Spatially-resolved, highly multiplexed (up to 800-plex) digital characterization of protein distribution and abundance in FFPE tissue sections using optical barcoding

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NanoString Technologies
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Definiens’ Symposium for Tissue Phenomics
Cambridge MA
<table>
<thead>
<tr>
<th>Year Range</th>
<th>Company</th>
<th>Role</th>
</tr>
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<tbody>
<tr>
<td>2012 – present</td>
<td>NanoString Technologies</td>
<td>SVP of R&amp;D</td>
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<tr>
<td>2000 – 2012</td>
<td>Life Technologies</td>
<td>CSO → CTO → VP → Head of Advanced Sequencing</td>
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<tr>
<td>1989 – 2000</td>
<td>Vanderbilt University Medical Center</td>
<td>Tenured Faculty</td>
</tr>
<tr>
<td>1981 – 1986</td>
<td>The Johns Hopkins University</td>
<td>PhD</td>
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</table>
Precision Oncology: More Biology, Smaller Samples

Smaller tumors

Smaller samples

More biology

- ER
- PR
- HER2
- Ki-67

Proteins

- ACTR3B
- ANLN
- BAG1
- BCL2
- BIRC5
- BLVRA
- CCNB1
- CCNE1
- CDC20
- CDC6
- CDC7
- CDCA1
- CDH3
- CENPF
- CEP55
- CXXC5
- EGFR
- EXO1
- FGFR4
- FOXA1
- FOXC1
- GPR160
- GRB7
- KIF2C
- KNTC2
- KRT14
- KRT17
- KRT5
- MAPT
- MDM2
- MELK
- MIA
- MLPH
- MMP11
- MYBL2
- MYC
- NAT1
- ORC6L
- PHGDH
- PTTG1
- RRM2
- SFRP1
- SLC39A6
- TMEM45B
- TYMS
- UBE2C
- UBE2T

FFPE tissue or Blood sample

nanoString TECHNOLOGIES
3D-Barcodes “Erase” the Boundaries: Profile DNA, RNA, & Protein Together
NanoString 3D-Barcode Chemistry

Half Site 50 bases

Target-specific Capture Probe

Half Site 50 bases

Barcode

Target-specific Reporter Probe
NanoString 3D-Barcode Chemistry: DNA, RNA, plus Proteins

Half Site 50 bases

Half Site 50 bases

Barcode

RNA or DNA Detection

Endogenous Nucleic Acid

Protein Detection

Unique ssDNA Tag

Photocleavable Linker

Primary Antibody

FOR RESEARCH USE ONLY. Not for diagnostic use.
No technique more digital than counting unamplified single-molecules

- Initial barcode chemistry pioneered in Leroy Hood’s lab at the Institute for Systems Biology (Seattle WA)

- Probes up to 800 different targets simultaneously, *any mixture of:*
  - DNA (CNVs, SNVs, IN-Dels)
  - RNA (mRNA, miRNA, fusions)
  - Proteins (including post-translational modifications)

Digitally count up to ~1 million molecules per sampling area
3D Biology: Definition

The ability to measure any combination of DNA, RNA, & protein simultaneously on a single system
3D Biology Workflow: Simultaneous Detection

- Low input requirements
- Simultaneous, single-lane digital counting of all analyte classes
- **NEW: All chemistries now enabled for spatially resolved detection**
“Boundary-less” view of biological response

Key changes linked to genotype

mRNA “explosion”

Key phosphorylation changes (decrease)

In a single nCounter lane, 6 SNVs, 180 transcripts, and 18 protein species are quantified simultaneously. The heatmaps represent average levels from biological triplicate samples after normalization to controls. Single and combination drug treatment was for 8 hours measured against treatment with vehicle (DMSO) alone.
3D Biology Case Study: unprecedented completely digital view of complex signaling cascades

