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	50	12.5 (no reaction)				x15	0.7x	—	6.5	0.290	1.89	17.98	
2	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	50	12.5 (no reaction)				x15	0.7x	—	6.5	0.272	1.77	18.10	
3	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	50	12.5 (no reaction)				x15	0.7x	—	6.5	0.284	1.85	18.25	
4	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	50	12.5 (no reaction)				x15	0.7x	—	6.5	0.284	1.85	17.99	
5	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	50	12.5 (no reaction)				x15	0.7x	—	13	0.356	4.63	17.71	
6	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	50	12.5 (no reaction)				x15	0.7x	—	13	0.332	4.32	17.87	
7	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x15	0.7x	—	8.5	0.324	2.75	18.13	
8	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x15	0.7x	+	4.25	0.458	1.95	17.51	
9	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x15	0.7x	+	8.5	0.242	2.06	18.42	
10	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x15	0.7x	—	17.0	0.164	2.79	18.83	
11	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x15	0.7x	+	8.5	0.324	2.75	17.95	
12	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x15	0.7x	+	17.0	0.196	3.33	18.82	
13	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	10	2.5 (no reaction)				x18	0.7x	+	8.5	1.07	9.10	16.08	
14	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	0.7x	+	8.5	1.30	11.1	15.71	
15	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.083	10	0.8 (no reaction)				x18	0.7x	+	8.5	3.06	26.0	14.37	
16	100	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.083	30	2.5 (no reaction)				x18	0.7x	+	8.5	1.00	8.50	16.45	
17	10	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	10	2.5 (no reaction)				x18	0.7x	+	8.5	0.162	1.38	19.55	
18	10	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	0.7x	+	8.5	0.206	1.75	18.77	
19	10	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.083	10	0.8 (no reaction)				x18	0.7x	+	8.5	0.490	4.17	17.91	
20	10	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.083	30	2.5 (no reaction)				x18	0.7x	+	8.5	0.236	2.01	19.08	
21	1 cell in DW	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.0475	0.807	25.76	
22	1 cell in DW	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.0719	0.61	25.12	
23	1 cell in DW	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.0801	1.36	21.52	
24	1 cell in DW	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.0871	1.48	22.28	
25	1 cell lysate	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.196	3.33	20.25	
26	1 cell lysate	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.160	2.72	20.20	
27	1 cell lysate	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.0975	1.66	20.86	
28	1 cell lysate	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	1x	+	17.0	0.258	4.39	19.66	
29	10	free	1.2	2.4	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	0.7x	+	8.5	0.172	1.46	18.82	
30	10	free	1.2	2.4	20	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7.5 (no reaction)				x18	0.7x	+	8.5	0.138	1.17	19.54	

31	10	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	1 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	0.7x	+	8.5	0.106	0.901	20.6 2
32	10	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	1 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	0.7x	+	8.5	0.069 5	0.591	21.6 8
33	10	free	1.2	2.4	8 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	1 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	0.7x	+	8.5	0.081 8	0.695	21.9 1
34	10	free	1.2	2.4	8 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	1 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	0.7x	+	8.5	0.071 0	0.604	21.6 8
35	30	free	1.2	2.4	1 5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	1 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	0.7x	+	8.5	0.454	3.86	17.3 4
36	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	1 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	0.7x	+	8.5	1.03	8.76	16.2 8
37	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.230	2.99	18.2 2
38	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.206	2.68	18.6 1
39	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.230	2.99	18.6 2
40	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.234	3.04	18.6 1
41	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.214	2.78	18.7 5
42	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.182	2.37	18.9 9
43	100	free	0.120	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.400	5.20	17.0 1
44	100	free	0.012	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.370	4.81	17.0 4
45	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.216	2.81	19.1 9
46	100	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.204	2.65	19.1 3
47	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.538	6.99	17.0 9
48	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.278	3.61	18.7 4
49	100	free	0.012	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.452	5.88	16.9 5
50	100	free	0.012	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.476	6.19	16.9 6
51	100	free	0.001 2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.334	4.34	17.6 5
52	100	free	0.001 2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.306	3.98	17.7 8
53	100	free	0.001 2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.286	3.72	18.1 6
54	10	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.228	2.96	19.3 5
55	10	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.210	2.73	19.3 8
56	10	free	1.2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.184	2.39	19.7 2
57	10	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.504	6.55	17.0 3
58	10	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.492	6.40	16.9 5
59	10	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.454	5.90	17.1 8
60	10	free	0.012	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.382	4.97	17.0 3
61	10	free	0.012	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.392	5.10	17.0 7
62	10	free	0.012	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.424	5.51	17.1 6
63	10	free	0.001 2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	N.D.	rVAL UEI	22.1 7
64	10	free	0.001 2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	N.D.	rVAL UEI	25.1 8
65	10	free	0.001 2	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min→on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 18	1x	+	13.0	0.140	1.82	19.0 0
66	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.450	5.85	16.9 7
67	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)		x 15	1x	+	13.0	0.442	5.75	17.1 5

68	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.398	5.17	17.1 5
69	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.448	5.82	16.9 1
70	100	free	0.12	2.4	8 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.474	6.16	16.8 0
71	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.370	4.81	17.2 8
72	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.466	6.06	17.0 4
73	100	free	0.12	2.4	8 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.328	4.26	40.0 0
74	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT*	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.426	5.54	17.0 9
75	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT*	5	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.362	4.71	17.3 0
76	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Homemade buffer same as SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.324	4.21	17.4 8
77	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	condition#80: 25Tris/30NaCl/3MgCl2 (mM,f.c.)	6.5	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.041 3	0.537	20.4 6
78	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.110	0.43	21.0 8
79	100	free	0.12	2.4	5 0	0	0	SMARTScribe RT	10	KCl substituted by NaCl, homemade buffer same as SSRT	2	2 0	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.710	9.23	16.4 5
80	10	free	2	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	65°C, 5min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.002 95	0.038 3	20.4 3
81	10	free	0.5	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	65°C, 5min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.214	2.78	18.5 2
82	10	free	0.12	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	65°C, 5min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.232	3.02	19.1 4
83	10	free	2	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	2 0	65°C, 5min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.116	1.51	22.3 3
84	10	free	2.00	2.4	5 0	0	0	Maxima H Minus RT	10	condition#80: 25Tris/30NaCl/3MgCl2 (mM,f.c.)	6.5	2 0	65°C, 5min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.110	1.43	21.4 5
85	10	free	0.48	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.288	3.74	17.5 4
86	10	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.280	3.64	18.1 0
87	10	free	0.48	1.5	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	50°C, 30min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.274	3.56	18.1 7
88	10	free	0.48	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	2 0	72°C, 3min →on ice 2min<	10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.766	9.96	17.0 4
89	10	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.832	10.82	16.9 9
90	10	free	0.48	1.5	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.744	9.67	16.9 9
91	10	free	0.48	2.4	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	2 0	no	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.734	9.54	18.1 0
92	10	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	no	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.696	9.05	18.3 0
93	10	free	0.48	1.5	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	no	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 18	1x	+	13.0	0.778	10.11	18.4 1
94	100	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.770	10.01	16.9 7
95	100	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.646	8.40	17.2 1
96	100	free	0.48	2.0	5 0	2	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.728	9.46	17.0 7
97	100	free	0.48	2.0	5 0	5	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.768	9.98	17.3 5
98	100	free	0.48	2.0	5 0	7	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	1.050	13.65	17.3 4
99	100	free	0.48	2.0	5 0	9	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)JRT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	1.040	13.52	17.6 7

