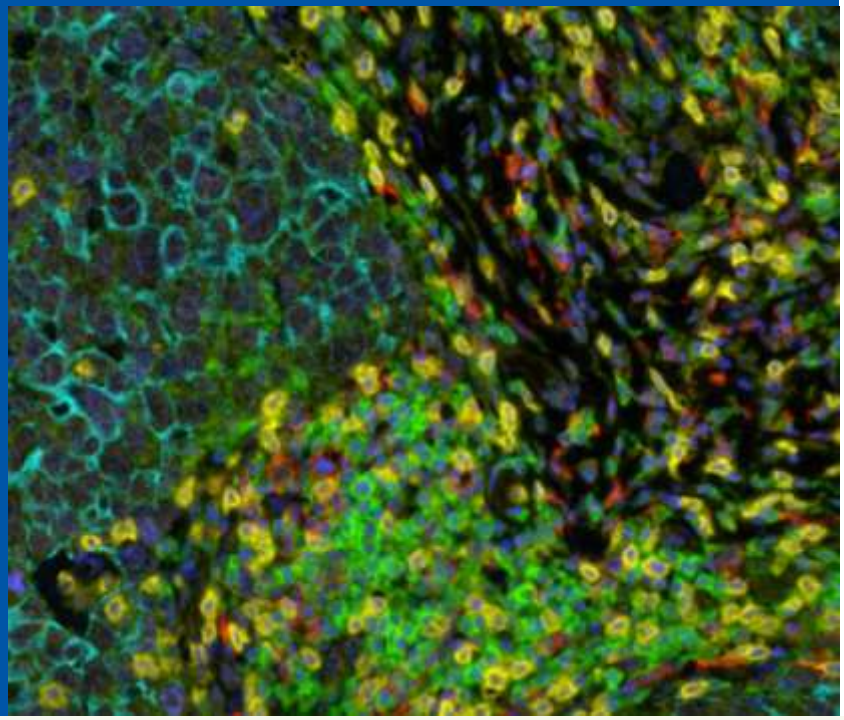


Immune Cell Phenotyping in Solid Tumors using Quantitative Pathology

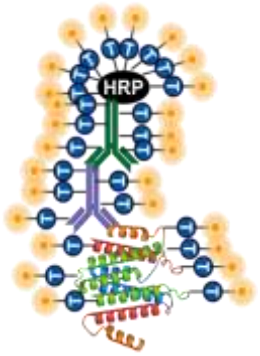
James R. Mansfield

Director of Quantitative Pathology Applications

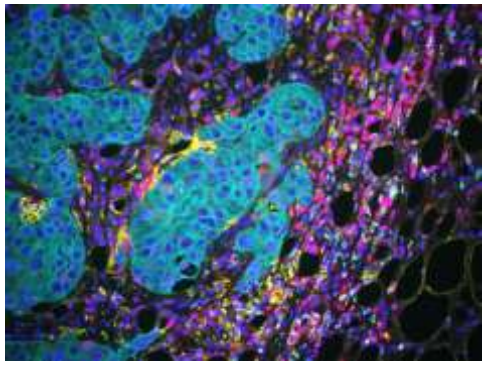
What is Quantitative Pathology?

Quantitative Pathology is the accurate measurement of protein distribution in tissue

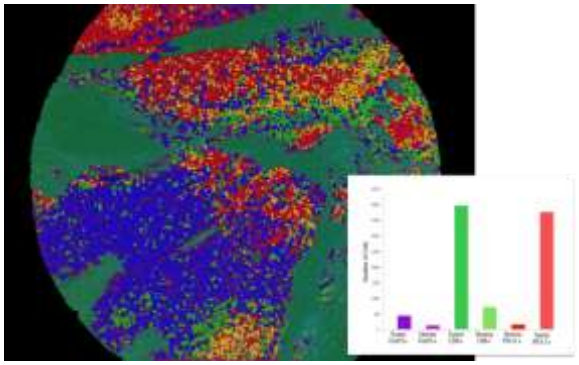
PerkinElmer's quantitative pathology solutions enable per-cell quantitation of one or many biomarkers in a single tissue section



