A duplex MIPs-based biological-computational cell lineage discovery platform

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[ABSTRACT]

Somatic mutations can reveal cell lineages during development, health, ageing and disease; however, the rare nature of such mutations makes identifying them time and resource consuming. Short Tandem Repeats (STRs) are highly mutable genomic elements composed of repetitive short motifs, widely distributed within the human's genome and as such, they are the most promising source for genomic variance among the cells of an individual. Earlier we presented a single-cell mutation discovery platform utilizing chip-based multiplex PCR targeting thousands of STRs. Here we present a significant improvement over the platform that combines efficient synthesis of duplex Molecular Inversion Probes (MIPs), high throughput targeting sequencing technologies together with tailored analysis and adaptive error correction all within an integrated bioinformatics Database Management System (DBMS). By applying this platform to various types of human cells, we demonstrated efficient acquisition of tens-of-thousands targets in single-cell whole-genome amplified DNA and discovered lineage relations among these cells.

[INTRODUCTION]

Cell lineaging aims to uncover the developmental history of organism cells back to their cell of origin. Caenorhabditis elegans is the only organism with a known cell lineage tree, derived from visual observation of its developmental process[1, 2]. Many fundamental open questions in human biology and medicine can be answered if the structure and dynamics of the human cell lineage tree could be[3].

Recently, the potential of CRISPR-Cas9 genome-editing systems based cell lineaging of model organisms has been demonstrated on cell lines and zebrafish[4-7]. Human lineages, on the other hand, could only be reconstructed utilizing somatic mutations that occur naturally during cell divisions, such as L1 retro transposition event, Copy Number Variants (CNV), Single Nucleotide Variant (SNV) and STRs (also termed as microsatellites)[8-15]. Among these, STRs are highly mutable and abundant[16, 17]. These features make STRs a promising mutational source for human cell lineaging.

Massive parallel target enrichment method for accurately genotyping STRs is not quite developed yet (Sup Table1). Our lab has developed a generic cell lineage analysis platform based on ~2,000 multiplexed PCRs targeting enriched pipeline enabled by Access Array (AA, Fluidigm) and Illumina Next Generation Sequencing (NGS) technologies. However, the initial setup is very expensive as synthesis of over ~2000 long PCR primers is required. Although PCR primers are a onetime purchase, scaling it up to tens of thousands of targets, the cost of primer synthesis alone becomes too high to consider. It will also require much larger multiplex groups which are prone to failure or more AA chips which cost also accumulates[18].

To overcome these limitations, we turned to Molecular Inversion Probes (MIPs, also termed Padlock Probes), which have the potential of much higher targeting throughput and better specificity. MIPs are single strand DNA molecules composed of two targeting arms and a linker between the two arms. The concept of MIPs was initially published by Ulf Landegren's group[19]. Since then, this technology was developed in two directions. The first was in situ molecular detections, including detecting specific RNAs, pathogenic somatic mutations etc[20-22]. The second is target enrichment of SNPs or bigger targets like exons. Single strand MIPs targeting SNV and exon built from a mixture of unique oligonucleotides synthesized using massively parallel microarray has been developed[23]. Shen *et al*. have developed single strand long MIPs (~325 bp) and succeeded in targeting 500~600bp targets in exons, they further upgraded to the first duplex MIPs pipeline[24, 25], which simplified the process of MIPs creation. The limitation of this procedure is the necessity to build the MIPs one by one for each target, which makes the procedure expensive and time consuming for large panels. Recently, Yoon *et al* described short duplex MIPs generated by microarray targeting SNPs in exons[26]. Single strand MIPs built one by one has been shown successfully targeting 102x tri- and hexa-nucleotide STRs in *A. thaliana*[27].

Thus previous work suggests that short duplex MIPs could be synthesized efficiently on a chip[23] and MIPs could detect STRs in a small scale[27]. Based on these publications, we developed our strategy to design high throughput standard-Illumina-NGS-compatible MIPs targeting STR regions, whose precursors could be synthesized on massively parallel microarray. To simplify our design further, we modified based on the biochemical pipeline developed by Shen *et al*. [25] to make our MIPs working in a duplex style. Following several round calibrations, we developed our duplex MIPs protocol. A duplex-MIPs-dedicated, STR-aware computational pipeline together with a Laboratory Information Management System (LIMS) software for

sample, data management, tracking, and production of lineage trees have been developed and integrated into one bioinformatics DBMS. This system enabled us to capture tens of thousands of STRs together with SNPs and conduct tailored analysis with adaptive error correction with high efficiency at a low cost.
In summary, we developed a duplex MIPs-based biological-computational cell lineage discovery platform, which integrated samples and data tracking, targets selection, MIPs design, NGS sequencing data management, tailored analysis and cell lineage reconstruction.

[RESULTS]

The workflow of duplex MIPs-based biological-computational cell lineage discovery platform

From our cell lineage database, highly mutable STR targets and other demanded targets were selected. The specific MIPs precursors were designed. The 150bp precursor is composed of a pair of universal adaptor, two 3bp unique molecular identifier (UMI), two target specific arms, one illumina sequencing compatible spacer (Figure1A). 4^6=4096 different UMI combinations can be used to detect capture events (Supplemental Figure2).

The MIPs precursors were synthesized on a microarray (Custom Array). Duplex MIPs were created from the synthesized oligoes by PCR amplification and MlyI digestion. As a quality control step of the duplex MIPs preparation, the sizes of precursors and duplex MIPs were both measured by Tape Station (Supplemental Figure1)**.** Purified by MinElute (Qiagen), the duplex MIPs were diluted to a working conc. 80nM or 8nM (Figure1B).

