mission bio

tapestri
APPLICATION NOTE

Single-cell DNA analysis with the Tapestri® Platform and nuclei from metastatic melanoma tissue

Takeaways

- Extracted nuclei from fresh frozen tissue generate high quality single-cell genomics data
- Single-cell data is highly correlated with matched bulk next generation sequencing (NGS) data for variant identification
- Single-cell data enables the unique reconstruction of tumor sample clonality and evolution unresolved by bulk sample analysis
- The Tapestri Platform is highly sensitive, detecting low-prevalence clones at a frequency of 0.15%, which were unreported in bulk NGS data

Abstract

Recent advancements in genomic analysis of tumors have revealed that cancer disease evolves by a reiterative process of somatic variation, clonal expansion and selection[1]. Therefore, intra- and inter-tumor genomic heterogeneity has become a major area of investigation.

While bulk NGS methods have significantly contributed to our understanding of cancer biology and genomics, the genetic heterogeneity of a tumor at the individual cell level is overlooked with the average readout provided by a bulk measurement. Very high level of bulk sequencing read depths are required to identify lower prevalence mutations, and confidence in confirming events at the 1% range or less remains a significant challenge. Furthermore, rare events and mutation co-occurrence within and between select cell populations are obscured with average signals. By way of example, a recent study highlighted key issues in false-positive rates in bulk exome studies.[2]

Single-cell analysis overcomes the limitations of bulk sample analysis and can provide unique insights into the cellular-level complexities of tumor heterogeneity and phylogenesis. Here, the use of the Mission Bio Tapestri Platform demonstrates the power of single-cell, targeted DNA sequencing in characterizing solid tumor tissue samples and understanding disease evolution. In a collaboration between Mission Bio and the Charles Swanton Laboratory at the Francis Crick Institute (London, England), single-cell targeted DNA analysis with the Tapestri Platform using sectioned melanoma metastatic tissues and normal liver tissue was performed. An analysis of samples from spatially-separated metastatic sites in a single subject revealed unique genomic signatures in each sample.

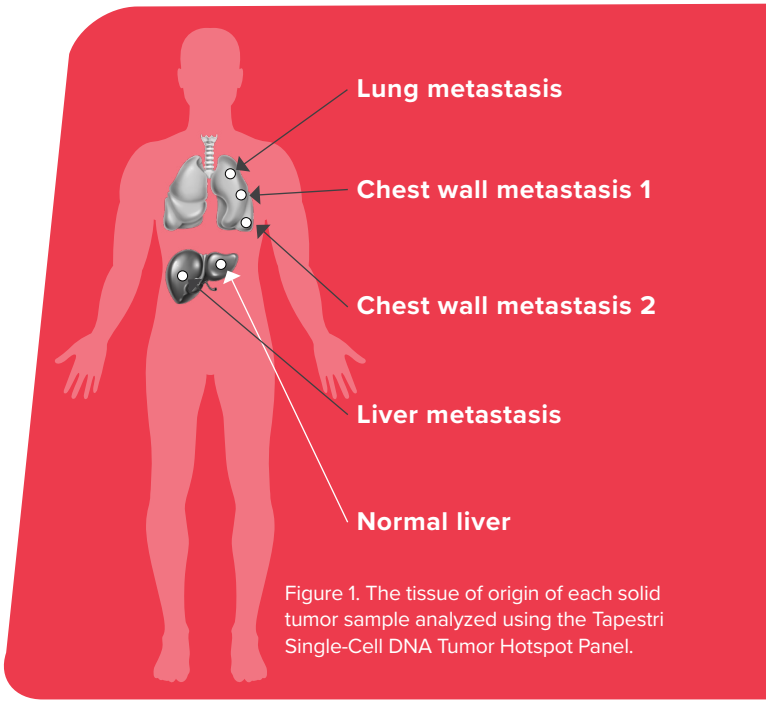

Figure 1. The tissue of origin of each solid tumor sample analyzed using the Tapestri Single-Cell DNA Tumor Hotspot Panel.