(2) Measure specific protein phosphorylation state changes

(3) Progressively follow downstream signaling

Transcription-factor “movies”
Multi-year collaboration in Biomarker signature development in Immuno-Oncology with MD Anderson announced 1-April-2015

April 1, 2015

NanoString Technologies and MD Anderson to Collaborate on Development of Multi-Omic Assays Simultaneously Profiling Gene and Protein Expression

Partners Aim to Jointly Discover and Validate Biomarker Signatures for Immuno-Oncology and Targeted Therapeutics

HOUSTON and SEATTLE, April 1, 2015 (GLOBE NEWSWIRE) -- The University of Texas MD Anderson Cancer Center and NanoString Technologies, Inc. (Nasdaq:NSTG), a provider of life science tools for translational research and molecular diagnostic products, today announced a multi-year collaboration to accelerate the development and adoption of a revolutionary new type of assay based on NanoString's nCounter® Analysis System. The collaboration will involve the development of "multi-omic" assays, which simultaneously profile both gene and protein expression, with a primary focus on identifying important biomarkers in the burgeoning field of immuno-oncology as well as extending programs for targeting therapeutics.
Immuno-oncology Gene Expression Profiling: origin of content (left) and application to developing combination-Rx signatures (right)

**Immuneome Compendium:**
Infiltrating Tumor Cell Type-Specific Expression

**Recent example data from GSK (Axel Hoos group)**


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NanoString gene expression profiling identifies potential combination therapy treatment biomarkers from clinical trial samples.

Gene Expression Profile from PBMC Lysates

Gene Expression Analysis

Non-Statistically Significant Changes

Log (Fold-Change, A+B vs. A)

Statistical Significance (Log_{10}(p-value))

Genes 1-7 are top biomarker candidates for combination therapy.
Merck identified four Keytruda NanoString signatures

**Companion Dx**

<table>
<thead>
<tr>
<th>nCounter-based Keytruda Predictive Signatures</th>
<th>IFN-γ (6 genes)</th>
<th>TCR signaling (13 genes)</th>
<th>Expanded immune (18 genes)</th>
<th>De novo (33 genes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best Overall Response</td>
<td>0.005</td>
<td>0.071</td>
<td>0.015</td>
<td>0.018</td>
</tr>
<tr>
<td>PFS</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Source: Seiwert et al. *Inflamed-Phenotype Gene Expression Signatures, and in Particular a γ-Interferon Signature, Predict Benefit From the Anti–PD-1 Antibody Pembrolizumab in PD-L1+ Head and Neck Cancer Patients. Poster 6017. ASCO 2015*

**Note:** Best overall response and PFS in patients with head and neck cancer, assessed by investigator. Nominal 1-sided P value from logistic or Cox regression for overall response and PFS, respectively, using signature scores as a continuous variable.
Diagnostics
NSTG multiplexed gene-expression assays translate all the way to the clinic.

Revlimid, pivotal phase 3 trial (April 2014)

Keytruda, (multiple tumor types) (Feb 2016)

Enzalutamide, pivotal phase 3 trial (Jan 2016)
Expansion of 3D Biology into Spatial-Domains: Design of a High-Plex Molecular Profiling Microscope for FFPE tissues and cells

**What the Data Look Like**

- High Expression
- Low Expression

**How it Works**

1. **Label FFPE Slide with Antibody Mix**
2. **Illuminate Region of Interest**
3. **Analyze Optical Barcodes on nCounter**

Markers:
- CD9
- CD4
- PD-L1
- CD28
- IL2R
High-Plex Digital IHC Microscopy using 3D Biology: *how it works*

1) **Process:** Apply high-plex antibody cocktail

2) **View:** Use visible wavelength low-plex imaging to establish tumor “geography.” Select regions-of-interest for high-plex profiling

3) **Profile:** UV-release high-plex oligo tags at select ROIs

4) **Plating:** Store released tags in microtiter plate, index, and hybridize to barcodes

5) **Digitally count** up to 1 million data points distributed over 800 analytes per ROI
NSTG High-Plex Microscope workflow vs. TSA-based multiplexing