Template DNA/WGA product were mixed together with duplex MIPs in separate wells. Then, a series of molecular biological reactions were applied: hybridization, gap filling, ligation, and digestion. Then, barcoding PCR was applied to each well using unique Illumina barcoding primers, to generate a sequencing library. Libraries were pooled and sequenced by the Illumina NGS platform; the raw reads were analyzed by our bioinformatics pipeline to detect mutations. The processes and sequencing results were documented into the cell lineage database. Somatic mutations were used to infer the cell lineage tree (Figure 1C). The whole workflow took around 5 days from hybridization to data analysis, with roughly 3-hour hands on time (Supplemental Figure3; detailed description of the duplex MIPs protocol in the methods section).

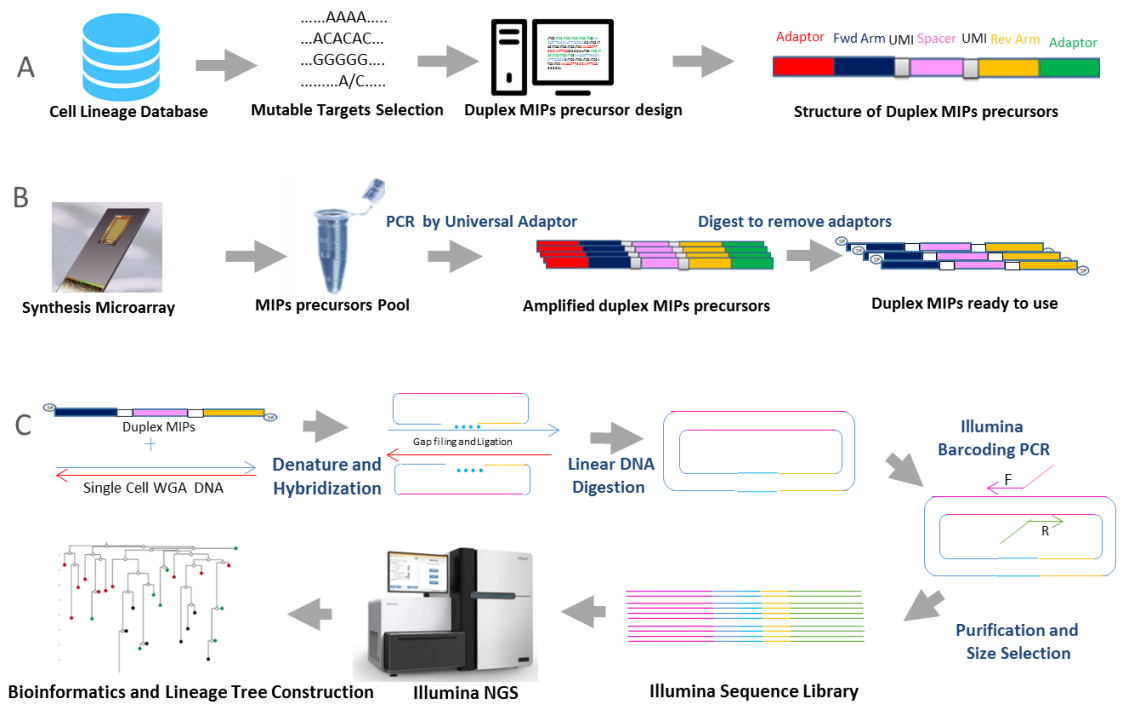

Figure1. Duplex MIPs based cell lineage platform workflow (A) Design of duplex MIPs precursor: Desired targets were selected from our cell lineage database; precursors were designed in the structure shown. (B) Duplex MIPs preparation: duplex MIPs precursors were synthesized on microarray and collected; then amplified by PCR as a pool with universal primers. PCR product was then digested to remove universal adaptors (red and green colors); the digested product was purified and diluted to obtain duplex MIPs that ready for use. (C) Duplex MIPs and template DNA were mixed. The targeting arms (blue and yellow) were annealed to the flanking regions of the targets. The MIPs were circularized by gap filling with DNA polymerase and ligase; linear DNA, including excess MIPs and template DNA, was removed by exonucleases. An Illumina sequencing library was generated by adding adaptors and barcodes using PCR, for each sample separately. Libraries were pooled and sequenced by the Illumina NGS platform and raw reads were analyzed to detect mutations. Mutation information was then used to infer the cell lineage tree.

Calibration of duplex MIPs pipeline

Three major steps, hybridization, gap-filling, digestion in the MIPs capture pipeline were calibrated in 18 different conditions. Hybridization was tested in 2, 4 and 18 hours; gap filling was tested in 1, 2 and 4 hours; while the digestion was tested in 1 and 2 hours. In the calibration experiment, 200 ng HeLa genomic DNA (NEB) and 80nM duplex MIPs were used for all reactions (corresponding to a ratio ~70:1 duplex MIPs to DNA template). The sequencing results of these conditions were used to evaluate the protocol. Among all the conditions, the protocol 18-4-1 showed the best success rate, ~83.5% and most loci captured, over 10,000. Thus, the protocol 18-4-1 was chosen as our standard protocol (Supplemental Table3).

To decide the proper range of library size, several ranges for BluePippin (Sage Science) size selection were chosen for comparison: 240-340bp, 270-310 bp, and 300 bp based on the designed amplicon size distribution. The sequencing result of seven single cell whole genome amplified samples and one bulk DNA sample, both 300 and 270-310 selection ranges had slightly better success rate compared to 240-340 range; But 240-340 size selection range was with much more loci captured. Therefore, 240-340 size selection range was chosen for later experiments (Supplemental Table4).

To further calibrate the impact of ratio between duplex MIPs concentration and template DNA amount, a systematic evaluation of the ratio was carried out. 0 to 2000 ng template Hela DNA, 0 to 80nM MIPs were tested in this calibration (Supplemental Table5 & Supplemental Table6). Samples with more than 8000 reads and over 4000 captured loci were chosen for efficiency comparison. 8 to 80nM duplex MIPs probes with 250 to 500 ng input DNA were the most robust range of capture efficiency for our pipeline (Supplemental Figure 4). Considered the yield of single cell WGA reaction, we decided to use 8nM MIPs and 250 to 500 ng single cell WGA DNA as the template DNA for our standard protocol. The final calibrated protocol described in details is in the methods section.