10 0	100	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	condition#80: 25Tris/30NaCl/3MgCl2 (mM,f.c.)	6.5	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.670	8.71	17.6 1
10 1	100	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	KCL substituted by NaCl, homemade buffer same as MaximaH(-)RT	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.586	7.62	18.0 0
10 2	100	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.766	9.96	17.4 7
10 3	100	free	0.48	2.0	5 0	0	0.5	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	N.D.	#VAL UE!	26.2 9
10 4	100	free	0.48	2.0	5 0	0	1	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	N.D.	#VAL UE!	27.5 3
10 5	100	free	0.48	2.0	5 0	2	0.5	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	N.D.	#VAL UE!	25.4 0
10 6	100	free	0.48	2.0	5 0	2	1	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	N.D.	#VAL UE!	26.8 4
10 7	100	free	0.48	2.0	5 0	5	0.5	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	N.D.	#VAL UE!	25.9 8
10 8	100	free	0.48	2.0	5 0	5	1	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	N.D.	#VAL UE!	26.5 6
10 9	100	free	0.48	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.566	7.36	18.8 0
11 0	100	free	0.48	2.0	5 0	2	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.582	7.57	18.9 0
11 1	100	free	0.48	2.0	5 0	5	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.530	6.89	19.0 7
11 2	100	free	0.48	2.0	5 0	7	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.630	8.19	19.1 3
11 3	100	free	0.48	2.0	5 0	9	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.660	8.58	19.5 0
11 4	100	free	0.12	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	1.21	15.7	17.9 5
11 5	100	free	0.12	2.0	5 0	2	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	1.45	18.9	17.8 3
11 6	100	free	0.12	2.0	5 0	5	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	1.55	20.2	17.9 0
11 7	100	free	0.12	2.0	5 0	7	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	1.23	16.0	18.6 8
11 8	100	free	0.12	2.0	5 0	9	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	1.52	19.8	17.9 1
11 9	100	free	0.12	2.0	5 0	0	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.684	8.89	17.5 9
12 0	100	free	0.12	2.0	5 0	2	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.726	9.44	17.5 2
12 1	100	free	0.12	2.0	5 0	5	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.608	7.90	18.2 8
12 2	100	free	0.12	2.0	5 0	7	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.554	7.20	19.0 1
12 3	100	free	0.12	2.0	5 0	9	0	Maxima H Minus RT	10	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x(twice)	+(1st only)	13.0	0.512	6.66	18.9 4
12 4	100	free	0.12	2.0	5 0	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)RT**	10	1 0	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min)→85°C, 5min	0.25	30	7. 5	(no reaction)					x 15	1x	+	13.0	0.740	9.62	16.1 5

125	100	free	0.12	2.0	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)					x15	1x	+	13.0	0.322	4.19	17.25
126	100	free	0.12	2.0	50	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)					x15	1x	+	13.0	0.680	8.84	16.58
127	100	free	0.12	2.0	50	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 3min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)					x15	1x	+	13.0	0.362	4.71	17.41
128	100	free	0.12	2.0	50	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)					x15	1x	+	13.0	0.648	8.42	16.49
129	100	beads	1.20	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	dN-SMART	10	1	+	(NFW)	x15	1x	+	13.0	0.078	1.02	23.92
130	100	beads	0.12	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	dN-SMART	10	1	+	(NFW)	x15	1x	+	13.0	0.114	1.48	23.31
131	100	beads	1.20	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	S3-randomer	10	1	+	(NFW)	x15	1x	+	13.0	0.116	1.51	24.00
132	100	beads	0.12	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	S3-randomer	10	1	+	(NFW)	x15	1x	+	13.0	0.178	2.31	23.14
133	100	beads	1.20	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			+	(NFW)	x15	1x	+	13.0	0.089	1.16	24.61
134	100	beads	0.12	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			+	(NFW)	x15	1x	+	13.0	0.036	0.47	22.98
135	100	free	0.12	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	dT			-		x15	1x	+	13.0	0.812	10.56	16.94
136	100	beads	3.6	2.0	40	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.464	6.03	17.82
137	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.634	8.24	16.64
138	100	beads	0.12	2.0	40	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.642	8.35	16.52
139	100	free	0.12	2.0	40	0	0	SMARTScribe RT	10	Attached buffer, w/ SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.446	5.80	17.22
140	100	beads	3.6	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.680	8.84	17.44
141	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.944	12.27	16.19
142	100	beads	0.12	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.776	10.09	16.38
143	100	free	0.12	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min→on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.744	9.67	16.74
144	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	S3-randomer	10	1	+	(RT buffer)	x15	1x	+	13.0	1.160	15.08	17.24
145	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	S3-randomer	1	1	+	(RT buffer)	x15	1x	+	13.0	0.450	5.85	17.33
146	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	S3-randomer	1	0.3	+	(RT buffer)	x15	1x	+	13.0	0.608	7.90	16.73
147	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			+	(RT buffer)	x15	1x	+	13.0	0.212	2.76	18.43
148	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.804	10.45	16.22
149	100	free	0.12	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.578	7.51	16.85
150	100	beads	1.2	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	S3-randomer	10	1	+	(RT buffer)	x15	1x	+	13.0	1.110	14.43	16.59
151	100	beads	1.2	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	dN-SMART	10	1	+	(RT buffer)	x15	1x	+	13.0	0.924	12.01	16.78
152	100	beads	1.2	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			+	(RT buffer)	x15	1x	+	13.0	0.474	6.16	17.10
153	100	beads	1.2	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-		x15	1x	+	13.0	0.860	11.18	16.04

154	100	free	0.48	2.0	40	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x	+	13.0	0.644	8.37	16.65
155	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	S3- randomer	10	1	+ (RT buffer)	x15	1x	+	13.0	0.458	5.95	17.30
156	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	dN- SMART	10	1	+ (RT buffer)	x15	1x	+	13.0	0.474	6.16	17.40
157	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			+ (RT buffer)	x15	1x	+	13.0	0.300	3.90	18.09
158	100	beads	1.2	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-	x15	1x	+	13.0	0.880	11.44	16.00
159	100	free	0.12	2.0	40	0	0	SMARTScribe RT	10	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-	x15	1x	+	13.0	0.648	8.42	16.62
160	100	free	0.12	2.0	40	0	0	SMARTScribe RT	2	KCL substituted by NaCl, homemade buffer same as SSRT	2	10	72°C, 3min →on ice 2min<	42°C, 90min→70°C, 10min	0.25	30	7.5	(no reaction)			-	x15	1x	+	13.0	0.452	5.88	17.18
161	100	free	0.48	2.0	50	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x(twice)	+(1st only)	13.0	0.246	3.20	17.99
162	100	free	0.48	2.0	50	2	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x(twice)	+(1st only)	13.0	0.272	3.54	17.90
163	100	free	0.48	2.0	50	5	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x(twice)	+(1st only)	13.0	0.366	4.76	17.69
164	100	free	0.48	2.0	50	7	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x(twice)	+(1st only)	13.0	0.414	5.38	17.63
165	100	free	0.48	2.0	50	9	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x(twice)	+(1st only)	13.0	0.484	6.29	17.76
166	100	free	0.12	2.0	50	0	0	Maxima H Minus RT	2	Attached buffer, w/ MaximaH(-)JRT**	10	10	72°C, 3min →on ice 2min<	42°C, 90min, 10x(50°C, 2min, 42°C, 2min) →85°C, 5min	0.25	30	7.5	(no reaction)			-	x15	1x(twice)	+(1st only)	13.0	0.402	5.23	17.65