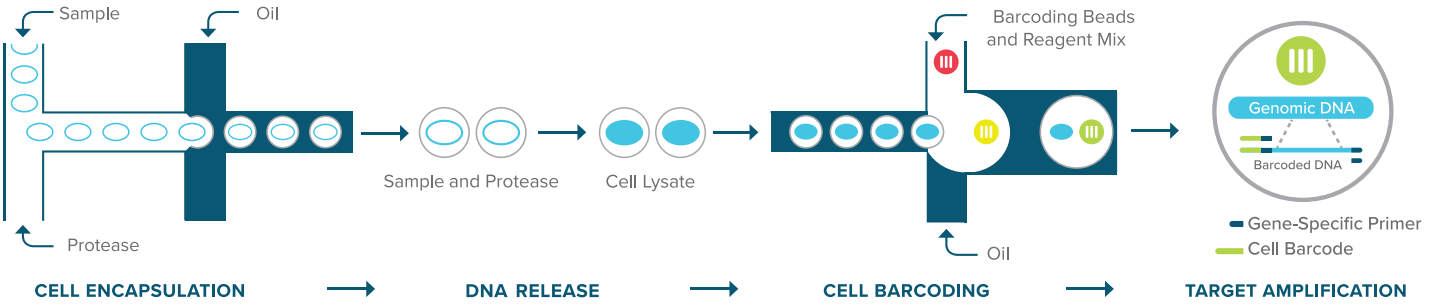

Figure 2. The Tapestri Microfluidic Workflow

Introducing the Tapestri Platform

The Tapestri Platform is a complete solution for high-throughput single-cell targeted DNA analysis, delivering high-quality variant calling of SNVs and indels. Leveraging droplet microfluidics and a novel two-step workflow, high-multiplex PCR, cell barcoding and targeted amplification is performed on thousands of individual cells simultaneously. Single-cell libraries are prepared using optimized Tapestri kits including cartridges and reagents along with the benchtop Tapestri Instrument, and then sequenced on an Illumina® platform. Data is then analyzed and visualized using the intuitive Tapestri Pipeline and Tapestri Insights Software.

Catalog panels are offered in hematology[3] and solid tumor profiling, and panels can also be customized based on specific regions of interest. Altogether, the Tapestri Platform enables the detection of mutation co-occurrence in subclonal populations, preservation of zygosity of mutations, and detection of rare clones down to 0.1%. Additionally, the workflow is amenable to a variety of sample types including cell lines, bone marrow aspirates, PBMCs isolated from whole blood, and nuclei isolated from fresh frozen solid tissue as well as archived solid tumors.

Here, we describe the results of profiling metastatic melanoma tissue samples using the Tapestri Platform with the Tapestri Single-Cell DNA Tumor Hotspot Panel and a universal nuclei isolation protocol.

Experiment and Methods

Tapestri Workflow with the Tumor Hotspot Panel

The Tapestri Platform is an end-to-end solution for single-cell DNA analysis. Using a novel two-step microfluidic workflow, thousands of individual cells - or nuclei - were first encapsulated in droplets by running the Tapestri cartridge on the Tapestri Instrument (Figure 2). Proteolytic action lyses the nuclei, exposing the DNA. Following lysate preparation, the proteases were inactivated via heat denaturation on a thermal cycler. In the second step on the Tapestri Instrument, individual nuclei lysates were returned to the same cartridge and then partitioned with barcoded beads and multiplex-PCR primers and reagents to target specific regions of the genome. Here, the Tapestri Single-Cell DNA Tumor Hotspot Panel was used, targeting 244 amplicons across 59 oncogenes and tumor suppressor genes relevant in a range of different solid tumors (Table 1).

After barcode incorporation and target amplification were complete, library amplification, purification, and quantification were performed prior to sequencing on Illumina platforms. Fastq files were processed through Tapestri Pipeline Software to produce Loom files, where reads are grouped by cell barcodes, thereby reconstructing the mutational profile for each cell. Loom files were then uploaded to desktop Tapestri Insights Software to facilitate data filtering, visualization, and interpretation.