Integrated bioinformatics Workflow Database Management System (DBMS) that enables efficient data management and reproducibility of analysis

A scalable architecture of Cell Lineage Discovery Workflow DBMS for collaborative cell lineage discovery was designed and implemented. The DBMS support (i) Computer storage and access to all workflow data, including data of each donor (anonymized), sample, cell, reagent type and location, measurement, sequencing run, analysis steps and algorithm runs. (ii) The application of any registered algorithm on any stored data. (iii) Version tracking of all workflow protocols and algorithms, and recording for each measurement, sequencing and algorithm-produced data which workflow version was used in its production. The design, as outlined in Figure 2, dissects both the biological and the computational workflow into atomic objects that are documented and referenced in all of experiments.

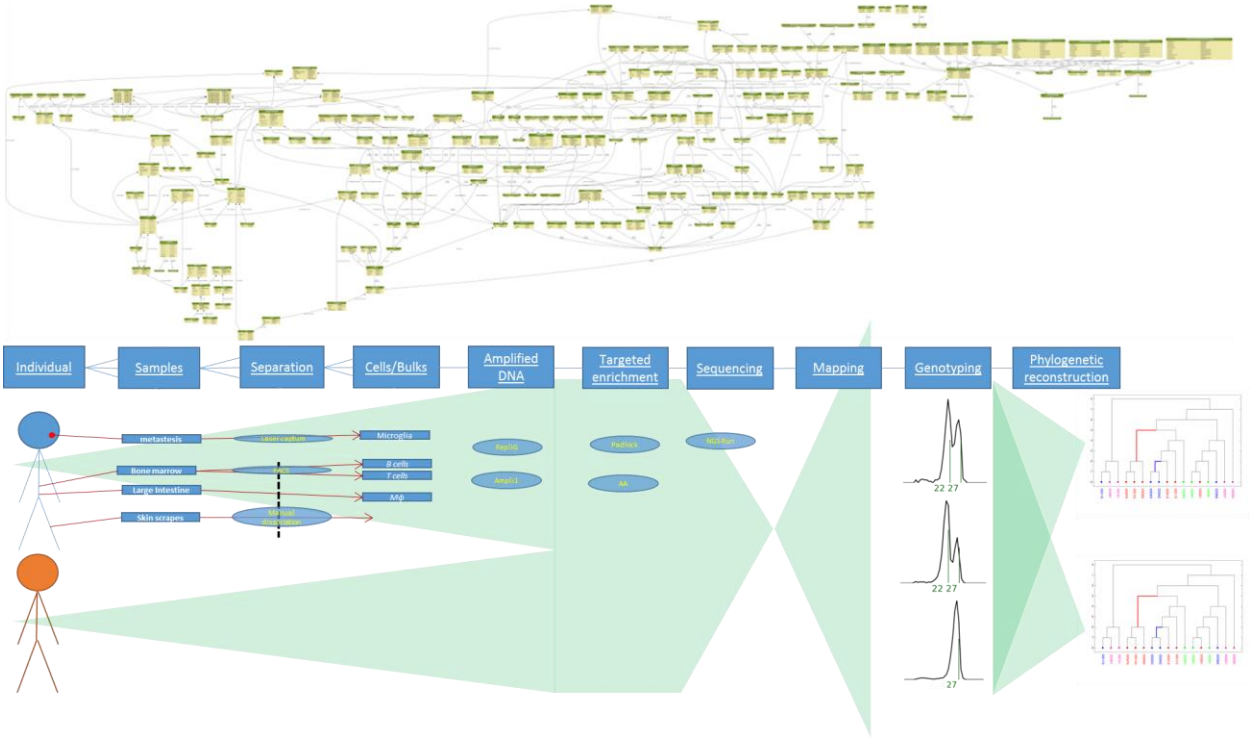

Figure 2. Outline of the Workflow Database and Management System | Top, Entity relation diagram (ERD) of the database structure. Bottom, schematic of the workflow from patient to tree through sampling documentation, library preparation, genotyping and phylogenetic analysis.

Comparison between duplex MIPs pipeline and Access Array pipeline

The cost between duplex MIPs pipeline and AA pipeline mainly differs in two aspects: the synthesis of oligoes or primers, reagents or chip for capture reactions per cell. As shown in Supplemental Figure4, the cost for synthesis was similar at 2000 loci scale, but when scaled up to 100,000 loci, the AA increased to 1,800,000$ roughly, while the duplex MIPs cost around 12,000$. The cost of reagents does not change much with scale up for duplex MIPs pipeline. For capture reaction per cell, more AA chips may be needed when scaled up, which increased the cost significantly. The initial cost of duplex MIPs pipeline was ~10,000$ for 12K loci panel (NGS run cost not included); capture reaction per cell was ~5.33$ (Supplemental Table 8).

The duplex MIPs pipeline uses a single PCR phase comparing to two in the AA pipeline[28]. A 15~20 cycles decrees inferred by the STR genotyping algorithm was shown in our pipeline (Figure 3C). This means less artificial noise was introduced into the STR repeats in the process of target enrichment that in turn translates into saving sequencing costs by allowing confident genotyping using less reads (Figure 3C).

DU145 *ex vivo* tree has been generated in our previous work that demonstrated cell lineage analysis using ~2000 MS loci[18]. Briefly, a single DU145 human male prostate cancer cell was cultured to generate a 9 generations *ex vivo* tree, each generation is of 12-15 cell divisions. Single cells were sampled in multiple stages of the tree and were subjected to WGA. The quality of the resulting genotypes was asserted by targeting the same single cell WGA DNA from *ex vivo* tree through the duplex MIPs pipeline[18]. The genotyping results reported by Biezuner *et al*[18] match those from our protocol in 99.2% of the cases. Our results demonstrate an improvement over the results reported by Biezuner *et al* using the AA chips[18] (Figure 3D). Duplex MIPs genotyping was performed with 5X as the minimal coverage against 30X reported for AA, further establishing the former introduces less noise.

