#2441 NanoString 3D Biology™ Technology: Simultaneous digital counting of DNA, RNA, and Protein

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Abstract

Development of improved cancer diagnostics and therapeutics requires detailed understanding of the genomic, transcriptomic, and proteomic profiles in the tumor microenvironment. Current technologies can excel at measuring a single analyte-type, but it remains challenging to simultaneously collect high-throughput DNA, RNA, and protein data from small samples.

We have developed an approach that uses optical barcodes to simultaneously profile DNA, RNA, and protein from as little as 5 ng DNA, 25 ng RNA, and 250 ng protein or just two 5 µm FFPE slides, and simplifies data analysis by generating digital counts for each analyte.

3D Biology™ Technology Overview

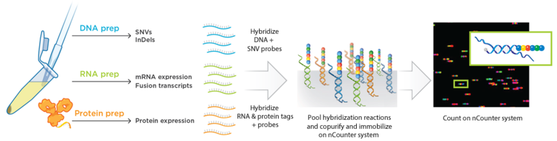


FIGURE 1. Principles of the multiplex 3D Biology assay. Up to 800 targets from any combination of DNA, RNA, and protein can be interrogated from a single sample by labeling with optical barcodes using either direct hybridization of barcode-conjugated oligonucleotides (for DNA and RNA) or via oligo-conjugated primary antibodies (for protein). Analyte types are then pooled into a single reaction and imaged on the nCounter® Analysis system. Quantification of the barcodes generates a representative count of the number of molecules of target that was present in the original sample.

3D Biology™ Technology Highlights

1. **Low sample input**

   5 ng DNA
   25 ng RNA
   250 ng Protein
   } As few as 5,000 cells or two 5 µM FFPE sections

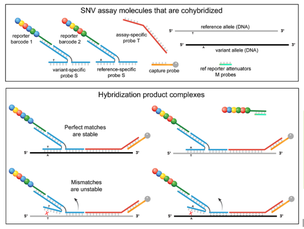
2. **3 analytes** measured from the same biological sample

   104 SNVs from 25 key driver genes
   Up to 770 mRNA targets
   30+ Proteins

3. **One instrument** provides digital readout of SNV, gene expression, and protein profiling from minimal sample, including FFPE tumor tissue

DNA: Single Nucleotide Variant (SNV) Detection

2A



A single nucleotide mismatch is thermodynamically unstable and disrupts probe hybridization.

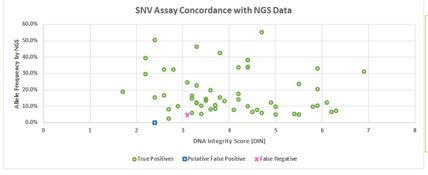
Hybridization complexes are captured on the imaging surface and digitally quantified to reveal relative differences between wild type (WT) and variant alleles.

2B

SNV probes are highly specific for their cognate variant allele targets.

Probe Name	Codon 12			Codon 13			COSM516	COSM520	COSM521	COSM522	COSM527	COSM527	COSM532	COSM517	COSM512	COSM518	COSM529	COSM528
Ref (exon 2)	G	T	G	G	C		81.9%	79.6%	78.0%	67.5%	79.4%	77.5%	66.1%	91.5%	69.7%	79.3%	74.7%	81.4%
COSM516 (G12C)	T						14.5%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%
COSM520 (G12V)		T					0.4%	16.9%	0.7%	0.4%	0.5%	0.5%	0.5%	0.7%	0.7%	0.5%	0.5%	0.5%
COSM521 (G12D)		A					0.4%	0.4%	18.5%	0.3%	0.4%	0.4%	0.3%	0.4%	0.5%	0.4%	0.3%	0.3%
COSM522 (G12A)		C					0.2%	0.3%	0.2%	29.5%	0.2%	0.2%	0.3%	0.2%	0.2%	0.2%	0.2%	0.2%
COSM527 (G13C)				T			0.1%	0.1%	0.1%	0.1%	17.2%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
COSM532 (G13D)					A		0.5%	0.5%	0.4%	0.4%	0.3%	19.4%	0.4%	0.5%	0.4%	0.4%	0.3%	0.4%
COSM517 (G12S)	A						0.6%	0.6%	0.6%	0.4%	0.5%	0.5%	31.6%	0.6%	0.4%	0.5%	0.4%	0.2%
COSM512 (G12F)	T	T					0.2%	0.2%	0.1%	0.1%	0.2%	0.2%	0.1%	4.4%	0.1%	0.1%	0.1%	0.1%
COSM518 (G12R)	C						0.2%	0.3%	0.3%	0.2%	0.2%	0.2%	0.2%	0.1%	27.1%	0.2%	0.2%	0.3%
COSM533 (G13A)					C		0.4%	0.4%	0.3%	0.3%	0.4%	0.4%	0.3%	0.5%	0.3%	17.6%	0.4%	0.4%
COSM529 (G13R)					C		0.2%	0.2%	0.1%	0.1%	0.1%	0.1%	0.1%	0.2%	0.1%	0.1%	22.0%	0.2%
COSM528 (G13S)					A		0.2%	0.2%	0.2%	0.1%	0.2%	0.2%	0.2%	0.1%	0.2%	0.2%	0.2%	15.4%