59 GENES - TUMOR HOTSPOT PANEL					
ABL1	CSF1R	FGFR1	IDH2	MLH1	RB1
AKT1	CTNNB1	FGFR2	JAK1	MPL	RET
ALK	DDR2	FGFR3	JAK2	MTOR	SMAD4
APC	EGFR	FLT3	JAK3	NOTCH1	SMARCB1
AR	ERBB2	GNA11	KDR	NRAS	SMO
ATM	ERBB3	GNAQ	KIT	PDGFRA	SRC
BRAF	ERBB4	GNAS	KRAS	PIK3CA	STK11
CDH1	ESR1	HNF1A	MAP2K1	PTEN	TP53
CDK4	EZH2	HRAS	MAP2K2	PTPN11	VHL
CDKN2A	FBXW7	IDH1	MET	RAF1	

Table 1. The Tapestri Single-Cell DNA Tumor Hotspot Panel gene list.

Nuclei extracted from melanoma metastases

To characterize the heterogeneity across metastatic lesions, the Francis Crick Institute provided tissues from 4 metastatic sites and 1 normal liver tissue from a single subject (Figure 1), where tissues were cryogenically frozen after isolation. Frozen tissue samples were sliced using sterile scalpels and every other serial fragment combined and processed following the Nuclei Extraction from Frozen Tissue for Single-Nuclei DNA Sequencing user guide.[4] The simple protocol adds less than 1 hour of hands-on time to the standard Tapestri workflow. On average, 3.4 x 10^6 (range: 1.4 – 7.7 x 10^6) nuclei were isolated from each tissue sample and ~150,000 nuclei were loaded per Tapestri run. Single-cell library QC was performed by running a portion of each library on an Illumina MiSeq® system, prior to full analysis using an Illumina HiSeq® 2500 system.

Results

High quality panel and sequencing data from fresh-frozen solid tumor nuclei

Extracted nuclei generated high quality sequencing data (Table 2). A total of 26,549 nuclei were sequenced from the 5 samples, with an average throughput of ~5,300 nuclei per sample. Additionally, panel uniformity was consistently above 85%, and the average depth of coverage of 78x per amplicon per nucleus was sufficient for high quality variant calling.

Tissue organ	Seq. read pairs	Nuclei	Panel uniformity	Reads per amplicon per nucleus
Chest wall metastasis 1	118M	4,346	86.1%	78x
Chest wall metastasis 2	113M	3,971	87.3%	85x
Lung metastasis	108M	5,772	91.4%	85x
Liver metastasis	131M	5,697	89.7%	65x
Normal liver	146M	6,943	90.2%	78x
Average	**123M**	**5,346**	**88.9%**	**78x**

Table 2. Panel performance and sequencing metrics for each sample. Panel uniformity is the percentage of panel where coverage depth is at least 20% of the mean coverage depth.

Single-cell data is highly correlated with bulk NGS data

Pathogenic variants detected by the Tapestri Platform were identical to those previously identified using bulk NGS sample analysis. Furthermore, compared to a pseudo bulk sample analysis, performed by informatically removing cell barcoding assignments from Tapestri single-cell data, aggregated variant allele frequencies (VAFs) of disease-related mutations correlated well with bulk data. Figure 3 illustrates the correlation for the lung metastasis sample, with a slope of 1.04, coefficient of determination R^2 of 0.97, and y-intercept of 0.01. The data demonstrates that

an ensemble of Tapestri single-cell data recapitulates bulk sample VAFs with high concordance.