Sequencing coverage distribution across panels was equivalent between Access Array and duplex MIPs. For each locus in the attempted panel (AA, Figure 3A or Duplex MIPs, Figure 3B), we counted the number of cells which present it with a coverage of over 30X. As the cells involved are actually the same, a similar distribution of sequencing depths indicated the loci retrieving performance of Duplex MIPs was not impaired by the ~10X scale up in the number of targets.

Finally, we compared the lineage reconstruction accuracy of AA and Duplex MIPs given the known lineage benchmark DU145 *ex vivo* tree, demonstrating improved lineage reconstruction accuracy (Figure 3E). Both reconstruction attempts were performed under naïve and equal parameters (detailed in methods section), the reconstructed lineage tree is shown in Figure 3F.

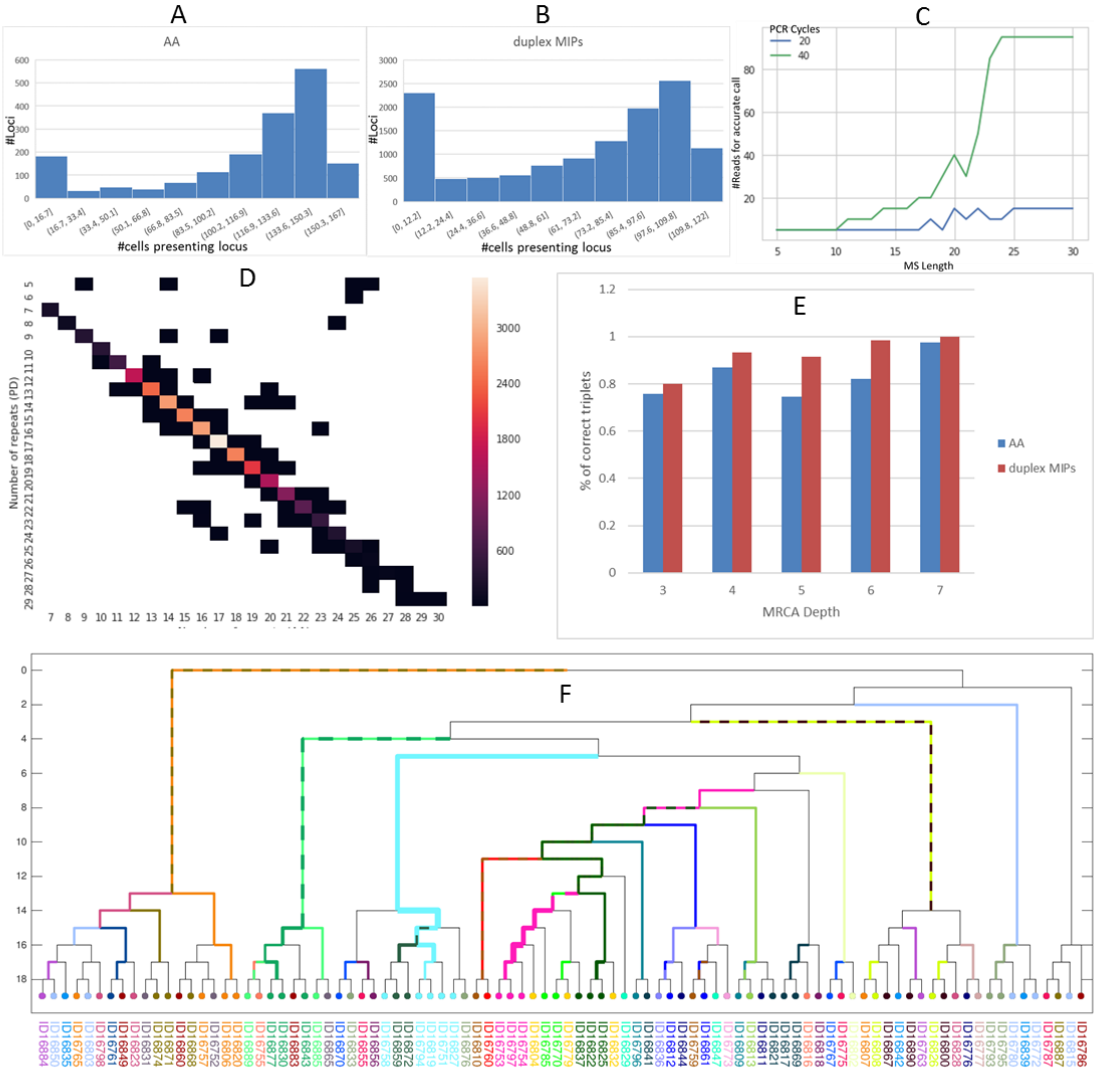

Figure3. *ex vivo* reconstruction and comparison between duplex MIPS pipeline and Access Array pipeline (A, B) STR signal comparison between Access Array and duplex MIPs. For each locus in the attempted panel, we count the number of cells, which present it with a coverage of over 30X. (C) Simulation analysis for the minimal number of reads required for accurate (less than 1 mistake in a 1000 attempts) genotyping of AC microsatellite given two PCR steps (AA protocol, estimated 40 amplification cycles) and a single PCR step (duplex MIPs protocol, estimated 20 amplification cycles). (D) Genotyping correlation, AA vs duplex MIPs. (E) Percentage of correct triplets as a function of the length between the two MRCAs of the triplet (higher is better). (F) Reconstructed Ex-Vivo lineage tree by duplex MIPs.