NanoString Multiplexed Digital IHC: up to 800 Targets

- Antigen Retrieval
- 1° Antibody Cocktail
- ROI Selection
- UV Cleavage
- Barcode Hybridization
- nCounter

Standard TSA-Based Multiplexed IHC: 5 Targets

- Target 1
  - Antigen Retrieval
  - 1° Antibody
  - Tyramide Reaction
  - 2° Antibody
- Target 2
  - Antigen Retrieval
  - Tyramide Reaction
  - 1° Antibody
  - 2° Antibody
- Target 3
  - Antigen Retrieval
  - Tyramide Reaction
  - 1° Antibody
  - 2° Antibody
- Target 4
  - Antigen Retrieval
  - Tyramide Reaction
  - 1° Antibody
  - 2° Antibody
- Target 5
  - Antigen Retrieval
  - Tyramide Reaction
  - 1° Antibody
  - 2° Antibody

Image

KEY

Antigen retrieval step (often microwave) heating
Step (2a): Visible wavelength Imaging for Tissue Morphology

Step (2b): Selecting ROI
Step (3) Photo-Cleavage at ROI and Step (4) Aspirate BCs in Microplate

Photo-cleave with UV

Collect & Index Tags

Tissue Section with Oligo-Tagged Antibodies
Step (5) Single-Molecule Counting, Microplate(i) = ROI(i), i = 1, n

Tonsil Tissue

nCounter Counts

Ki-67
CD3

Region 5
Region 8
Region 9
Region 10

Region 5
Region 8
Region 9
Region 10
Is the microscope linear & quantitative? What is the sensitivity limit?
Illumination Area ∝ Single-Molecule Counts: Proves MOA of Microscope

Extremely High Linearity for CD3 in Lymph Node

LOD = 26 µm
R² = 0.9968

Background + 2 x SD

Counts vs. UV Illumination Area (mm²)
Limit-of-detection ~ 25 μm²

Signal Linearity for CD3 in Lymph Node

Counts

UV Illumination Area (mm²)

LOD = 25 μm

Background + 2 x SD
Prototype Detection Limit Nearing Single-Cell

T cells in a Melanoma Sample

25 µm Diameter
# 30-Plex Protein Profiling in FFPE (Counting Key IO Targets in Tonsil)

<table>
<thead>
<tr>
<th>Target</th>
<th>Counts</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7-H3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
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</tr>
<tr>
<td>CD8</td>
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<tr>
<td>CD4</td>
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<tr>
<td>Vista</td>
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</tr>
<tr>
<td>CD45</td>
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<tr>
<td>CD45RO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone H3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomal S6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **CD3-enriched regions**
- **Ki67-enriched regions**
- **Mixed regions**
- **Connective Tissue**
Comparison of High-Plex Microscope vs Clinical Readout of HER2-status in Breast Cancer TMAs

Breast Tumor TMAs with Graded HER2

Fluorescent HER2 Staining in Graded TMAs
Step (2): Visible wavelength imaging of HER2 in TMAs

Example Tissue with ROI

48 ROIs Selected for Digital Multiplexed IHC
Step (5): Digital counting of HER2, Comparison with Her2 (ASCO-CAP) pathology status (Her2 +, ++, +++)

**Her2 nCounter Counts versus Fluorescence Staining Intensities**

- **nCounter Counts** vs. **Sum Pixel Intensities (x10^9)**
  - 0
  - 1+
  - 2+
  - 3+

**R^2 = 0.9232**

**Her2 Counts versus Her2 Status (ASCO-CAP Guidelines)**

- **Equivocal Test Result**
  - **IHC +**
  - **R^2 = 0.511**

FOR RESEARCH USE ONLY. Not for diagnostic use.
Immuno-Therapy Profiling: Spatial Distribution is Important
30-Plex Immuno-profiling in Melanoma biopsy: clear quantitation of high-infiltration vs. low-infiltration regions of tumor
Path Forward for high-plex microscope: incorporating digital-micromirror device (DMD) for Fast, Single-Cell Resolution profiling