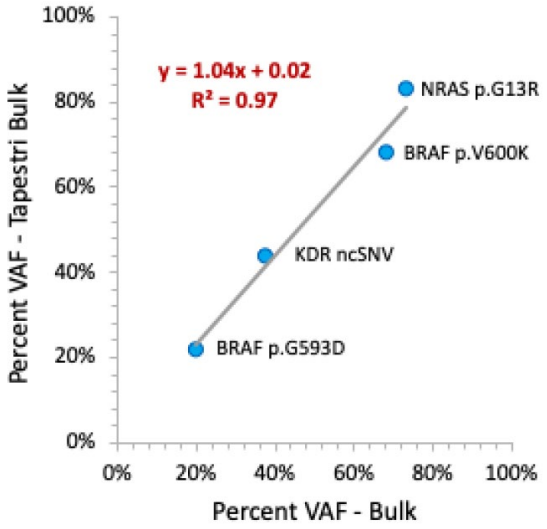

Figure 3. The percent VAF calculated from Tapestri Platform single-cell data converted to bulk correlates strongly with data previously obtained from bulk sample analysis (lung metastasis).

True clonal architecture detected and phylogeny reconstructed across metastatic lesions

Single-cell analysis of the different metastatic tumor samples revealed numerous unique insights into tumor heterogeneity, clonal architecture, and phylogeny of disease. The clonal composition in each sample was determined by the unique set of mutations that co-occured within each clone.

Table 3 outlines the mutation assignment across all cells in all samples. A total of 5 different clones across the 5 different tissues were identified, which were defined by 6 putative disease-related variants. A candidate founder clone was also defined (double-mutant for BRAF p.V600K and KDR ncSNV), based on the mutation pattern present in all clones.

Two clonal evolution trajectories were inferred from the data. The first branch was defined by the acquisition of an oncogenic NRAS p.Q61L mutation of the founder clone, resulting in clone 1. . As cancer progresses, the mutant allele of the oncogenic gene may undergo gene amplification and/or the wild-type allele may be lost, which may explain the formation of clone 2 (BRAF p.V600K/KDR ncSNV/NRAS p.Q61L), with no detectable wild-type NRAS alleles. Clone 2 then acquired a MTOR ncSNV mutation to form quadruple-mutant clone 3.

The second branch was characterized by the acquisition of oncogenic NRAS p.G13R mutation to form clone 4, which evolved into a quadruple-mutant clone 5 with the acquisition of BRAF p.G593D.

The increasing VAF of the BRAF p.V600K mutation along both branches, only attainable through single-

Clones	Wild-type Clone	Founder Clone	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5
Tissues containing clone	All	N/A	Liver Chest wall 1 Chest wall 2	Liver Chest wall 1 Chest wall 2	Chest wall 1	Lung	Lung
MTOR ncSNV	WT	WT	WT	WT	HET	WT	WT
NRAS p.Q61L	WT	WT	HET	HOM	HOM	WT	WT
KDR ncSNV	WT	HET	HET	HET	HET	HET	HET
BRAF p.V600K	WT	HET	HET	HET	HET	HET	HET
NRAS p.G13R	WT	WT	WT	WT	WT	HOM	HOM
BRAF p.G593D	WT	WT	WT	WT	WT	WT	HET

Table 3. All clones identified across all tissue samples using the Tapestri Platform. The inferred putative founder clone is based on the presence of the mutational pattern in all clones. WT is wild-type for that allele, HET is heterozygous for that mutant allele, and HOM is homozygous for that mutant allele.

cell analysis, revealed another layer of genetic heterogeneity, which highlights the possibility of somatic gene amplification representative of disease progression. Figure 4 illustrates the model as a phylogenetic tree.