Cell lineage reconstruction of a melanoma patient (YUCLAT)

Single cell WGA DNA was obtained from both metastases cancer cells and normal peripheral blood lymphocytes (PBL) donated by a single melanoma patient as described in YUCLAT Krauthammer *et al*[29]. Cells were then processed using our platform for their cell lineage tree reconstruction (Figure 4). The reconstructed tree demonstrated an effective *in vivo* separation, validating both the expected grouping suggested by the samples origin as well as the SNP based clustering. Furthermore, a promising symbiosis of SNP and STR data was suggested by the additional clustering revealed in Figure 4C.

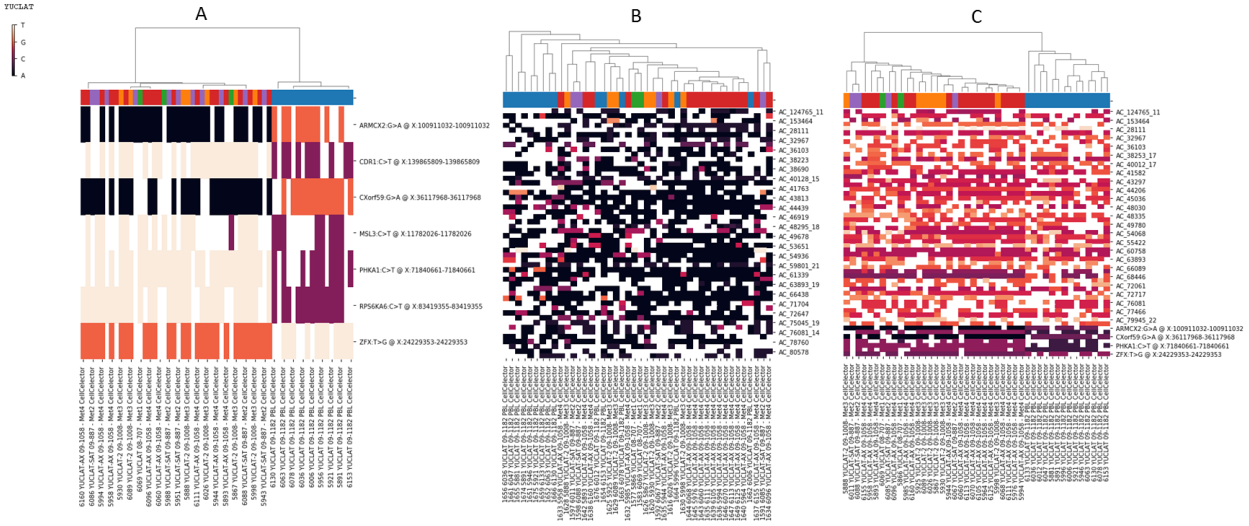

Figure 4. Cell lineage reconstruction of melanoma and normal lymphocytes from the same patient (YUCLAT) | (A,B) Combined heatmap and dendrograms, top: phylogenetic dendrogram that best mediates the clustering constraints imposed by the signal presented in the heatmaps (white indicated missing data, colors represent ACTG bases in A and STR repeat number differences in B, C). Between the heatmap and the dendrogram, a color-coded labels band highlights the original cells grouping labels. SNP based reconstruction shows clustering of healthy PBL from other Melanoma metastases (B) STR based reconstruction agrees with A and adds a cluster of Metastasis4 (C) Combined SNP and STR reconstruction agrees with both A and B and suggests a cluster of Metastasis3.

[DISCUSSION]

Several protocols for STRs genotyping or target sequencing were developed (Supplemental Table1), most of them designed for bulk DNA samples. Although they suit their needs well, a scalable high throughput method for cell lineage reconstruction from whole genome amplified DNA is still lacking. During the past decade, two STRs target enrichment platforms for single cell whole genome amplified DNA have been development in our lab: the first one was targeting 128 STRs based on 4X multiplex PCR, genotyping them by their fragment length detected by capillary electrophoresis[14]. To overcome the limitation from the low throughput of capillary electrophoresis, the second generation, NGS based protocol was developed with up to 50X multiplex PCR on AA chips[18]. This protocol could target over 2000 STRs for 48 samples on one AA chip. This Access Array based protocol was also too expensive to scale to target more than 100,000 STRs due to the accumulating cost of primer synthesis and AA chips[18]. Based on the MIP biochemical pipeline published by Shen *et al.*[25], we have developed a modified protocol: based on a microarray synthesized oligo pool, we generated a duplex MIPs based STRs target sequencing platform, which was demonstrated in 4,000 (data not shown) and 12K targets scale. Since the duplex MIPs could be generated from massive array based synthesis cheaply as an initial one- time purchase which is 10K$ for 100K and 2K$ for 12K, and the capture reaction cost does not change with the increment of targeting panel, the cost and scalability of STRs target enrichment could be significantly improved (Supplemental Figure6). The amplification cycles during sequencing library construction were reduced by 15~20 cycles compared to AA chip based pipeline, this allows us to obtain accurate STR genotyping with less reads as amplification based *in vitro* mutations are reduced. Currently, with a 12K panel, 150~200 cells can be sequenced in one NextSeq run. With more biochemical calibrations and improvement of the bioinformatics MIP arm design and sequencing analysis, future plans are to fit more single cells into a single sequencing run, and to increase the targets scale to 100K targets, thus improve the resolution of the tree[18].

Reconstructing a perfect lineage tree remains challenging. However, several issues we could make our effort to address. The artificial noise during both whole genome amplification can be reduced by Nano liter scale reaction WGA volume on droplet or Fluidigm chip[30, 31]; reduce amplification time and lower cell lysis temperature[32]. The artificial noise introduced by target enrichment process can also be reduced by use less barcoding PCR cycles. We could also use an improved single cell WGA kit to increase the uniformity of amplification. To aid bi-allelic or multi-allelic loci genotyping, duplex MIPs targeting SNV-STRs region within one amplicon can be designed. The STRs selection strategy can also improve by filtering out close bi-allelic loci like (AC)X14, (AC)X15. To do this, a more accurate STR annotated human reference genome may be needed[33].