FIGURE 2. A) Diagram of SNV probe architecture to differentiate WT from mutant allele. **B**) KRAS exon 2, codons 12 and 13, contain several known SNV. gDNA standards containing several of these mutations (COSMIC ID) were pooled and tested using the nCounter® Vantage 3D™ SNV Assay. Specificity is determined by the percentage of counts for a given probe to its intended target compared to counts for all KRAS targets.

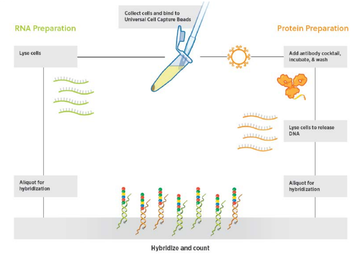


nCounter Vantage 3D SNV calls are concordant with NGS over a wide range of allele frequencies and DNA Integrity Scores.

FIGURE 3. gDNA extracted from 45 different FFPE tumor samples with known SNV content (determined by NGS) was tested by nCounter Vantage 3D SNV Assay using a panel of 104 SNV probes. True positive: variant detected by NGS and nCounter. Putative False Positive: variant detected by nCounter but not NGS. False Negative: variant detected by NGS but not nCounter.

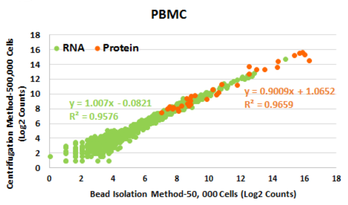
Multiplexed RNA and Protein Analysis

4A



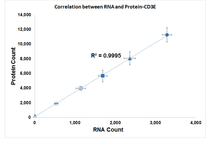
As few as 20,000 cultured cells (50,000 primary cells) can be collected by the Universal Cell Capture Beads, and processed for both RNA and protein quantification.

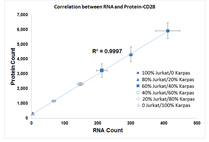
4B



RNA:Protein data from Universal Cell Capture of 50,000 PBMC correlates highly to 500,000 cells pelleted by centrifugation.

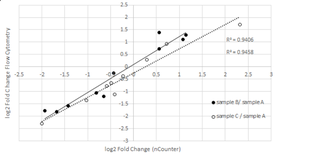
4C





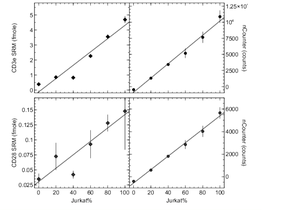
mRNA and protein expression of Jurkat-specific CD3E and CD28 are highly correlated in titration of Jurkat into Karpas cells.

4D



High fold-change correlation between flow cytometry and nCounter protein expression.

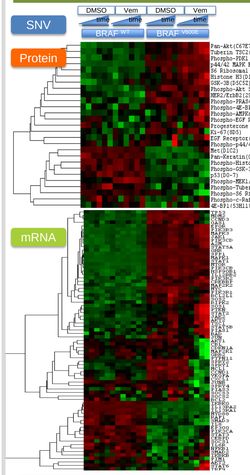
4E



nCounter protein count linearity is higher than SRM mass spectrometry for CD3E and CD28 in Jurkat cell model.