Multiplexed IHC staining method



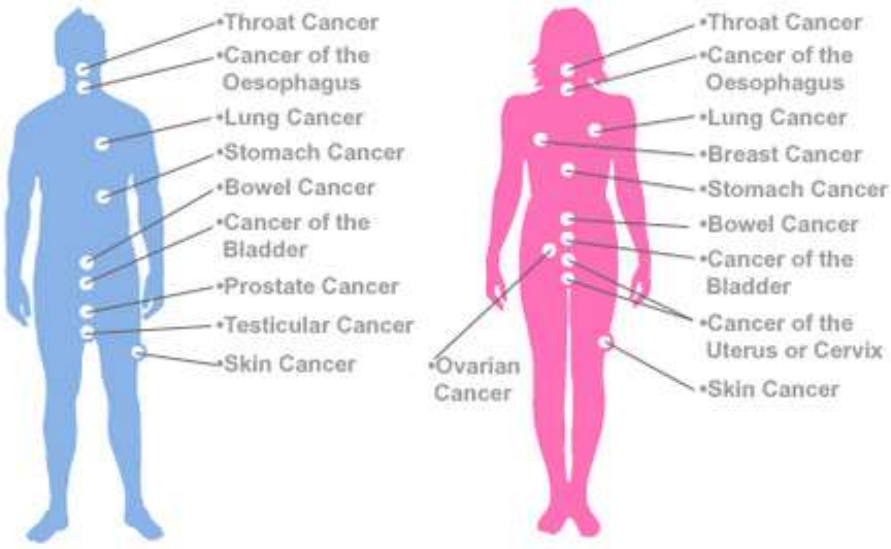
Multispectral imaging of multiple labels on the same tissue section



Cellular expression analysis in discrete tissue compartments

Research Use Only
Not for use in diagnostic procedures.

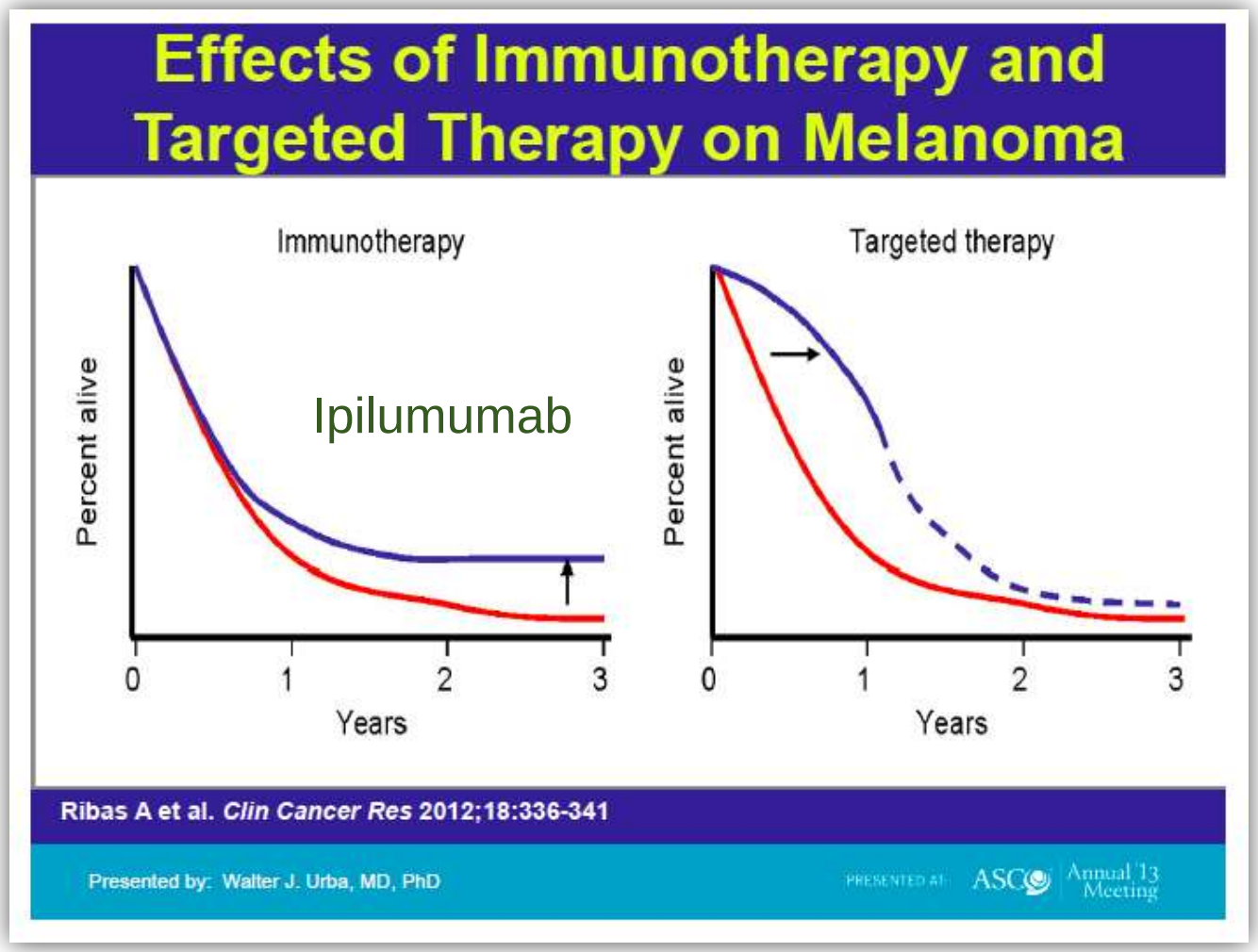
Cancer Immunotherapy: a potential cure?