In summary, we have developed an easily initiated, scalable, cost effective platform for massive parallel STRs mutation discovery integrated in a bioinformatics Database Management System. This platform features efficient synthesis of duplex MIPs based high throughput targeting sequencing technologies, adaptive error correction, tailored sequencing analysis and lineage reconstruction modules. It supports quick development iterations with customizable targets integrations including STRs, SNVs *etc*. on demand. By applying this platform to various types of human cells, we demonstrated capabilities that tens-of-thousands hyper-mutable STR targets in single cell whole genome amplified DNA could be acquired efficiently and discovered lineage relations among these cells.

With the advancement of single cell analysis methodologies, the cost of human cell lineage tracing can be reduced and the accuracy and resolution can be improved. What's more, the cell states, single cell spatial information, genomic functions could be integrated into the cell lineage tree. Together, this will help us better understand the development of human in both health and disease status.

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Online Method

Melanoma patient single cells WGA DNA

YUCLAT metastatic melanoma and Peripheral Blood Lymphocytes (PBL) were collected from a 64-yr-old male patient by the Tissue Resource Core of the Yale SPORE in Skin Cancer with the participant's signed informed consent according to Health Insurance Portability and Accountability Act (HIPAA) regulations with a Human Investigative Committee protocol as described.[1] Single cell WGA DNA was prepared in our previous work[2].

Single cell WGA DNA from DU145 *ex vivo* tree

DU145 *ex vivo* tree and single cell WGA DNA of this *ex vivo* tree were prepared with a modified RepliG Mini protocol in our previous work[2]. The profile of these single cells was retrieved from our cell lineage database.

Duplex MIPs Pipeline Reagents

Reagents	Catalog #
KOD Hot Start DNA Polymerase	Merck 71086
dNTP 25mM each	Bioline BIO-39053
Betaine solution 5M	Sigma B0306 1VL
Phusion High-Fidelity DNA Polymerase	NEB M0530L
Ampligase 10X Reaction Buffer 5ml	EPICENTRE A1905B
Ampligase DNA Ligase W/O Buffer	EPICENTRE A1905B
Exonuclease I (E.coli) - 15,000 units	NEB M0293L
Exonuclease III (E.coli) - 25000 units	NEB M0206L 1
RecJf - 1,000 units	NEB M0264S
Exonuclease T - 1,250 units	NEB M0265L
T7 Exonuclease - 5,000 units	NEB M0263L
Lambda Exonuclease	EPICENTRE LEO32K
NEBNext Ultra II Q5 Master Mix	NEB M0544S
MinElute PCR Purification Kit (250)	QIAGEN 28006
Qubit® dsDNA HS Assay Kit	Life Technologies Q32854
Agencourt Ampure XP Beads	Beckman Coulter A63881
2% Agarose, dye-free, w/ internal standards	Sage Science ES-BDF2003

KOD Hot Start Real Time PCR Mix 5X (KOD 5X MIX)

First, SYBR 1:100 was prepared by mixing 10 µl from stock SYBR green I (Lonza, 10,000X) and 990 µl Dimethyl Sulfoxide (DMSO) (Sigma).

The KOD 5X Mix was prepared according to the table below.

Reagents	Stock Conc.	Final Conc.	1X (µl)
DDW			0.27
KOD Buffer 10X (Merck)	10X	1X	2.5
$MgSO_4$ 25mM (Merck)	25mM	7.5mM	1.5
dNTP 25mM each (Bioline)	25mM	7.5mM	0.2
KOD Enzyme 1 U/µl (Merck)	1 U/µl	0.1 U/µl	0.5
SYBR 100X (Lonza)	100X	1X	0.025
Total Volume			5

Duplex MIPs Generation

(A). PreAmp PCR on oligo pool:

Oligo pool received from provider (Custom Array, Inc.) was diluted to 1ng/µl as PCR template, amplification primers designed to fit the universal adaptors were used for PreAmp PCR in LightCycler 480 (LC480, Roche) according to the setup shown below. SYBR green was added to track the amplification.

PreAmp PCR primers:

OM4_Mly_F: GTCTATGAGTGTGGAGTCGTTGC
OM4_Mly_R: CTAGCTTCCTGATGAGTCCGATG

PreAmp PCR condition:

Reagents	Stock Conc.	Final Conc.	1X (µl)
Template	1 ng/µl	0.2 ng/µl	1.8
OM4_Mly_F	10 pmol/µl	0.3 pmol/µl	1.35
OM4_Mly_R	10 pmol/µl	0.3 pmol/µl	1.35
KOD 5X MIX	5X	1X	9
DDW			31.5
Total Volume			45

PreAmp PCR program:

Temperature	Time	Cycles
95 ºC	2 min	
95 ºC	20 sec	X12 cycle
60 ºC	10 sec	
70 ºC	5 sec	
70 ºC	50 sec	
4 ºC	Hold	

PreAmp PCR product was purified by MinElute PCR purification kit (Qiagen), its concentration was measured by Qubit dsDNA HS Assay Kit (Life Technologies). The purified PreAmp PCR product was diluted to 1ng/µl as the template for next step, the production PCR.

(B). Production PCR (48 reactions)
48 reactions of production PCR in a 96 well plate (Roche) were performed according to the setup below in LC480. The amplification was tracked with SYBER.

Reagents	Stock Conc.	Final Conc.	1X (µl)
Template	1 ng/µl	0.2 ng/µl	1.8
OM4_Mly_F	10 pmol/µl	0.3 pmol/µl	1.35
OM4_Mly_R	10 pmol/µl	0.3 pmol/µl	1.35
KOD 5X MIX	5X	1X	9
DDW			31.5
Total Volume			45

Production PCR program

Temperature	Time	Cycles
95 ºC	2 min	
95 ºC	20 sec	X12 cycle
60 ºC	10 sec	
70 ºC	5 sec	
70 ºC	50 sec	
4 ºC	Hold	

Every four wells of PCR product were merged together and purified by one MinElute column according to the manufacturer's protocol. Elution was in 45µl DDW, and all products were pooled; 1µl of the pool was used to check the concentration by NanoDrop spectrophotometers (Thermo Scientific) according to the manufacturer's protocol. The pool was diluted to ~30ng/µl based on measured concentration. 20µl of sample was kept for quality control; the rest was processed to next step.