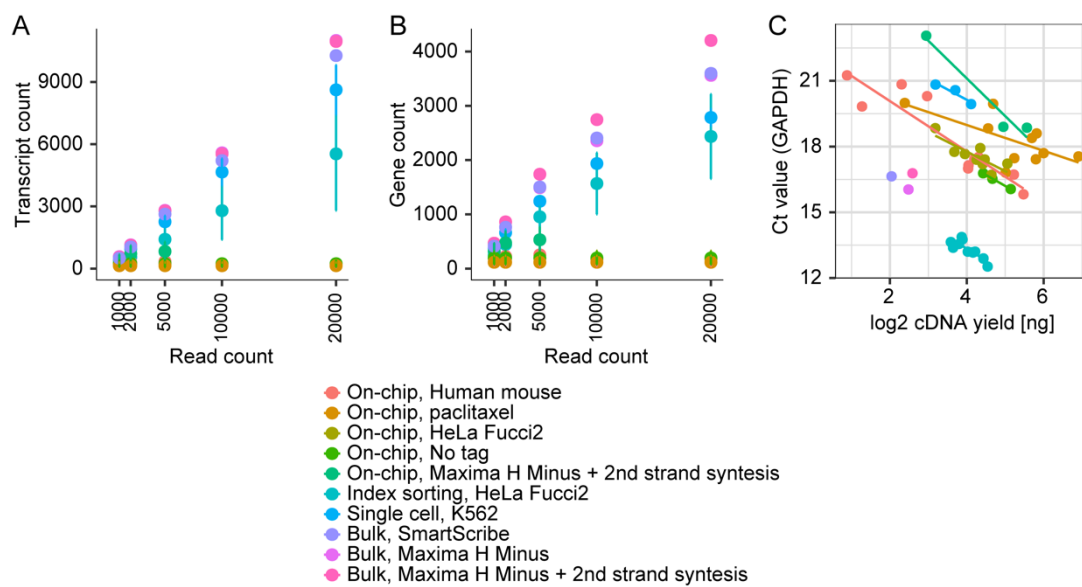


Figure S 1 Comparison of library preparation protocol in Table S6

A. B. Scatter plot showing transcript counts (A) and gene counts (B) against read count, comparing different library preparation protocols. Values are listed in Table S7. C. Scatter plot showing cDNA yields and Ct values of GAPDH after reverse transcription and PCR amplification, comparing different library preparation protocols

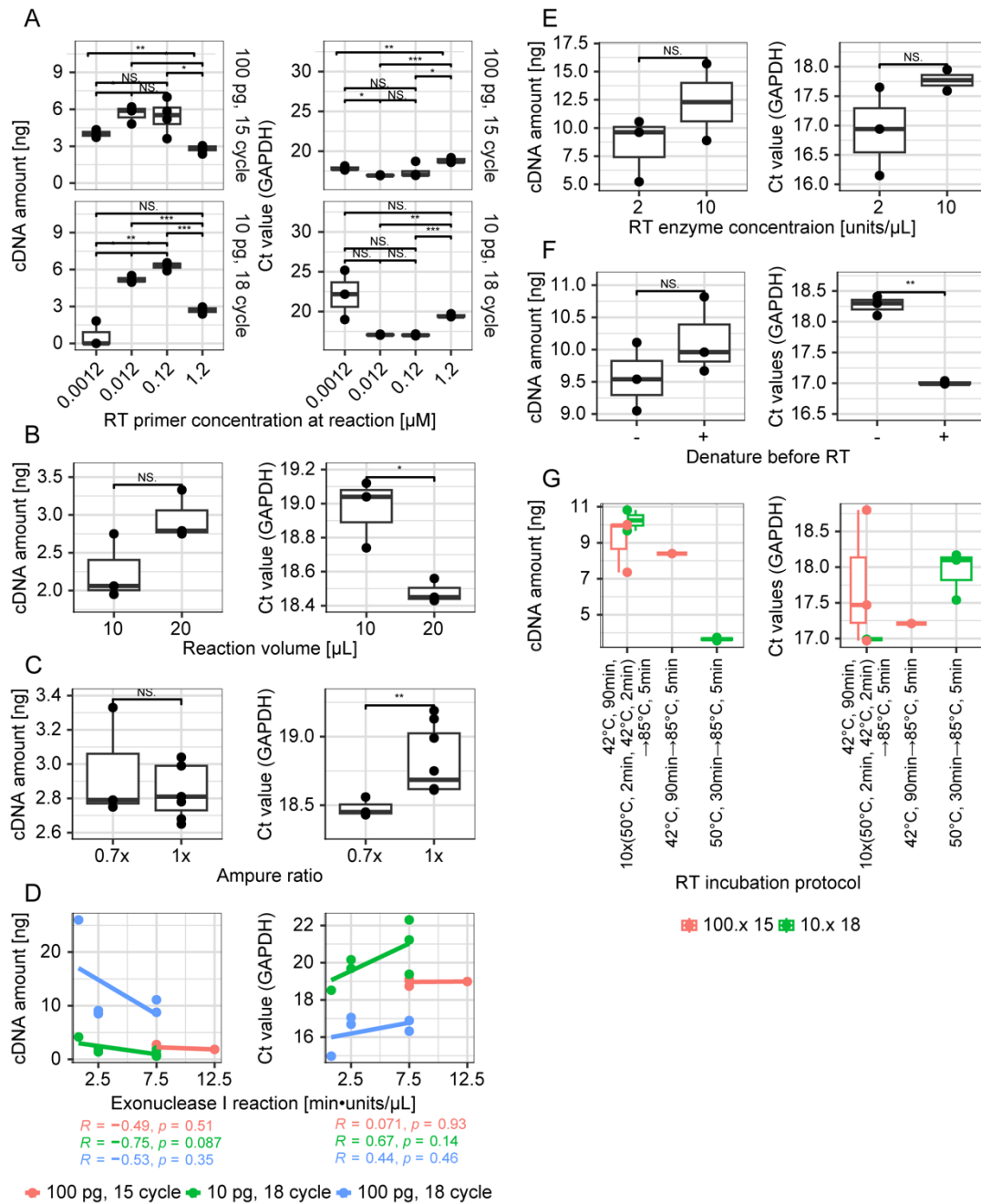


Figure S 2 Optimization of library preparation protocols.

cDNA yields and Ct values of GAPDH after reverse transcription and PCR amplification, comparing different conditions. Data from Table S8, for which experimental conditions were available, were selected and presented in the figures. We selected Sample ID 37-66 for (A), 7-12 for (B), 10-12, 37-42, 45-46 for (C), 4, 7-9, 13-20, 31-32, 36 for (D), 114, 119, 124, 135, 166 for (E), 88-93 for (F), 85-87, 89-90, 94-95, 102, 109 for (G). The Ct values were adjusted to facilitate comparison of different

elution volumes, with C_t values recalculated to be equivalent to an elution volume of 13 μL for (B, C, D).