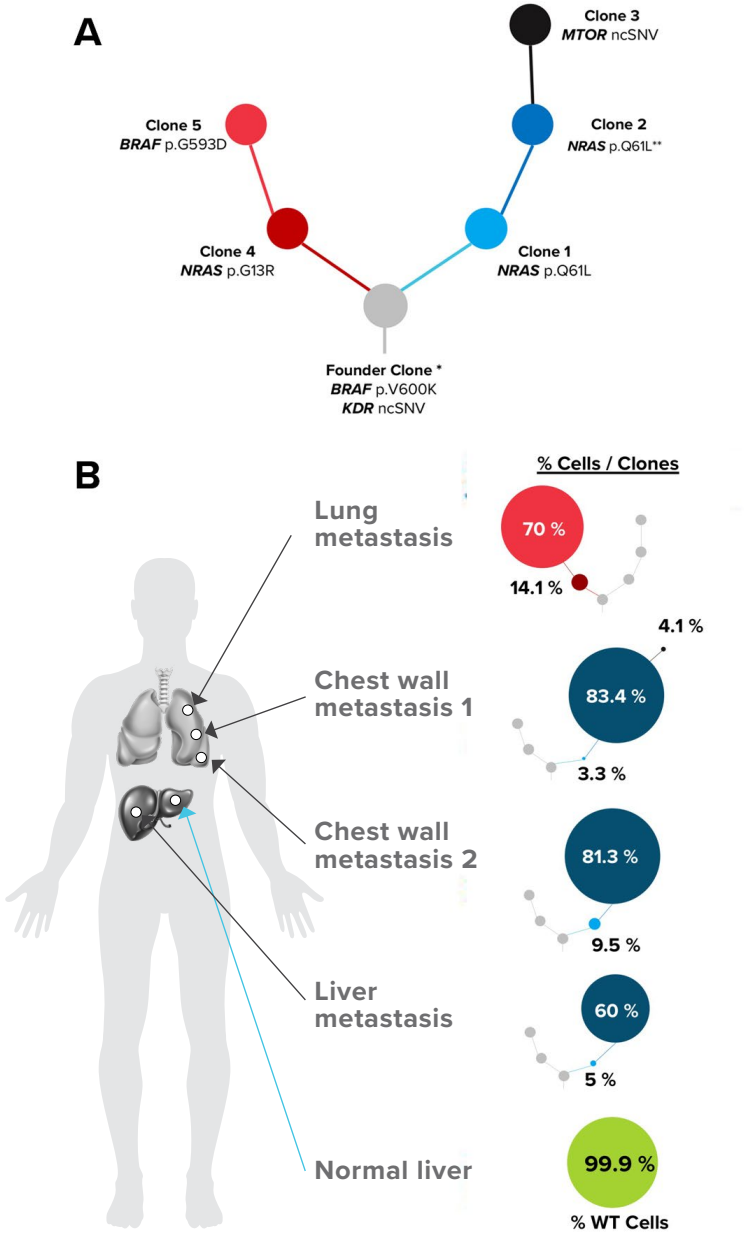

Figure 4. A. A phylogenetic tree illustrates the evolutionary path of each clone. B. Clonal distribution in all tissue samples. *founder clone. **Unlike in clone 1, the wild-type allele was not detected in clone 2.

Detection of rare clones

Significantly, the Tapestri Platform detected low-prevalence clones in supposedly normal tissue, which was not entirely surprising given the degree of metastatic disease in this subject. Triple-mutant clones (clones 1 and 2) were detected in the normal liver sample, indicating the presence of metastatic disease in supposedly normal tissue (Figure 4). These clones were detected at a combined frequency of 0.15% (5 cells) and were not detected by prior bulk sample analysis. Detection of rare clones is extremely important when tracking therapeutic response and monitoring disease remission and relapse.