(C). The diluted DNA from last step was mixed with reagents according to the table below and incubated at 37 °C overnight, then deactivated at 80 °C for 20min and finally kept at 4 °C.

Reagents	Stock Conc.	Final Conc.	1X (µl)
DNA	30 ng/µl	25.2 ng/µl	84
10X NEB Smarter Buffer	10X	1X	10
MlyI	10 U/µl	0.6 U/µl	6
Total Volume			100

Digested DNA was cleaned by MinElute. If more than one PCR was performed, all elution samples were merged into one tube. Concentration was measured using by Qubit dsDNA HS (High Sensitivity) assay kit according to the manufacturer's protocol.

(D). Tape Station size check
The cleaned digested product was the final duplex MIPs. Its size (~105bp) was measured together with an undigested sample from step (C) (~150bp) by Tape Station (Agilent) (Supplemental Figure1).

(E). Duplex MIPs working solution preparation
Based on length of 105 bp and the measured concentration, the final duplex MIPs was diluted to 5.8ng/µl working solution, equivalent to 80nM (80fmol/µl). Adjusted to 8nM by 1:10 dilution where needed. Working solutions were stored in -20°C freezer.

Duplex MIPs-based targeted enrichment pipeline

(A). Hybridization
Single cell WGA product concentration is generally 100-200 ng/µl. 200~500 ng single cell WGA DNA (~2µl) was used as template for each reaction. Reaction mix was prepared according to the table below:

Reagents	Stock Conc.	Final Conc.	1X (µl)
Single Cell WGA DNA	100ng/µl	20 ng/µl	2
Duplex MIPs	8fmol/µl	0.8fmol/µl	1
Ampligase Buffer	10X	1X	1
Betaine	5M	0.9 M	1.8
DDW			4.2
Total Volume			10

For a big batch experiment, hybridization mix was prepared based on the above table without DNA according to the sample numbers. 8µl hybridization mix was distributed to a 96-well plate, 2µl DNA or DDW was added to each well and mixed by liquid handling system (Evoware, Tecan) or manually.

The reaction plate was put into a PCR machine with 100°C lid temperature, and then heated to 98°C for 3 minutes, followed by a gradual decrease in temperature of 0.01°C per second to 56°C and incubated at 56°C for 17 hours.

Optional: if your PCR machine could not decrease as slow as 0.01°C/second, alternative strategy could be applied: the reaction plates was heated to 98°C and kept for 3 minutes, decreased by 0.1°C every cycle as slow as possible and was kept 15 second at this temperature. Cycling until 56°C and incubated at 56°C for 17 hours.

An example in our PCR machine was shown below.

Step	Temperature	Time	Cycles
1	97.9 ºC	3 min	
2	97.9 ºC	15 sec	X420
	decrease as slow as 0.1ºC/sec		
	decrease by 0.1ºC/sec every cycle		
3	56 ºC	17 hour	
4	56 ºC	Pause for adding gap filing mix	

(B). Gap filling

The gap filling mix was prepared half an hour before hybridization finished as below and kept at 56°C on the heat block.

Reagents	Stock Con.	Final Conc.	1X (µl)
dNTP	2mM	0.3 mM	1.5
NAD	10mM	2 mM	2
betaine	5M	1.1 M	2.2
Ampligase buffer	10X	1X	1
Ampligase	5U/µl	0.5 U/µl	1
Phusion	2U/µl	0.8 U/µl	0.4
DDW			1.9
Total Volume			10

The reaction plate was transferred from the PCR machine to a 56°C heat block when the hybridization step finished. 10µl of gap filling mix was added to each well, carefully mixed by pipette, sealed tightly and quickly and put it back to the PCR machine for 56°C incubation for 4 hour, then 68°C for 20 minutes and 4°C until next step.

Optional: After the gap filling, the reaction plate can be stored at 4°C fridge for up to two days.

(C). Digestion of linear DNA:

The digestion mix was prepared as below 15 min before gap filling step finished.

Reagents	Stock Con. (U/µl)	Final Conc. (U/µl)	1X (µl)
exo I	20	3.5	0.175
exo III	100	18	0.18
exo T7	10	4	0.4
exo T	5	0.4	0.08
RecJf	30	3	0.1
lambda exo	10	0.2	0.02
DDW			1.045
Total Volume			2

The reaction plate was taken off from PCR machine, the cover was carefully removed. 2µl of the digestion mix was added to each well and mixed. The reaction plate was spin down and sealed, then incubated at 37°C for 60 minutes, 80°C for 10 minutes and 95°C for 5 minutes.

Pause Point: the reactions can be stored at -20°C for months after the digestion step.

Sequencing library preparation

(A).Sample specific barcoding PCR

The barcoding PCR was using the same dual-index barcoding primers designed and published by Biezuner *et al.* [2] with a modification of the PCR enzyme to NEBNext Ultra II Q5. Here just showed the structure of the dual-index Illumina barcoding primers used in the experiments, details of their sequences could be found in the supplemental information by Biezuner *et al.* [2].

i5-index-primer: AATGATACGGCGACCACCGAGATCTACAC[i5-8bp-index]ACACTCTTTCCCTACACGACGCTCTTCCG;
i7-index-primer: CAAGCAGAAGACGGCATACGAGAT[i7-8bp-index]GTGACTGGAGTTCAGACGTGTGCTCTTCCG;

2µl product from last step and unique barcoding pair of primers for each sample were used in the barcoding PCR according to the table below.