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

Research Use Only
Not for use in diagnostic procedures.

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

Research Use Only
Not for use in diagnostic procedures.

Cancer Immunotherapy – in the news

Merck spotlights promising data on 'breakthrough' cancer immunotherapy MK-3475

November 18, 2013 | By John Carroll

Merck ($MRK) 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 | Insurability | 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

Like 40 Tweet 18

MD Anderson News Release 11/07/13

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.


James Allison, Ph.D.

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 ipilumumab (Yervoy®) for treatment of metastatic melanoma in 2011.

Topics: R&D | Venture Capital

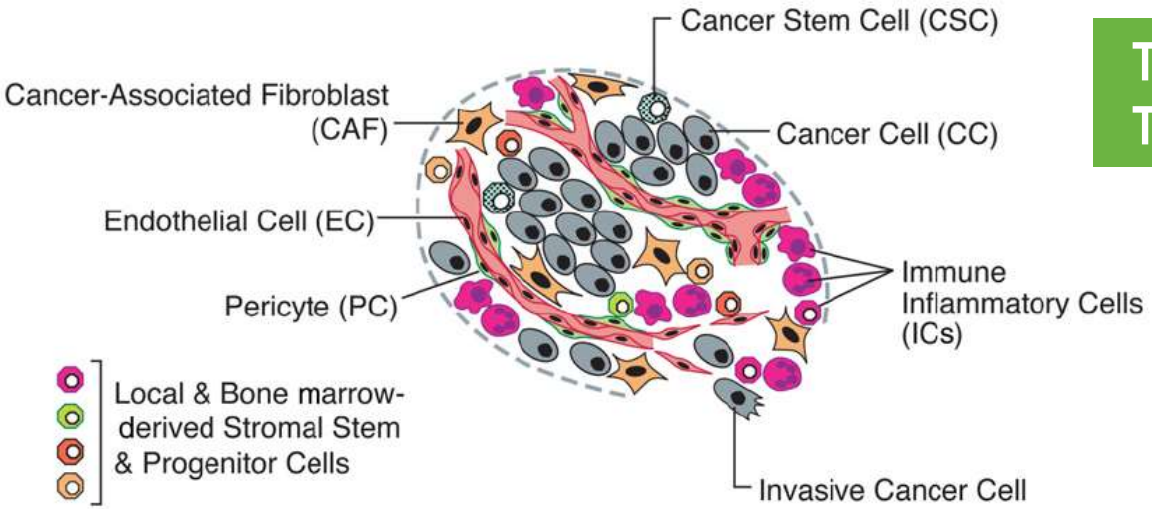
Immune Design recruits new team, lands $32.5M for cancer immunotherapies

October 30, 2013 | By John Carroll

Topical area, global interest

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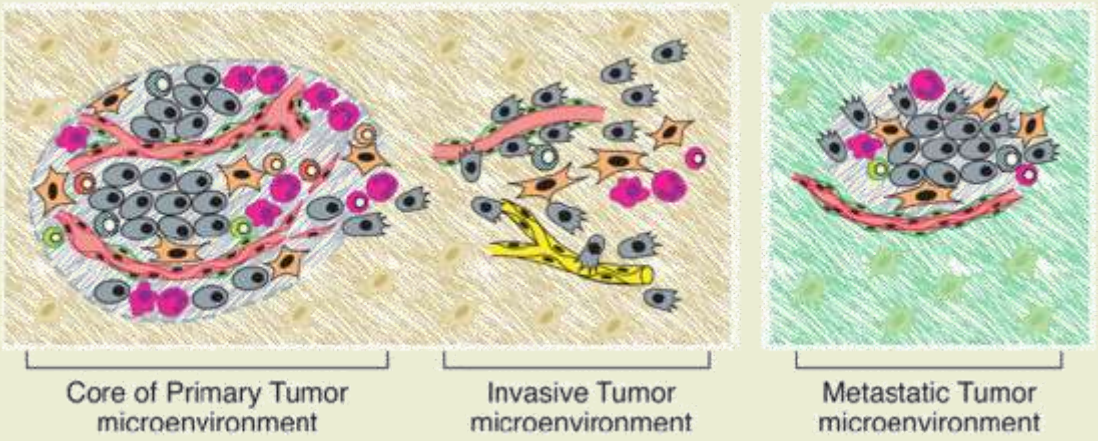
The Hallmarks of Cancer: The tumor microenvironment …



