Immune Cell Phenotyping in Solid Tumors using Quantitative Pathology

James R. Mansfield
Director of Quantitative Pathology Applications
What is Quantitative Pathology?

Quantitative Pathology is the accurate measurement of protein distribution in tissue.

PerkinElmer’s quantitative pathology solutions enable per-cell quantitation of one or many biomarkers in a single tissue section.

Multiplexed IHC staining method

Multispectral imaging of multiple labels on the same tissue section

Cellular expression analysis in discrete tissue compartments
Cancer Immunotherapy: a potential cure?

Cancer immunotherapy aims to help the body recognise cancer so that the immune system can attack it and eradicate it like an infection.
Cancer immunotherapy is providing durable benefits.

The immune system is the “agent” that can improve outcome leading to lasting cures in people with metastatic solid cancer.
Cancer Immunotherapy – in the news

Merck spotlights promising data on ‘breakthrough’ cancer immunotherapy MK-3475

November 18, 2013 | By John Carroll

Merck (SMRK) highlighted evidence of a rising response rate to its closely watched PD-1 immunotherapy MK-3475, spotlighting an 81% overall survival rate for advanced melanoma patients after 12 months of therapy with 41% of all patients demonstrating tumor shrinkage—rising to an impressive 51% for one group at the high end of the dose range. About one in 10 patients were ranked as "complete" responders to the therapy, with 88% of all 49 responders showing no signs of disease progression.

The new data offer some additional encouragement for the team handling the Phase Ib trial, which has enrolled more than 1,000 patients--extremely unusual for that stage of development. These checkpoint receptor inhibitors are designed to expose cancer cells to an immune system attack, and initial results indicate that this could offer a major advance in treating cancer.

AstraZeneca to collaborate with Advaxis for cancer immunotherapy

Reuters Jul 22, 2014, 12:09PM IST

Tags: tumours | Test definition | Pfizer | Incurability | immunotherapy | Drugs | AstraZeneca | Advaxis

LONDON: AstraZeneca is casting its net wider in the hot cancer immunotherapy field through a clinical trial collaboration with US biotech firm Advaxis that will test drugs from both companies in combination.

Britain-based AstraZeneca, the target of an unsuccessful $118 billion takeover bid by Pfizer earlier this year, is banking on widespread use of its immunotherapy drugs, which boost the body's immune system, to fight a range of tumours.

The Economist honors cancer immunotherapy pioneer James Allison

2013 Innovation Award for bioscience goes to MD Anderson scientist

MD Anderson News Release 1/07/13.

For basic science research that opened a completely new approach for treating cancer, The Economist has named James Allison, Ph.D., professor and chair of Immunology at The University of Texas MD Anderson Cancer Center, as its 2013 Innovations Award winner in Bioscience.

Allison identified an immune checkpoint molecule that turns off T cells – white blood cells that are the attack dogs of the immune system – before they can mount a successful response to tumors that they are primed to destroy.

An antibody that blocks that immune checkpoint molecule, unleashing a T cell attack, became the first drug to ever extend survival for patients with late-stage melanoma. The U.S. Food and Drug Administration approved ipilimumab (Yervoy®) for treatment of metastatic melanoma in 2011.

Immune Design recruits new team, lands $32.5M for cancer immunotherapies

October 30, 2013 | By John Carroll

Topics: R&D | Venture Capital

Research Use Only
Not for use in diagnostic procedures.
The Hallmarks of Cancer: The tumor microenvironment…

The Cells of the Tumor Microenvironment

Some cells enable tumor growth, others attack the tumor… how do we know which cells are which?

“…the biology of a tumor can only be understood by studying the individual specialized cell types within it”

Hanahan, Weinberg, Cell, 2011
Standard approaches to phenotyping immune cells in solid tissues

Flow cytometry of disaggregated fresh tissue identifies phenotypes but not distribution

Pathology shows distribution of cells, but not phenotypes

Are the cells in the tumor or the stroma? Are they at the margins? Are classes grouped together?

Which are regulatory T cells, which are activated T cells and how do they relate?
Immune Cell Phenotyping in Solid Tissues

Our approach allows different phenotypes to be visualized and quantified simultaneously in the same tissue section, enabling researchers to study the relationships and distribution of these cells within tumors and within the tumor microenvironment.