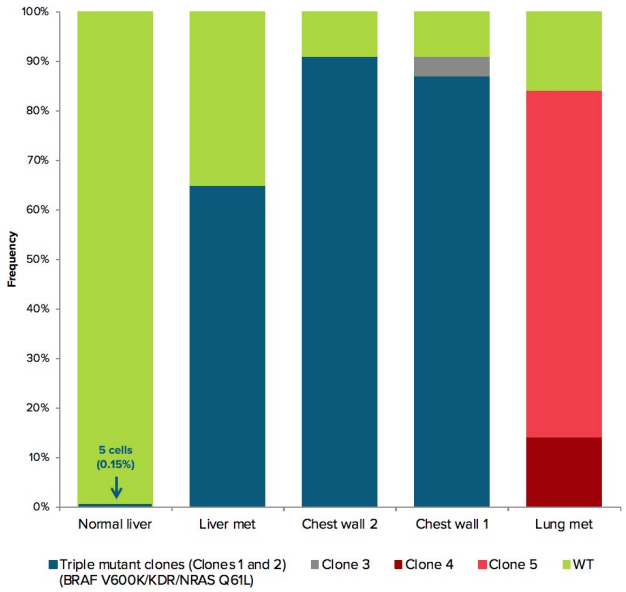

Figure 5. Triple-mutant clone distribution in all tissue samples. The detection of triple-mutant clones at extremely low prevalence (a total of 5 cells) in the normal tissue sample highlights the power of single-cell analysis in detecting rare metastatic cells in a background of wild-type cells.

Accurate measurement of tumor purity

While tumor purity is typically determined by a pathologist using visual or image analysis of tissue sections, here the tumor purity was unambiguously determined by the distribution of mutated and wild-type cells in each sample. Figure 6 summarizes the distribution of wild-type cells in each sample identified by single-cell analysis using the Tapestri Platform, and gives a more accurate measurement of tumor purity compared to histological analysis.

Conclusions

- Nuclei extraction and library construction was demonstrated across a range of solid tumor samples.
- Single-cell analysis correlated strongly with bulk sample analysis, enabling confident comparison with previously-acquired results.
- Single-cell analysis unambiguously identified the clones in each sample, enabling the reconstruction of clonal phylogeny.
- The different clonal lineage observed in the lung tissue highlights complex disease progression.
- Rare clones - present at 0.15% -were detected, which is critical when monitoring disease progression.
- Tumor purity was measured at the single-cell genetic level.

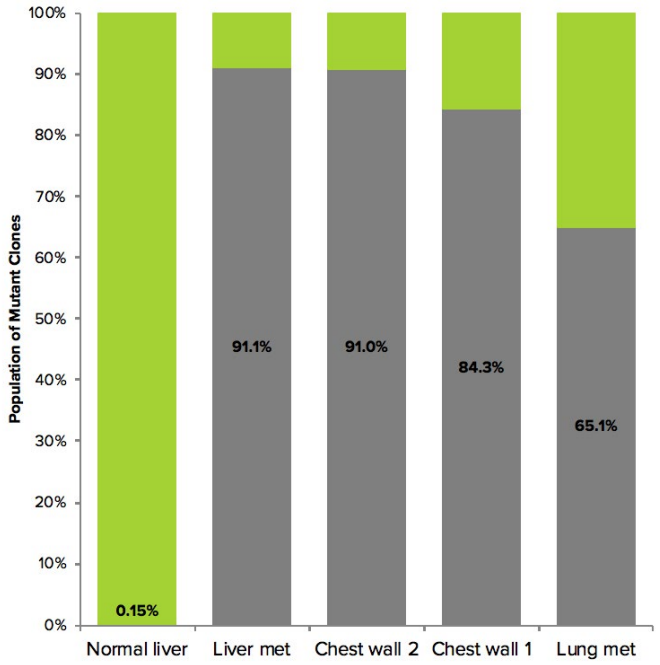

Figure 6. The distribution of mutant cells in all samples (shown in gray) indicate varying levels of tumor purity. Using single-cell genotype data the wild-type cells can be directly detected and removed from downstream analysis.

References

1. Vogelstein *et al.*, Cancer Genome Landscapes. Science, **339**, 1546-1558 (2013).
2. Shi *et al.,* Reliability of Whole-Exome Sequencing for Assessing Intratumor Genetic Heterogeneity. Cell Reports, **25**, 1446–1457 (2018).
3. Pellegrino *et al.*, High-throughput single-cell DNA sequencing of acute myeloid leukemia tumors with droplet microfluidics. Genome Res. **28**, 1345-1352 (2018).
4. *Nuclei Extraction from Frozen Tissue for Single-Nuclei DNA Sequencing,* Mission Bio 2018 user guide.

QUESTIONS?

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