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**"



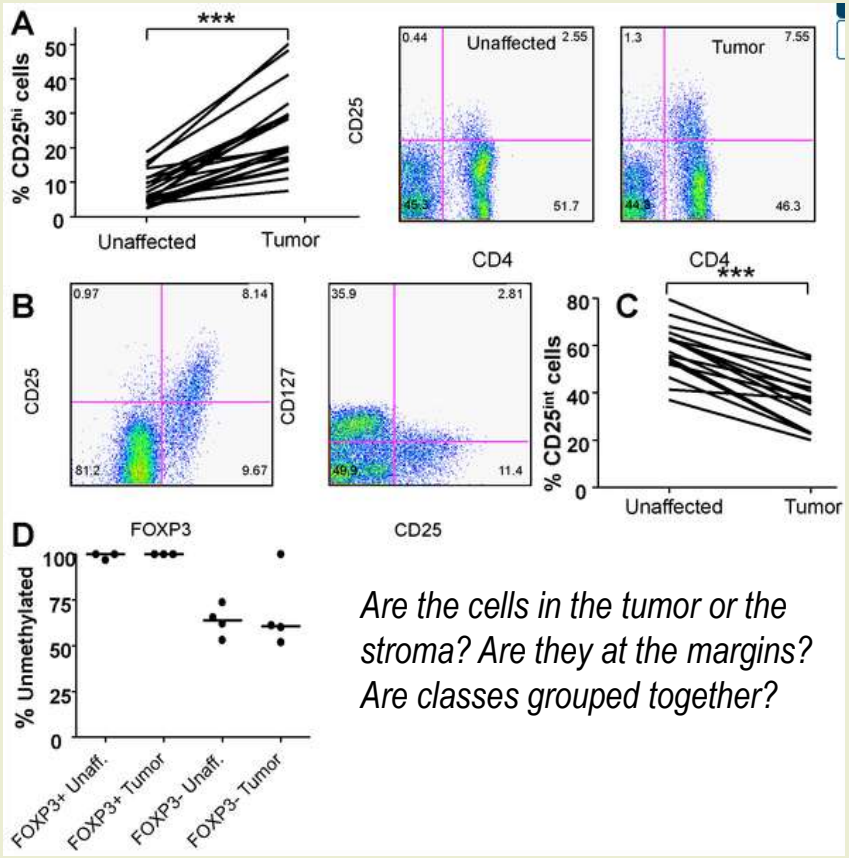
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Hanahan, Weinberg, Cell, 2011

… is an important aspect of cancer

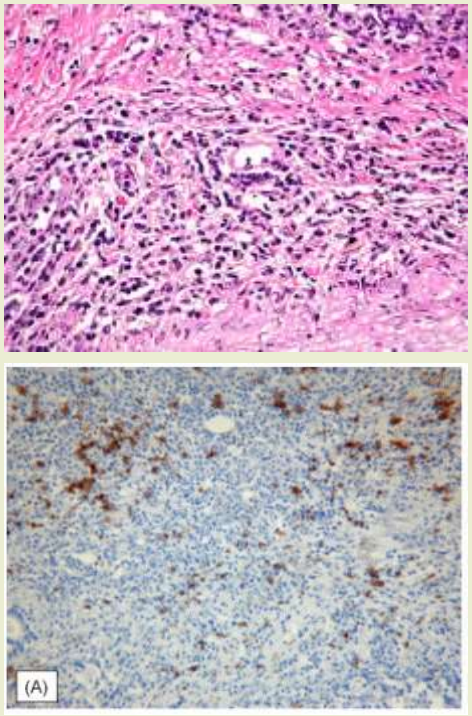
Standard approaches to phenotyping immune cells in solid tissues

Flow cytometry of disaggregated fresh tissue Identifies phenotypes but not distribution