Reagents	Stock Conc.	Final Conc.	1X (µl)
Template	NA	NA	2
dual-index Illumina primers	5 pmol/µl each	0.5 pmol/µl each	2
NEBNext Ultra II Q5 Master Mix	2X	1X	10
SYBER 10X	10X	0.5X	1*
DDW			5
Total Volume			20

* Final Conc. 0.25X SYBER used in early calibration experiments.

Barcoding PCR program

Temperature	Time	Cycles
98 ºC	30 sec	
98 ºC	10 sec	X5 cycle
56 ºC	30 sec	
65 ºC	45 sec	
98 ºC	10 sec	X15 cycle
65 ºC	75 sec	
65 ºC	5 min	
4 ºC	Hold	

(B). Diagnosis Sequencing Sample pooling and Purification

Barcoding PCR product was cleaned in 96-well plate by 0.8x AMPure XP SPRI magnetic beads (Beckman Coulter) according to manufactory's manual by Tecan liquid handling system, eluted in

40µl DDW. Equal volume of purified samples was pooled by Echo (Echo550, Labcyte). The pool was concentrated by MinElute according to manufactory into 35µl DDW.

(C). Size Selection for Diagnosis Sequencing

3ul of the concentrated pool was kept for later quality control. 30µl of the concentrated pool was used on a 2% V1 cassette BluePippin (Sage Science) with setting range 240-340bp according to manufactory's protocol. The size selected elution was collected and cleaned by MinElute into 15µl DDW. The concentration was measured by Qubit dsDNA HS (High Sensitivity) assay kit. The size distribution of the concentrated pool before and after BluePippin was measured by Tape Station dsDNA chip (Supplemental Figure5). The size selected pool with a single peak around 300bp was used to prepare 12µl of 4nM (4fmol/µl) library for Illumina NGS calculated based on the measured concentration and the average size on Tape Station.

(D). Diagnosis Sequencing

10pM library was sequenced on MiSeq 1M nano or 4M micro flow cell with 151x2 pair-end run parameters according to manufactory manual, the default sequencing primers were used.

(E). Based on the diagnosis sequencing result, the volume for each sample to equalize the reads was calculated to create production sequencing Echo pooling table. According to this table, the purified samples from step (B) were pooled by Echo550 and concentrated by into 35µl DDW. The pool was used to prepare the production-sequencing library similar process in step (C).

(F). Production sequencing

1.8~2.2pM library was sequenced on NextSeq500 flow cell with 151x2 pair-end run parameters according to manufactory manual, the default sequencing primers were used.

(Optional) If the production sequencing did not generate enough reads for some samples, another round of NextSeq could be conducted using the same library to get more reads.

Target Specific duplex MIPs design and preparation

Hyper-mutable STRs selection criteria: Several types of STRs were chosen based on the hg19 reverence human genome annotation. AC-type STRs longer than 10 repeats, AG-type STRs longer than 10 repeats, A-type STRs longer than 6 repeats and G-type STR longer than 6 repeats were selected. SNVs targets were chosen based on cancer related highly mutable regions or known cancer associated regions.

Amplicon criteria: Amplicons contains TTAA sequence were ruled out to fit Ampli1 WGA kit. Amplicon size was designed around ~150bp.

Primer-3 based python script was used to design the targeting primers. Only top-scored primers were chosen as candidates for duplex MIPs precursor design. From these candidates, several more filters were used in the designing: both forward primer and reverse primer shall be unique across the human genome; precursors with MlyI digestion recognition site shall be ruled out; the total

length of the precursors shall not be longer than 150bp, which is the maximum limit of the oligo synthesis provider.

Duplex MIPs structure:

Mly1_F='TATGAGTGTG**GAGTC**GTTGC'

Mly1_R='GCTTCCTGAT**GAGTC**CGATG'

5`[Mly1_F]+[FW_PRIMER_SEQUENCE]+[NNN]+[AGATCGGAAGAGCACACGTCTGAACTCTTTCCCTACACGACGCTCTTCCGATCT]+[NNN]+[Reverse Complement(RV_PRIMER_SEQUENCE)]+[Reverse Complement(Mly1_R)]-3`

Duplex MIPS precursors sequence example:

5`TATGAGTGTGGAGTCGTTGCTACTTGGTGGCTAATTCAGCAGGNNNAGATCGGAAGAGCACACGTCTGAACTCTTTCCCTACACGACGCTCTTCCGATCTNNNTTGCAAGCTCCCTCTGAAAAGTTCATCGGACTCATCAGGAAGC3`

Database Management System (DBMS)

Our computational workflow can be divided into two parts, (1) Sample documentation from Individual to NGS barcoded sample that later annotates the reconstructed cell lineage trees (2) Analysis workflow that starts with raw genomic sequencing data and ends with cell lineage trees, including mutation analysis and lineage tree reconstruction (Supplemental Figure7).
All computational tools mentioned in the text can be found in https://github.com/ofirr/clineage
Although this git folder is, still being improving by adding detailed introductions of the whole pipeline systematically.

Ex-vivo reconstruction parameters for Figure 3.

AA data[2] was genotyped with a minimal coverage of 30X reads, a confidence threshold of 0.05 (correlation above 0.95) between the measured histogram and the reported model. Duplex MIPs data was genotyped with a minimal coverage of 5X and the same confidence threshold as AA.
In both attempts, low coverage samples and loci were filtered out, leaving the top 75% of the cells and loci as input. In both attempts, the reconstruction was performed using the Neighbor Joining algorithm with the absolute distance function.

1. Krauthammer, M. et al. Exome sequencing identifies recurrent mutations in NF1 and RASopathy genes in sun-exposed melanomas. *Nat Genet* **47**, 996-1002 (2015).
2. Biezuner, T. et al. A generic, cost-effective, and scalable cell lineage analysis platform. *Genome Res* **26**, 1588-1599 (2016).