- Wide-field illumination
- DMD points UV-photocleavage light to any “geography” of the tumor (even down to single cells) in a completely automated manner (any pattern)
- Hundreds-of-DMD-pixels per 10 µm diameter cell, allow for highly flexible UV-photocleavage and molecular profiling
Path Forward for high-plex microscope: incorporating digital-micromirror device (DMD) for Fast, Single-Cell Resolution profiling

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Hundreds-of-DMD-pixels per 10 µm diameter cell, allow for highly flexible UV-photocleavage and molecular profiling
Automated, Single-Cell UV-Photocleavage & Molecular Profiling

**Single Cell**

**5 Dispersed Cells**

- Cell boundaries
- Photo-cleaved area
Automated, Single-Cell UV-Photocleavage & Molecular Profiling

Late-Breaking: Come to Association of Molecular Pathology Meeting (AMP, Nov 10-12 2016, Charlotte NC) or Soc. Immuno. Therapy Cancer (SITC, Nov 9-13 2016, Natl Harbor MD) to see a full-workup of this data (and more...)
Key Features of NanoString High-Plex Digital IHC Microscope

- **Multiplex up to 800 analytes** on one tissue slice in a **single pass** with 3D-Biology capability

- **Completely non-destructive**: nothing-but-light ever touches the sample. Enables “multi-cycle (n)”, n X 800-plexing

- **1 million digital counts per ROI**, with up to 6 logs (base 10) dynamic range*

- **Many fewer steps and less hands-on time** than TSA-based multiplexing

- **Limit of detection to reach single-cell** soon (with DMD, sans DMD 1-4 cells)

*(only have control samples up to ~ 10^5 completed)

Commercial Time-Line for this technology not yet disclosed. Initial Pilot Projects with outside groups performed in H2 2016
Questions?

Email: jbeechem@nanostring.com
Cancer Immune Response/Therapy Focused Technology Development

- 770 gene expression panel (plus 30 user-defined add-ins)
- Working directly with many leaders in the field, including Cancer Immunologist Dr. Jerome Galon of INSERM Lab of Integrative Cancer Immunity (example papers to the right).

High-Level Content:
- Immune cell markers
- Specific cancer related immune responses
- Adaptive immune response
- Innate immune response
- Adaptive immune repression
- Immune cell activation
- Interferon Type 1 response
- Cancer cell antigens
NanoString immuno-profiling in translational research and clinical studies

Sample 1: (typically) pre-treatment FFPE biopsies

Solid Tumor and Infiltrating Immune Cells

Sample 2-"n": (typically) PBMC fractions

Immune Cells in Blood
### 3D Biology: Digital barcode-counting multiplexed protein assays for key IO-targets

770 RNA and 30+ protein measurements across the Cancer Immunity Cycle

<table>
<thead>
<tr>
<th>Stage of Cycle</th>
<th>Associated Proteins</th>
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<tbody>
<tr>
<td>(2) Antigen presentation</td>
<td>CD4, CD40, CD40L</td>
</tr>
<tr>
<td>(3) Priming and activation</td>
<td>PD-1, PD-L1, PD-L2, IL2R, NCAM, GITR, OX40, CD27, CD28, CD127, CD137</td>
</tr>
<tr>
<td>(4-5) Trafficking and infiltration</td>
<td>CD9</td>
</tr>
<tr>
<td>(6-7) Recognition of and killing cancer cells</td>
<td>PD-1, PD-L1, PD-L2, BTLA, HLA-DRA</td>
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<tr>
<td>Immune modulation</td>
<td>PD1, ICOS, KIR3DL1, NKp46, CTLA-4, CD3E, CD8A, CD14, CD19, CD33, CD68, CD163, CD45RO, NT5E</td>
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