In standard FFPE biopsies

Research Use Only
Not for use in diagnostic procedures.
Quantitative Pathology Solutions

Labeling Strategies
- Multicolor IHC and IF
  - Opal™ Multiplexing Kits
  - TSA Plus™ reagents

Imaging Strategies
- Quantitative Multispectral Imaging
  - Vectra® Intelligent Imaging System
  - Mantra™ Quantitative Pathology Imaging System

Image Analysis Strategies
- Advanced Image Analysis Software
  - inForm® Tissue Finder™

Automation Strategies
- Whole slide and TMA imaging
  - Vectra® Intelligent Imaging System

For each piece of the puzzle

Research Use Only
Not for use in diagnostic procedures.
Opal™ Multiplexed IHC Protocol

Heat-induced epitope retrieval (HIER) treatment (in this case using a microwave oven) between detection of each target eliminates antibody cross reactivity
- MWT removes $1^\circ$ & $2^\circ$ antibodies
- TSA signal stays because it is covalently bound

4-plex staining protocol <12 hours versus expectation of multiple days

- Multispectral imaging
  - Enables quantitation of overlapping fluorophores
  - Removes limits to multiplexing

- Segmented and scored with InForm

- Scalable approach for higher level multiplexing

$\alpha$ Vasopressin (rabbit)
$\alpha$ Tyrosine Hydroxilase (rabbit)
$\alpha$ CRH (rabbit)

Melanoma TMA core: multiplexed labeling, conventional imaging
Melanoma TMA core: spectrally separated, autofluorescence removed

Unmixed composite:
- DAPI – blue
- CD8 – green
- CD34 – yellow
- FoxP3 – purple
- PD-L1 – red
Melanoma TMA core: automated identification of tissue morphology

Tissue segmentation:
- Tumor region – red
- Stroma region – green
- Other - blue
Melanoma TMA core with multiplexed immunofluorescence staining

Per-cell quantitation
Blue – CD8-/FOXP3-
Green – CD8+/FOXP3-
Red – CD8-/FOXP3+
Yellow – CD8+/FOXP3+
Melanoma TMA core with multiplexed immunofluorescence staining

Compartment-specific cell counts
Melanoma TMA core with multiplexed immunofluorescence staining

Per-cell intensity quantitation

PD-L1 0-3+ Positivity:
- Blue – 0+ (73.13%)
- Yellow – 1+ (24.06%)
- Orange – 2+ (2.39%)
- Red – 3+ (0.42%)

H-Score = 30
**Quantitative Pathology vs. Flow Cytometry vs. High-Content Imaging**

**Flow Cytometry**
- Multiple markers (up to 20) from individual cells measured one at a time
- Requires homogenous samples
- Blood and other fluids
- Disassociated tissue samples

**No context**

**Vectra (tissue sections)**
- Multiple markers (up to 7-8) from individual cells in an intact FFPE tissue section
- Quantitative autofluorescence removal
- Tissue segmentation user-trainable for many different morphologies
- Cell segmentation (nuclear, cytoplasmic and membrane)

**In context**

**High-Content Imaging**
- Multiple markers (4-5) from individual cells in culture
- Per-cell and per-cell-compartment expression
- A range of cell cycle and cell morphology algorithms

**No context**
Case Studies
Application to Breast Cancer ER/PR/Her2/ki67

Collaborator: Dr. Kent Osborne, Director of Baylor Cancer Institute, Houston, TX
Application to ER/PR/Her2/ki67

Multiple labels on a single section
Pathology training

Tumor recognition
Expression in context
Application to ER/PR/Her2/ki67: measuring positivities

green = ER
yellow = PR
purple = ki67
red = Her2
blue = DAPI

ER vs Her2 co-expression

PR vs ki67 co-expression

<table>
<thead>
<tr>
<th>ER vs Her2</th>
<th>PR vs ki67</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single Positive 1</strong></td>
<td><strong>Single Positive 1</strong></td>
</tr>
<tr>
<td>68.94 %</td>
<td>4.38 %</td>
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<tr>
<td><strong>Double Positive</strong></td>
<td><strong>Double Positive</strong></td>
</tr>
<tr>
<td>0.83 %</td>
<td>0.00 %</td>
</tr>
<tr>
<td><strong>Double Negative</strong></td>
<td><strong>Single Positive 2</strong></td>
</tr>
<tr>
<td>29.92 %</td>
<td>93.15 %</td>
</tr>
<tr>
<td><strong>Single Positive 2</strong></td>
<td><strong>Double Negative</strong></td>
</tr>
<tr>
<td>0.32 %</td>
<td>2.47 %</td>
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</table>
Breast Cancer Example
CD4 / CD8 / CD20 / cytokeratin / DAPI