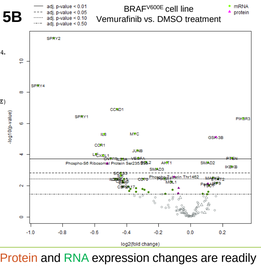
FIGURE 4: **(A)** Workflow of multiplexed RNA and protein analysis.**(B)** Correlation of RNA and protein measurements between the universal cell capture and centrifugation methods. 50,000 PBMCs (universal cell capture) or 500,000 PBMCs (centrifugation method) were used. **(C)** Correlation between CD3E and CD28 RNA and protein measurements in the Jurkat and Karpas cell mixtures at various ratios. Data is shown as the mean of 4 replicates **(D)** Comparison of nCounter and flow cytometry quantification. HEK-293 cell lines transiently transfected with 10 protein epitopes are assayed with oligo-conjugated nCounter antibodies, and in parallel flow cytometry using matched unconjugated primary antibody and fluorescently labeled secondary antibodies. **(E)** Comparison of nCounter and SRM mass spectrometry quantification. CD3 and CD28 are expressed by Jurkat cells, but not Karpas cells. Cell lines were mixed in defined ratios prior to SRM or NanoString profiling. Absolute quantification for SRM was provided by spiking heavy isotope labeled AQUA peptides into the mixes as standards for absolute quantification. Error bars: one standard deviation of the replicate experiments.

3D Biology Application



SNV, protein and RNA gene expression are simultaneously measured to profile melanoma cell lines +/- treatment with BRAF^{V600E} specific inhibitor Vemurafinib. Precise digital readout allows for easy visualization of protein and RNA expression differences for pathway analysis.

5B



Significant Protein and RNA expression changes are readily identified from digital counts using the nSolver analysis program.

FIGURE 5. Application of 3D Biology Technology. **(A)** BRAF^{WT} and BRAF^{V600E} cell lines, treated with DMSO or Vemurafinib, are profiled for 104 SNV, 30 protein, and 192 mRNA targets. Only data for genes with >100 digital counts shown. **(B)** Volcano plot of 8hour Vemurafinib treatment vs. DMSO, showing significance of protein and mRNA fold-changes as a result of treatment.

Methods

SNV Assay: Genomic DNA was purified from single, 5 µM FFPE tissue sections or 25,000 cultured cells using the Qiagen AllPrep kit. Purified DNA was quantified by Qubit fluorometric assay. Exactly 5 ng sample DNA was used as input in the SNV amplification reaction. DNA integrity scores were determined by Agilent TapeStation analysis.

Cell collection and RNA/Protein sample lysis:

PBMC samples (Centrifugation method): 500,000 PBMCs were collected by centrifugation at 400 RCF for 5 minutes. Cell pellets were lysed in 77 µL Qiagen buffer RLT (6500 cells/µL RLT). 1.5 µL, or 10,000 cell equivalents, were input into the nCounter hybridization. Universal Cell Capture method: 50,000 PBMC were collected using the nCounter Vantage 3D Universal Cell Capture Kit. Briefly, PBMC cell suspension is incubated with Universal Cell Capture beads (anti-β2M conjugated magnetic beads) and collected on a 96-well plate magnet, supernatant is removed and Fc Receptor Blocking solution added. An aliquot of 50,000 blocked cells is collected by magnet and a portion are incubated with Antibody mix, collected and lysed in buffer LH with another portion dedicated to direct lysis with buffer LH for RNA analysis. 10,000 cell equivalents of lysate are added to the RNA hybridization, while 800 cell equivalents are added for protein hybridization.

Cell line samples: Jurkat and Karpas cell lines were cultured separately, harvested, and mixed at indicated percentages to create mock fold-change samples. BRAF^{WT}, BRAF^{V600E} heterozygous, and BRAF^{V600E} homozygous mutant cell lines (SKMEL2, SKMEL5, SKMEL28) were cultured from 10min to 24 hours in presence of 0.1% DMSO or 250 nM Vemurafinib (1mM stock in DMSO, Selleckchem Houston, TX). NanoString has validated the RNA:protein assay with as little as 20,000 cultured cells collected by Universal Cell Capture.

Conclusions

NanoString 3D Biology Technology enables highly sensitive and reproducible digital profiling of DNA, RNA, and protein from small amounts of cells and tumor FFPE tissue. We have presented data demonstrating several key attributes of this experimental workflow:

- ✓ High SNV probe specificity that performs comparably to the NGS benchmark
- ✓ Protein detection that correlates highly with both flow cytometry and SRM mass spectrometry
- ✓ Recapitulation of known genotype-dependent gene expression and protein phosphorylation changes in a melanoma cancer model