Appendix Protocol 1

Preparation of cDNA library

- Standard cDNA Synthesis -

A. Reverse transcription and proxy extension with template switching (see also SMART-seq v4 Ultra Low Input RNA Kit and SMART-Seq V4 3' DE Kit)

1. Regents

Component	Storage	Company	Product#	Note
100 U/μL SMARTScribe™ Reverse Transcriptase	-20°C	Takara Bio	639536	●
5X First-Strand Buffer	-20°C	Takara Bio	639536	●
20 mM DTT	-20°C	Takara Bio	639536	●
RNase Inhibitor (40 U/μL)	-20°C	Takara Bio	2313A	
10X lysis buffer	-20°C	Takara Bio	635013	
dNTP mix	-20°C	ThermoFisher Scientific	R0192	
Nuclease free water	Room temp	Invitrogen	10977023	

- ...Components of the kit of “SMARTScribe™ Reverse Transcriptase”.

Primers

- Barcoded RT primers (Eurofin. The cell barcode is underlined. Replace the barcode with other cell IDs.)

AAGCAGTGGTATCAACGCAGAGTACGACGCTCTCCGATCTACCTCGTTG
ANNNNNNNNTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN

- Template switching oligo: /Bio/-AAGCAGTGGTATCAACGCAGAGTACrGrG+G (order to Qiagen; Takara is the distributor in Japan, 5' biotinylated; + LNA; r ribonucleotide)
- Thaw 5X First-Strand Buffer at room temperature. Thaw other reagents on ice except for enzymes. Gently mix them and spin down. Store all on ice.
 - Preheat the thermal cycler to 72°C.
 - Prepare a 10x reaction buffer

10x reaction buffer for 10 lysis

Lysis buffer	9.5 μ L
RNase Inhibitor (40U/ μ L)	0.5 μ L
Total	10 μ L

Mix briefly, then spin down.

NOTE: Lysis buffer contains a detergent (NP-40), it is critical to avoid bubbles when mixing.

- Prepare sample

Sample components

Sample	1–2.75 μ L	1	Total
Nuclease-free water	Up to 2.75 μ L	3.5	
10x reaction buffer	0.5 μ L	1	
Total	3.25 μ L	6.5	

Pipette to mix sample.

- Add 1 μ L of RT primer (1.2 μ M) to the sample.

Sample solution	3.25 μ L	6.5	final 0.12 μ M
1.2 μ M RT primer	1 μ L	2	
Total	4.25 μ L	8.5	

- If you are working with cells, incubate at room temperature (or 25°C) for 5 min.
- Incubate the tube at 72°C in a preheated, hot-lid thermal cycler for 3 min. Then place the sample on ice for 2 min.

Room temperature	5 min
72°C	3 min
On ice	2 min

NOTE: Prepare your master mix while (step 7, 8) while your tubes are incubating. The enzyme will be added just before use (step 11). Steps 12–13 below are critical for first strand cDNA synthesis and should not be delayed after completing step 8.

- Preheat the thermal cycler to 42°C.

10. Prepare enough template switch master mix for all reactions, plus 10% the total reaction mix volume.
11. Add 1 μL of SMARTScribe Reverse Transcriptase to the master mix (total 5.75 μL , final 10 U/ μL). Add the reverse transcriptase to the master mix just prior to use, making sure to gently mix the reverse transcriptase tube without vortexing before adding it.

Template switch master mix: 5.75 μL per reaction

	10 μL rxn	20 μL rxn	Final co nc.
-5X First-Strand Buffer	2 μL	4	1X
-10 mM dNTP	1 μL	2	1 mM
-40 U/ μL RNase Inhibitor	0.25 μL	0.5	1 U/ μL
-20 mM DTT	1 μL	2	2 mM
-48 μM template switching oligo	0.5 μL	1	2.4 μM
/Bio/-			
AAGCAGTGGTATCAACGCAGAGTACrG			
rG+G			

SMARTScribe Reverse Transcriptase	1 μL	2	10 U/ μL
Total	5.75 μL	11.5	

12. Add 5.75 μL of the master mix to the sample (4.25 μL) to be 10 μL of the total volume.

Master mix	5.75 μL	11.5
Sample solution	4.25 μL	8.5
Total	10 μL	20

13. Place the tubes in a thermal cycler with a heated lid, preheated at 42°C.

Thermal cycler program for reverse transcription

42°C	90 min
70°C	10 min
4°C	∞

STOPPING POINT: The tubes can be stored at 4°C overnight.

B. Digesting Primers by Exonuclease I

1. Regents

Component	Storage	Company	Product#
Exonuclease I	-20°C	Takara Bio	2650A

2. Add 1 μL of 2-fold diluted the Exonuclease I (2.5 U/ μL) to Reaction mixture after RT (10 μL), tube 11 μL of total volume.

Reaction mixture after RT	10 μL	
2.5 U/ μL Exonuclease I	1 μL	final 0.23 U/ μL
Total	11 μL	

3. Place the tube in a thermal cycler.

Thermal cycler program for Exonuclease I treatment	
37°C	30 min
80°C	20 min
4°C	∞

C. PCR amplification of cDNA

IMPORTANT: The following table provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for the first-strand cDNA synthesis. Typical cycle numbers are provided as a rough guide for working with small amounts of RNA. Optimal parameters may vary for different templates, different cell types, and different thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles.

Cycling guidelines based on amount of starting material				
Input Amount of Total RNA	Input Amount of Cells	Typical Number of PCR Cycles		
		Individual	6 reactions in pool	12 reactions in pool
10 ng	1,000 cells	7–8	4–5	3–4
1 ng	100 cells	10–11	8–9	7–8
100 pg	10 cells	14–15	11–12	10–11
10 pg	1 cell	17–18	14–15	13–14

1. Regents

Component	Storage	Company	Product#
SeqAmp™ DNA Polymerase	-20°C	Takara Bio	638504
Nuclease free water	Room temp	Invitrogen	10977023

Primers

- PCR primer2: AAGCAGTGGTATCAACGCAGAGT

2. Thaw all the reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
3. Prepare enough PCR Master Mix for all the reactions, plus 10% of the total reaction mix volume. Combine the following reagents in the order shown:

PCR Maser Mix (14 µL/reaction)		
2X SeqAmp PCR Buffer	12.5 µL	f. 1X
12 µM PCR primer2	0.5 µL	f. 0.24 µM
SeqAmp DNA Polymerase	0.5 µL	f. 0.025U/ µL
Nuclease Free Water	0.5 µL	

Total	14 μ L
-------	------------

NOTE: Remove the SeqAmp DNA polymerase from the freezer, gently mix the tube without vortexing, and add to the Master Mix just before use. Mix the Master Mix well without vortexing and spin the tube briefly to collect the contents at the bottom of the tube.