Helper T cell ($T_H$), Cytotoxic T cell ($T_C$) and B Cell Panel
T<sub>H</sub>, T<sub>C</sub> & B cell panel in breast cancer

As seen by microscope

Spectrally unmixed

Simulated IHC view

CD4, CD8, CD20, CK, DAPI
\( \text{TH}, \text{TC} \) and B cell panel in breast cancer

Stromal immune cells

Tumor immune cells

CD4+ T cells: Green
CD8+ T cells: Red

Two views of the same sample

Showing both stromal and tumor immune cell distributions
**Quantitative image analysis - Results**

The relative distances of lymphocytes from the tumor-stroma interface was analyzed.

<table>
<thead>
<tr>
<th>Green</th>
<th>Blue</th>
<th>Red</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER+ cases</td>
<td>Her2+ cases</td>
<td>triple negative cases</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Avg. CD8+ Dist. to Tumor Edge (μ)</th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Within Tumor</td>
<td>Within Stroma</td>
<td>Within Tumor</td>
</tr>
<tr>
<td>BRER-01-6H</td>
<td>57.1</td>
<td>29.1</td>
<td>0</td>
</tr>
<tr>
<td>BRER-02-3G</td>
<td>8.2</td>
<td>32.6</td>
<td>0</td>
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<tr>
<td>BRER-03-2P</td>
<td>20.7</td>
<td>20.5</td>
<td>8</td>
</tr>
<tr>
<td>BRER-04-1N</td>
<td>18.2</td>
<td>34.2</td>
<td>0</td>
</tr>
<tr>
<td>BRHR-03-2C</td>
<td>N/A</td>
<td>18.8</td>
<td>0</td>
</tr>
<tr>
<td>BRHR-04-2J</td>
<td>14.4</td>
<td>40.2</td>
<td>0</td>
</tr>
<tr>
<td>BRHR-09-2B</td>
<td>57.4</td>
<td>52.2</td>
<td>0</td>
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<tr>
<td>BRHR-10-2C</td>
<td>N/A</td>
<td>29.0</td>
<td>0</td>
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<tr>
<td>BRTN-21-2J</td>
<td>19.6</td>
<td>26.3</td>
<td>8</td>
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<td>BRTN-22-2L</td>
<td>17.8</td>
<td>24.3</td>
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<td>19.0</td>
<td>46.2</td>
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<td>BRTN-24-2H</td>
<td>N/A</td>
<td>23.9</td>
<td>8</td>
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</table>

Hoyt, et al, 2014
Quantitation of activated (CD69) and regulatory (FOXP3) T cells in follicular lymphoma

Richard Byers, et al, U Manchester; Chris van der Loos, AMC Amsterdam; James Mansfield, PerkinElmer
Enumerating regulatory and activated T cells in follicular lymphoma

Follicular lymphoma TMA
A single section of a tissue microarray containing 70 follicular lymphoma cores from 42 males, 28 females, 17 alive and 53 dead at the end of follow-up, survival range 2-171 months (median 55 months).

CD3: DyoBlue
CD69: DAB
FOXP3: Fast Red
Counterstain: Methyl green

CD3: all T cells
CD3+/FOXP3+: “Tregs”
CD3+/CD69+: Tacts

Richard Byers, U Manchester
Lilli Nelson, U Manchester
Chris van der Loos, AMC Amsterdam
James Mansfield, PerkinElmer

Looking for CD3+/FOXP3+ and CD3+/CD69+ cells
Two cases: different outcomes

Cases:
- similar number of cells
- similar number of T cells (CD3+)
- different number of Tregs (CD3+/FOXP3+)
- very different survival times

Sample 1
- Extra-follicular cells: 1473
- CD3+/FOXP3-: 13.9%
- CD3+/FOXP3+: 12.3%
- Survival: 171+ months