4. Add 14 μ L of PCR Master Mix to each tube containing 11 μ L of first-strand cDNA product from Section B (total 25 μ L). Mix the Master Mix well and spin the tube briefly to collect the contents at the bottom of the tubes.
5. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

Thermal cycler program for PCR amplification of cDNA

95°C	1 min	
X cycles:		
98°C	10 s	]
65°C	30 s	
68°C	4 min	
72°C	10 min	
4°C	∞	

STOPPING POINT: The tubes may be stored at 4°C overnight.

D. Purification with AMPure & QC with Qubit and Bioanalyser & qPCR

D-1. Purification of cDNA with AMPure

1. Regents

Component	Storage	Company	Product#
Agencourt AMPure XP PCR Purification	4°C	Beckman Coulter	
Ethanol (99.5) for Molecular Biology	Room temp	Wako	052-07221
Nuclease free water	Room temp	Invitrogen	10977023
Elution buffer (10 mM Tris-HCl, pH8.5)	Room temp	Takarabio	ST0770

2. Pool the differently-indexed PCR products from Step C in a single 1.5 mL tube, if you need.
3. Prepare fresh 80% ethanol. You will need 0.4 mL per tube.
4. Add 0.5 μ L of 10x Lysis buffer to cDNA amplification mix.
5. Add 0.7x AMPure to cDNA amplification mix. ()
6. Mix by vortexing or pipetting the entire volume up and down at least 10 times to mix thoroughly.
7. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.
8. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for ~5 minutes or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
9. While the samples are on the magnetic separation device, pipette out the supernatants.
10. Keep the samples on the magnetic separation device. Add 200 μ L of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 seconds and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
11. Repeat Step 9.
12. Briefly spin the samples to collect the liquid from the side of the wall. Place the samples on the magnetic device for 30 seconds, then remove all the remaining ethanol with a pipette.
13. Allow the samples to dry at room temperature for 2.5-3 minutes or longer until the pellet is no longer shiny and before a crack appears.

14. Once the beads are dry, add 8.5 μL of Elution Buffer (10 mM Tris-HCl, pH 8.5) to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads.
15. Incubate at room temperature for 2 minutes to rehydrate.
16. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic device for 2 minute or longer until the solution is completely clear.
17. Transfer clear supernatant containing purified cDNA to a nuclease-free non-sticky tube.

D-2. Quantify the yield with Qubit

Range: over 10 pg/ μL to 100 ng/ μL

1. Regents

Component	Storage	Company	Product#
Qubit dsDNA HS Assay kit	4°C	ThermoFisher Scientific	Q32845

2. Follow the protocol of Qubit dsDNA HS Assay kit.

D-3. qPCR

1. Regents

Component	Storage	Company	Product#
TaqMan Universal Master Mix II, no UNG	4°C	ThermoFisher Scientific	4440040
TaqMan Gene Expression Assays (20X), GAPDH	-20°C	ThermoFisher Scientific	Hs02758991_g1
TaqMan Gene Expression Assays (20X), ACTB	-20°C	ThermoFisher Scientific	Hs01060665_g1

2. Prepare the reaction mix for each sample using the components listed below.

qPCR Mix (20 μL /reaction)

TaqMan Universal Master Mix II, no UNG (2X)	5 μL	f. 1X
TaqMan Gene Expression Assay (20X)	0.5 μL	f. 1X
cDNA sample	1 μL	
Nuclease Free Water	3.5 μL	
Total	10 μL	

- Calculate the volume of each component of the PCR reaction mix by multiplying the volume of each component by the number of replicates for each sample.
 - Prepare 110% of the required volume to account for pipetting error.
3. Cap tubes.
 4. Vortex the tube briefly to mix the solutions.
 5. Centrifuge the tubes to spin down the contents and eliminate any air bubbles from the solutions.
 6. Load the tubes into the real-time PCR system and run the following program:

Thermal cycler program for qPCR amplification of cDNA

95°C	10 min
40 cycles:	
95°C	15 s
60°C	1 min
4°C	∞

D-3. Quality check by Bioanalyzer

Chip: High sensitivity DNA Chip

Range: 100-5000 pg/μL, dsDNA

E. cDNA Library Preparation

E-1. Tagmentation of cDNA

1. Regents

Component	Storage	Company	Product#
Nextera XT DNA Library Preparation Kit	-20°C	illumina	
Index primers	-20°C	Eurofins	

- Thaw all reagents required for tagmentation on ice. Gently vortex, spin down briefly, and store them on ice.
- Prepare tagmentation mixtures in individual 0.2 mL RNase-free PCR tubes on ice as shown in the table below.

Sample components

TD Buffer	10 μ L	} Total 5 μ L
cDNA	xx μ L (400 pg)	
Nuclease Free Water	Up to 5 μ L	
ATM	5 μ L	
Total	20 μ L	

- Place the tubes in a preheated thermal cycler with heated lid that has the following program running.

Thermal cycler program for PCR amplification of cDNA

55°C	5 min
10°C	∞

- Once the sample reaches 10°C, immediately add 5 μ L of NT Buffer. Mix the tubes by vortexing gently, and spin down briefly to collect the contents at the bottom.
- Incubate the tubes at RT for 5 min and then place it on ice. The total volume to be 25 μ L.

E-2. 3' cDNA library construction with Nextera PCR

1. Thaw NPM (Nextera PCR Master Mix) and Forward and Reverse Index Primers.
Gently vortex each to mix and spin down briefly and store on ice.
2. Prepare enough PCR Master Mix for all reactions, plus one extra reaction volume.
Combine the following reagents in the order shown.

Master Mix	
NPM	15 μ L
*PCR primer_i5_(1~8) (10 μ M)	1 μ L
*PCR primer_i7_(1~12) (10 μ M)	1 μ L
Nuclease Free Water	8 μ L
Total	25 μ L

* List of PCR primers is described in Table S2.

3. Add 25 μ L of the Master Mix to the sample (25 μ L) to be 50 μ L of the total volume.
4. Place the tubes in a thermal cycler.

Table S12. PCR cycle with 50 μ L of reaction volume

72°C	3 min	
95°C	30 s	
12 cycles:		
95°C	10 s	]
55°C	30 s	
72°C	30 s	
72°C	5 min	
4°C	∞	

E-3. Purification with AMPure & QC with Qubit and Bioanalyser

1. Add 0.6x AMPure XP to cDNA amplification mix and purify by eluting with ~8 μ L of RSB.
2. Quantify the yield with Qubit dsDNA HS Assay Kits.
3. In detail, please follow the procedures described in Step D.

Appendix Protocol 2

Preparation of cDNA library

- Second-Strand Synthesis-Based cDNA Synthesis

-

A. Reverse transcription and 2nd strand synthesis with template switching (see also Seq-Well S3 protocol)

14. Regents

Component	Storage	Company	Product#	Note
Maxima H Minus Reverse Transcriptase (200 U/μL)	-20°C	Thermo Scientific™	EP0751/ EP0752/ EP0753	●
5X RT Buffer	-20°C			●
RNase Inhibitor (40 U/μL)	-20°C	Takara Bio	2313A	
10X Lysis Buffer	-20°C	Takara Bio	635013	
dNTP Mix (10 mM each)	-20°C	ThermoFisher Scientific	R0192	
Nuclease free water	Room temp	Invitrogen	10977023	

- ...Components of “Maxima H Minus Reverse Transcriptase”.

Primers

- Synthetic acrylamide beads with barcode RT primer
- Template switching oligo: /Bio/5'-
AAGCAGTGGTATCAACGCAGAGTACrGrG+G -3' (order to Qiagen; Takara is the distributor in Japan, 5' biotinylated; + LNA; r ribonucleotide)

- Thaw 5x RT Buffer at room temperature. Thaw other reagents on ice except for enzymes. Gently mix them and spin down. Store all on ice.
- Preheat the thermal cycler to 72°C.

17. Prepare a 10 x Reaction buffer

10 x Reaction buffer for 20 samples

10 x Lysis Buffer	9.5 μ L
RNase Inhibitor (40U/ μ L)	0.5 μ L
Total	10 μ L

Mix briefly, then spin down.

NOTE: Lysis buffer contains a detergent (NP-40), it is critical to avoid bubbles when mixing.

18. Prepare sample

Sample components

Sample	1 μ L	
12 μ M RT primer beads	1 μ L	final 1.2 μ M
10 mM dNTP	0.5 μ L	final 0.5 mM
10 x Reaction buffer	0.5 μ L	final 0.5 x
Nuclease-free water	2 μ L	
Total	5 μ L	

Pipette to mix sample.

19. Incubate the tube at 72°C in a preheated, hot-lid thermal cycler for 3 min. Then place the sample on ice for >2 min.

72°C	3 min
on ice	> 2 min

NOTE: Prepare your master mix while (step 6) while your tubes are incubating. The enzyme will be added just before use (step 10). Steps 11–12 below are critical for first strand cDNA synthesis and should not be delayed after completing step 6.

20. Set the thermal cycler program and standby at 42°C.
21. Prepare enough template switch master mix for all reactions, plus 10% the total reaction mix volume.
22. Dilute Maxima H Minus Reverse Transcriptase (200 U/ μ L) by 1/5 with Nuclease-free water to 40 U/ μ L.

23. Add 0.5 μL of Maxima H Minus Reverse Transcriptase to the master mix (total 5 μL , final 2 U/ μL). Add the reverse transcriptase to the master mix just prior to use, making sure to gently mix the reverse transcriptase tube without vortexing before adding it.

Template switch master mix: 5 μL per reaction

	/10 μL , rxn	final conc.
5 x RT Buffer	2 μL	1 x
RNase Inhibitor (40 U/ μL)	0.25 μL	1 U/ μL
48 μM template switching oligo	0.42 μL	2 μM
Nuclease-free water	1.83 μL	2.4 μM
Maxima H Minus Reverse Transcriptase (40 U/ μL)	0.5 μL	2 U/ μL
Total	5 μL	

24. Add 5 μL of the master mix to the sample (5 μL) to be 10 μL of the total volume.

Master mix	5 μL
Sample solution	5 μL
Total	10 μL

Mix well by pipetting, then spin down briefly.

25. Place the tubes in a thermal cycler with a heated lid, preheated at 42°C.

Thermal cycler program for reverse transcription

Temperature	Time	cycles
42°C	90 min	1
50°C	2 min	10
42°C	2 min	
85°C	5 min	1
4°C	∞	1

STOPPING POINT: The tubes can be stored at 4°C overnight.

B. Exonuclease I treatment to remove excess oligos

4. Regents

Component	Storage	Company	Product#
Exonuclease I	-20°C	Takara Bio	2650A

5. Add 0.5 μL of Exonuclease I (5 U/ μL) to reaction mixture after RT (10 μL) to be 10.5 μL of the total volume.

Reaction mixture after RT	10 μL	
Exonuclease I (5 U/ μL)	0.5 μL	final 0.24 U/ μL
Total	10.5 μL	

Mix well by pipetting, then spin down briefly.

6. Place the tube in a thermal cycler.

Thermal cycler program for Exonuclease I treatment	
37°C	30 min
80°C	20 min
4°C	∞

C. Ribonuclease H treatment to remove RNA

1. Regents

Component	Storage	Company	Product#
Ribonuclease H (RNase H)	-20°C	Takara Bio	2150A

2. Add 0.5 μL of RNase H (60 U/ μL) to reaction mixture after Exonuclease I treatment (10.5 μL) to be 11 μL of the total volume.

(Reaction mixture after Exonuclease I)	10 μL	
RNase H (60 U/ μL)	0.5 μL	final 2.7 U/ μL
Total	11 μL	

Mix well by pipetting, then spin down briefly.

3. Place the tube in a thermal cycler.

Thermal cycler program for Exonuclease I treatment

37°C	30 min
65°C	20 min
4°C	∞

D. Second-Strand Synthesis: using dN-SMART/S3-Randomer and Klenow enzyme for the extension. (Both full-length and partial cDNAs will be converted to double-stranded cDNA.)

1. Regents

Component	Storage	Company	Product#
DNA Polymerase I, Large (Klenow)	-20°C	New England	M0212S
Fragment		Biolabs	

Primers

- a. S3-Randomer: 5'- AAGCAGTGGTATCAACGCAGAGTGANNNGGNNNB -3' (34-mer)
 - b. dN-SMART: 5'- AAGCAGTGGTATCAACGCAGAGTNNNGGNNNB -3' (32-mer)
2. Add 1.35 μL of dNTP (10 mM) and 1.4 μL of 100 μM S3-Randomer/dN-SMART to reaction mixture after RNase H treatment (11 μL).
 3. Finally, add 0.5 μL of Large (Klenow) Fragment (5,000 U/mL) to be 14.25 μL of the total volume.

(Reaction mixture after RNase H)	11 μ L	
10 mM dNTP	1.35 μ L	final 0.95 mM
100 μ M S3-Randomer/dN-SMART	1.4 μ L	final 9.8 mM
Large (Klenow) Fragment (5,000 U/mL)	0.5 μ L	final 0.18 U/ μ L
Total	14.25 μ L	

Mix well by pipetting, then spin down briefly.

- Place the tube in a thermal cycler.

Thermal cycler program for Exonuclease I treatment

37°C	1 h
75°C	20 min
4°C	∞

E. Buffer change

- Prepare Wash buffer.

Wash buffer		
5 x RT Buffer	200 μ L	final 1 x
10 x Lysis Buffer	50 μ L	final 0.5 x
Nuclease-free water	750 μ L	
Total	1000 μ L	

- Following incubation, the beads were washed three times with 100 μ L of Wash buffer.
- 1st wash:** Add 100 μ L of Wash Buffer directly to the reaction mixture (14.25 μ L) to suspend the beads, then centrifuge at 4,700 rpm (2,100 x g) for 3 minutes at room temperature to precipitate the beads.

NOTE: After adding Wash Buffer, mix the beads thoroughly without pipetting to avoid adsorption of the beads on the pipette tip. Mix by tapping or inverting the tube.