Sample 2
- Extra-follicular cells: 1917
- CD3+/FOXP3-: 19.66%
- CD3+/FOXP3+: 0.66%
- Survival: 54 months
53 samples from 40 patients were automatically analyzed using this methodology and the number of FOXP3+/CD3+ Treg cells in each determined, in both T-cell (CD3+) rich and poor areas. The number of Tregs cells were used in Kaplan-Meier survival analysis, demonstrating association of higher numbers of Tregs with favourable outcome in both T-cell rich (extra-follicular) and poor (intra-follicular) areas (shown with data split at median for CD3+/FOXP3+ Treg score). This meant patients were divided into groups determined by their Treg numbers. Kaplan-Meier demonstrated that patients with Treg numbers in the top 75%, 50% and 25% all had significant survival advantages over those with lower numbers when divided into two groups based on these proportions.

\[ p = 0.0036 \]

\[ p = 0.0034 \]
Melanoma Example
PD-L1, FOXP3, CD8, CD34, DAPI

Melanoma samples courtesy Bernie Fox, Providence Cancer Center, Oregon
PD-L1 Antibody (Clone E1L3N) courtesy Cell Signaling Technology, Inc.
Multiplexed marker analysis of melanoma TMA core

- PD-L1 / Cy5
- CD34 / coumarin
- CD8 / FITC
- FOXP3 / Cy3
- DAPI

Spectrally separated
H&E view
PDL1 view
CD8 view
FOXP3 view
CD34 view
Multiplexed four marker analysis of melanoma TMA core

Green = tumor compartment
Blue = stroma and inflammation
Purple = vessels

Automated tissue segmentation
PDL1 heat map; H score 229
CD8 density 441 cell/mm²
Our latest 6-plex assay

Breast Cancer

CD4  CD8  CD20  PD-L1  FOXP3  AE1/AE3  DAPI

Research Use Only
Not for use in diagnostic procedures.
# Example cancer immunology panels on Vectra

<table>
<thead>
<tr>
<th>Panel Description</th>
<th>Panel Details</th>
<th>Contributors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast / Ovary TILs:</td>
<td>CD4 / CD8 / CD20 / cytokeratin / DAPI</td>
<td>Dr. Michael Feldman, University of Pennsylvania</td>
</tr>
<tr>
<td>Melanoma Tregs:</td>
<td>CD3 / CD8 / FOXP3 / S100 / hematoxylin</td>
<td>Dr. David Rimm, Yale University</td>
</tr>
<tr>
<td>Tumor-draining lymph node and breast cancer:</td>
<td>CD3 / CD8 / PD-1 / PD-L1 / cytokeratin / hematoxylin</td>
<td>Dr. Peter Lee, City of Hope Research Center</td>
</tr>
<tr>
<td>Follicular lymphoma:</td>
<td>CD4 / CD25 / FOXP3 / hematoxylin</td>
<td>Dr. Richard Byers, University of Manchester, UK</td>
</tr>
<tr>
<td>Melanoma:</td>
<td>PD-L1 / FOXP3 / CD8 / CD34 / DAPI</td>
<td>Dr. Bernie Fox, Providence Cancer Institute</td>
</tr>
<tr>
<td>Melanoma:</td>
<td>CD3 / CD4 / CD8 / dendritic cell markers</td>
<td>Dr. Carl Figdor, University of Nijmegen</td>
</tr>
<tr>
<td>Melanoma:</td>
<td>CD3 / CD8 / PD-L1</td>
<td>Drs. Pardoll, Topalian, Taube, Johns Hopkins</td>
</tr>
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Quantitative Pathology Solutions

Contract discovery services

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Automation Strategies
- Whole slide and TMA imaging
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For each piece of the puzzle
Thank you for your attention!

**PerkinElmer offers:**

- A selective and specific staining protocol for multiplexed IHC
- Multispectral imaging systems for measuring individual labels
- Software to analyze cellular expression in discrete tissue compartments
- Technical expertise in reagents, imaging and analysis
- Contract discovery services for assay development and slide analysis

Quantitative pathology enables per-cell quantitation of many biomarkers in a single FFPE tissue section for cancer immunology.
Thank you for your attention!

Any questions?