- After centrifugation to precipitate the beads, leave more solution (~40 μ L) to avoid sucking up the beads and remove the supernatant (~75 μ L) by slowly aspirating with a fine tip.
- 2nd/3rd wash:** Repeat steps 3 and 4, and discard 100 μ L of the supernatant from step 4 in the 2nd/3rd wash.
- Finally, the volume is adjusted to 11.5 μ L with the Wash Buffer.

F. PCR amplification of cDNA

IMPORTANT: The following table provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for the first-strand cDNA synthesis. Typical cycle numbers are provided as a rough guide for working with small amounts of RNA. Optimal parameters may vary for different templates, different cell types, and different thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles.

Cycling guidelines based on amount of starting material				
Input Amount of Total RNA	Input Amount of Cells	Typical Number of PCR Cycles		
		Individual	6 reactions in pool	12 reactions in pool
10 ng	1,000 cells	7–8	4–5	3–4
1 ng	100 cells	10–11	8–9	7–8
100 pg	10 cells	14–15	11–12	10–11
10 pg	1 cell	17–18	14–15	13–14

6. Regents

Component	Storage	Company	Product#
SeqAmp™ DNA Polymerase	-20°C	Takara Bio	638504

Primers

- PCR primer2: AAGCAGTGGTATCAACGCAGAGT
- Type m primer: AGACGTGTGCTCTTCCGATCT

- Thaw all the reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
- Prepare enough PCR Master Mix for all the reactions, plus 10% of the total reaction mix volume. Combine the following reagents in the order shown:

PCR Maser Mix (13.5 µL/reaction)		
2X SeqAmp PCR Buffer	12.5 µL	f. 1X
12 µM PCR primer2	0.5 µL	f. 0.24 µM
0.45 µM Type m primer	0.5 µL	f. 0.009 µM
SeqAmp DNA Polymerase (1.25U/µL)	0.5 µL	f. 0.025U/ µL
Total	13.5 µL	

NOTE: Remove the SeqAmp DNA polymerase from the freezer, gently mix the tube without vortexing, and add to the Master Mix just before use. Mix the Master Mix well without vortexing and spin the tube briefly to collect the contents at the bottom of the tube.

9. Add 13.5 μL of PCR Master Mix to each tube containing 11.5 μL of second-strand cDNA product from Section E (total 25 μL). Mix the Master Mix well by pipetting and spin the tube briefly to collect the contents at the bottom of the tubes.
10. Place the tubes in a preheated thermal cycler with a heated lid and run the following program:

Thermal cycler program for PCR amplification of cDNA

95°C	1 min	
X cycles:		
98°C	10 s	]
65°C	30 s	
68°C	4 min	
72°C	10 min	
4°C	∞	

STOPPING POINT: The tubes may be stored at 4°C overnight.

G. Purification with SPRIselect & Quality Check (QC) with Qubit and Bioanalyser & qPCR

G-1. Purification to separate tag-derived cDNA (<180 bp) from mRNA-derived cDNA (>300 bp) using SPRIselect

18. Regents

Component	Storage	Company	Product#
SPRIselect	4°C	Beckman Coulter	B23318
Ethanol (99.5) for Molecular Biology	Room temp	Wako	052-07221
Elution buffer (10 mM Tris-HCl, pH8.5)	Room temp	Takara Bio	ST0770

19. Perform SPRI selection to separate mRNA-derived and tag-derived cDNAs. Transfer the PCR products (25 µL) from Step F in a single 1.5 mL tube.

NOTE: DO NOT DISCARD SUPERNATANT FROM 0.6X SPRI. THIS CONTAINS THE Tags!

20. Prepare fresh 80% ethanol. You will need 1 mL per sample.
21. Add 0.5 µL of 10x Lysis buffer to cDNA amplification mix.
22. Add 0.6x SPRIselect (15 µL) to cDNA amplification mix.
23. Mix by vortexing or pipetting the entire volume up and down at least 10 times to mix thoroughly.
24. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.
25. Briefly spin the samples to collect the liquid from the side or wall of the tube or sample. Place the samples on the magnetic separation device for ~5 minutes or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
26. While the samples are on the magnetic separation device, carefully transfer the supernatant to a new 1.5 mL tube without disturbing the pellet.

NOTE: Cleanup of this supernatant containing tag-derived cDNA continues in step

18.

27. Keep the samples on the magnetic separation device. Add 200 µL of freshly made 80% ethanol to each sample without disturbing the beads. Wait at least 30 seconds and carefully remove the supernatant containing contaminants with a pipette. DNA will remain bound to the beads during the washing process.
28. Repeat Step 10.

29. Spin down briefly to collect the liquid from the side or wall of the tube. Place the samples on the magnetic device for 30 seconds, then remove all the remaining ethanol with a pipette.
30. Remove from the magnet and air dry for 2-3 minutes at room temperature or longer until the pellets are no longer shiny and before they crack.

NOTE: Do not over dry beads as this will decrease elution efficiency.

31. Once the beads are dry, add 13 μ L of Elution Buffer (10 mM Tris-HCl, pH 8.5) to cover the bead pellet and mix thoroughly to resuspend the beads.
32. Incubate at room temperature for 2 minutes to rehydrate.
33. Spin down briefly to collect the liquid from the side or wall of the tube. Place the samples back on the magnetic device for 2 minute or longer until the solution is completely clear.
34. Transfer the clear supernatant containing the purified cDNA to a nuclease-free tube.
35. **(Continue from step 9)** Vortex to resuspend the SPRIselect reagent. Add 1.4x SPRIselect (35 μ L) to 40 μ L of the transferred supernatant to obtain a final SPRI volume of 2x SPRI.
36. Mix by vortexing or pipetting the entire volume up and down at least 10 times to mix thoroughly.
37. Incubate for 8 minutes at room temperature to allow the short cDNA to bind to the beads.
38. Spin down briefly to collect the liquid from the side or wall of the tube. Place the tube on the magnetic separation device for ~5 minutes or longer, until the solution is completely clear.
39. Carefully remove and discard the supernatant.
40. Add 200 μ L of 80% ethanol to the tube without disturbing the pellet and wait for 30 seconds.
41. Carefully remove the 80% ethanol wash.
42. Repeat steps 23 and 24 for a total of 3 washes.
43. Spin down briefly to collect any liquid remaining of the tube. Place on the magnetic device for 2 minute or longer.
44. Carefully remove and discard any remaining ethanol wash.
45. Remove from the magnet and air dry for 2-3 minutes.

NOTE: Do not over dry beads as this will decrease elution efficiency.

46. Add 11 μ L of Elution Buffer (10 mM Tris-HCl, pH 8.5) to cover the bead pellet and mix thoroughly to resuspend the beads.
47. Incubate at room temperature for 2 minutes to rehydrate.
48. Spin down briefly to collect the liquid in tube. Place the tube on the magnetic separation device for ~5 minutes or longer, until the solution is completely clear.
49. Transfer the clear supernatant to a new tube.

G-2. Quantify the yield with Qubit

- Range : over 10 pg/uL to 100 ng/uL

3. Regents

Component	Storage	Company	Product#
Qubit dsDNA HS Assay kit	4°C	ThermoFisher Scientific	Q32845

4. Follow the protocol of Qubit dsDNA HS Assay kit.

G-3. qPCR

7. Regents

Component	Storage	Company	Product#
TaqMan Universal Master Mix II, no UNG	4°C	ThermoFisher Scientific	4440040
TaqMan Gene Expression Assays (20X), hGAPDH	-20°C	ThermoFisher Scientific	Hs02758991_g1

8. Prepare the reaction mix for each sample using the components listed below.

qPCR Mix (10 µL/reaction)

TaqMan Universal Master Mix II, no UNG (2X)	5 µL	f. 1X
TaqMan Gene Expression Assay (20X)	0.5 µL	f. 1X
cDNA sample	1 µL	
Nuclease Free Water	3.5 µL	
Total	10 µL	

- Calculate the volume of each component of the PCR reaction mix by multiplying the volume of each component by the number of samples.
- Prepare 110% of the required volume to account for pipetting error.

9. Cap tubes.

10. Vortex the tube briefly to mix the solutions.

11. Centrifuge the tubes to spin down the contents and eliminate any air bubbles from the solutions.

12. Place the tubes in the real-time PCR system and run the following program:

Thermal cycler program for qPCR amplification of cDNA

95°C	10 min
40 cycles:	
95°C	15 s
60°C	1 min
4°C	∞

G-3. Quality check by Bioanalyzer or TapeStation

- Chip: High sensitivity DNA Chip
- Range: 100-5000 pg/μL, dsDNA
- Dilution buffer: Elution buffer

NOTE: Up to 10 mM Tris salt is acceptable.

H. cDNA Library Preparation

H-1. Tagmentation of cDNA

7. Regents

Component	Storage	Company	Product#
Nextera XT DNA Library Preparation Kit	-20°C	illumina	FC-131-1096
Index primers	-20°C	Eurofins	

8. Thaw all reagents required for tagmentation on ice. Gently vortex and spin down briefly, and store them on ice.
9. Prepare tagmentation mixtures in individual 0.2 mL RNase-free PCR tubes on ice as shown in the table below.

Sample components

Tagment DNA Buffer (TD)	10 µL
cDNA + Nuclease Free Water	5 µL (1 ng)
Amplicon Tagmentation Mix (ATM)	5 µL
Total	20 µL

10. Place the tubes in a preheated thermal cycler with a heated lid and run the following program.

Thermal cycler program for tagmentation of cDNA

55°C	5 min
10°C	∞

11. Once the sample reaches 10°C, immediately add 5 µL of Neutralization buffer (NT). Mix the tubes by pipetting gently, and spin down briefly to collect the contents at the bottom.
12. Incubate the tubes at room temperature for 5 min and then place it on ice. The total volume to be 25 µL.

H-2. 3' cDNA library construction with Nextera PCR

5. Thaw Nextera PCR Mix (NPM) and Forward and Reverse Index Primers. Gently vortex each to mix and spin down briefly and store on ice.
6. Prepare enough PCR Master Mix for all reactions, plus one extra reaction volume. Combine the following reagents in the order shown.

Master Mix

Nextera PCR Mix (NPM)	15 μ L	
*PCR primer_i5_(1~8) (10 μ M)	1 μ L	f. 0.2 μ M
*PCR primer_i7_(1~12) (10 μ M)	1 μ L	f. 0.2 μ M
Nuclease Free Water	8 μ L	
Total	25 μ L	

* List of PCR primers is described in appendix S1.

- Add 25 μ L of the Master Mix to the sample (25 μ L) to be 50 μ L of the total volume.
- Place the tubes in a thermal cycler and run the following program.

PCR cycle with 50 μ L of reaction volume		
72°C	3 min	
95°C	30 s	
12 cycles:		
95°C	10 s	]
55°C	30 s	
72°C	30 s	
72°C	5 min	
4°C	∞	

H-3. Purification with AMPure XP & QC with Qubit and Bioanalyser

- Reagents

Component	Storage	Company	Product#
AMPure XP Reagent	4°C	Beckman Coulter	A63881

- Add 0.6x AMPure XP to cDNA amplification mix and purify by eluting with 6.5 μ L of Resuspension Buffer (RSB).
- Quantify the yield with Qubit dsDNA HS Assay Kits.
- In detail, please follow the procedures described in Step G.

I. Short-tag Library Preparation

I-1. Real-time PCR based on SYBR Green I to determine library PCR cycles

1. Regents

Component	Storage	Company	Product#
KAPA HiFi HotStart ReadyMix	-20°C	Roche Diagnostics	07958927001: KK2601
SYBR™ Green I Nucleic Acid Gel Stain	-20°C	TaKaRa	5761A
ROX	-20°C	BioRad	1725858

- Thaw KAPA HiFi HotStart ReadyMix and gently vortex and spin down briefly, and store it on ice.
- Thaw SYBR™ Green I and ROX at room temperature. Gently mix them and spin down.
- Dilute SYBR™ Green I to 1000x and ROX to 2.5x with nuclease-free water.
- Prepare the reaction mix for each sample using the components listed below.

Reaction mix component: 10 µL per reaction

	/10 µL, rxn	final conc.
KAPA HiFi HotStart ReadyMix (2x)	5 µL	1 x
*PCR primer_i5_(1~8) (10 µM)	0.25/0.5/0.9 µL	f. 0.25/0.5/0.9 µM
* TruSeq DNA Methylation primer_i7_(1~8) (10 µM)	0.25/0.5/0.9 µL	f. 0.25/0.5/0.9 µM
SYBR™ Green I (x1/1000)	0.5 µL	f. x1/20000
ROX (x1/2.5)	0.5 µL	f. x1/0
Template (purified tag cDNA)	1 µL	
Nuclease-free water	2.5/2/1.2 µL	
Total	10 µL	

* List of PCR primers is described in appendix S1.

- Place the tubes in StepOne™ Real-Time PCR Systems and run the following program.

PCR cycle with 10 µL of reaction volume

98°C	2 min
2 cycles:	
98°C	20 s
74°C	30 s

28 cycles:

98°C

20 s

72°C

30 s

7. From the above results, read the number of cycles of the SYBR dye fluorescence signal at 1/14 of the Rn value, which indicates a plateau in the SYBR dye fluorescence signal, and then determine the number of cycles of library preparation.

I-2. Short-tag Library construction with PCR

1. To prepare the library, perform the PCR for the number of cycles determined above without SYBR and ROX.
2. Once the sample reaches 10°C, immediately add 5 µL of Neutralization buffer (NT). Mix the tubes by pipetting gently, and spin down briefly to collect the contents at the bottom.
3. Incubate the tubes at room temperature for 5 min and then place it on ice. The total volume to be 25 µL.

I-3. Purification with SPRIselect & QC with Qubit and Bioanalyser

1. Add 1.5x SPRIselect to the cDNA amplification mix, purify twice, and finally elute with 8 µL of Resuspension Buffer (RSB).
2. Quantify the yield with Qubit dsDNA HS Assay Kits.
3. In detail, please follow the procedures described in Step G.

J. Library quantification

1. Regents

Component	Storage	Company	Product#
KAPA Library Quantification Kits	-20°C	Roche Diagnostics	7960204001 : KK4835

2. Follow the protocol of KAPA Library Quantification Kits.