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

Pathology shows distribution of cells, but not phenotypes

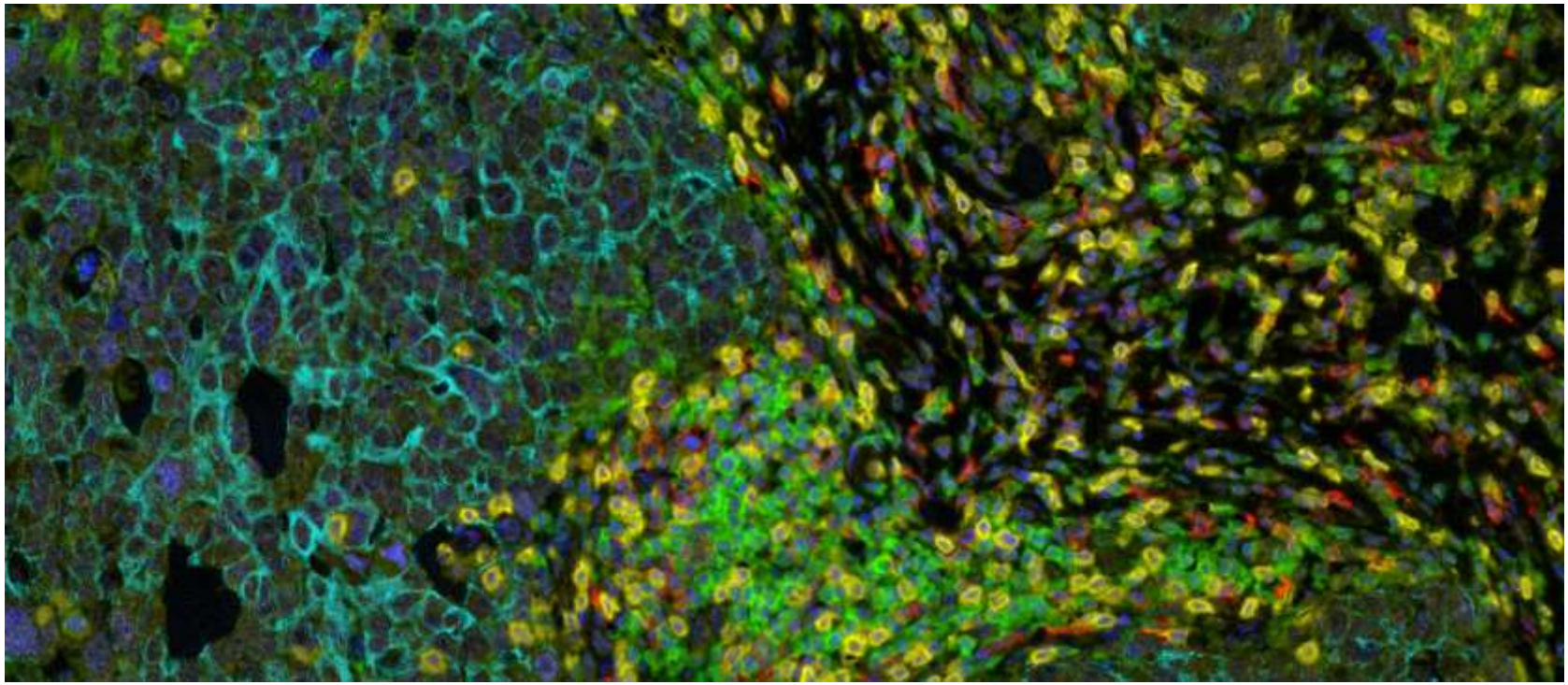


Which are regulatory T cells, which are activated T cells and how do they relate?

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Morphology with no phenotype, or phenotype with no morphology

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

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In standard FFPE biopsies

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

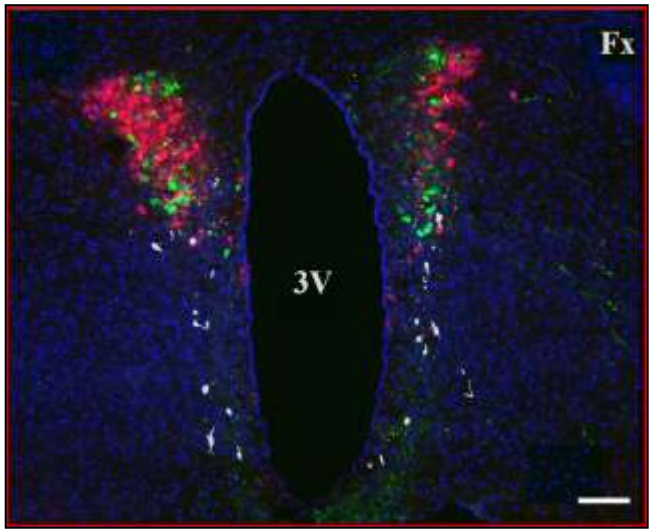
Whole slide and TMA imaging

Vectra® Intelligent Imaging System

Research Use Only
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For each piece of the puzzle

Opal™ Multiplexed IHC Protocol



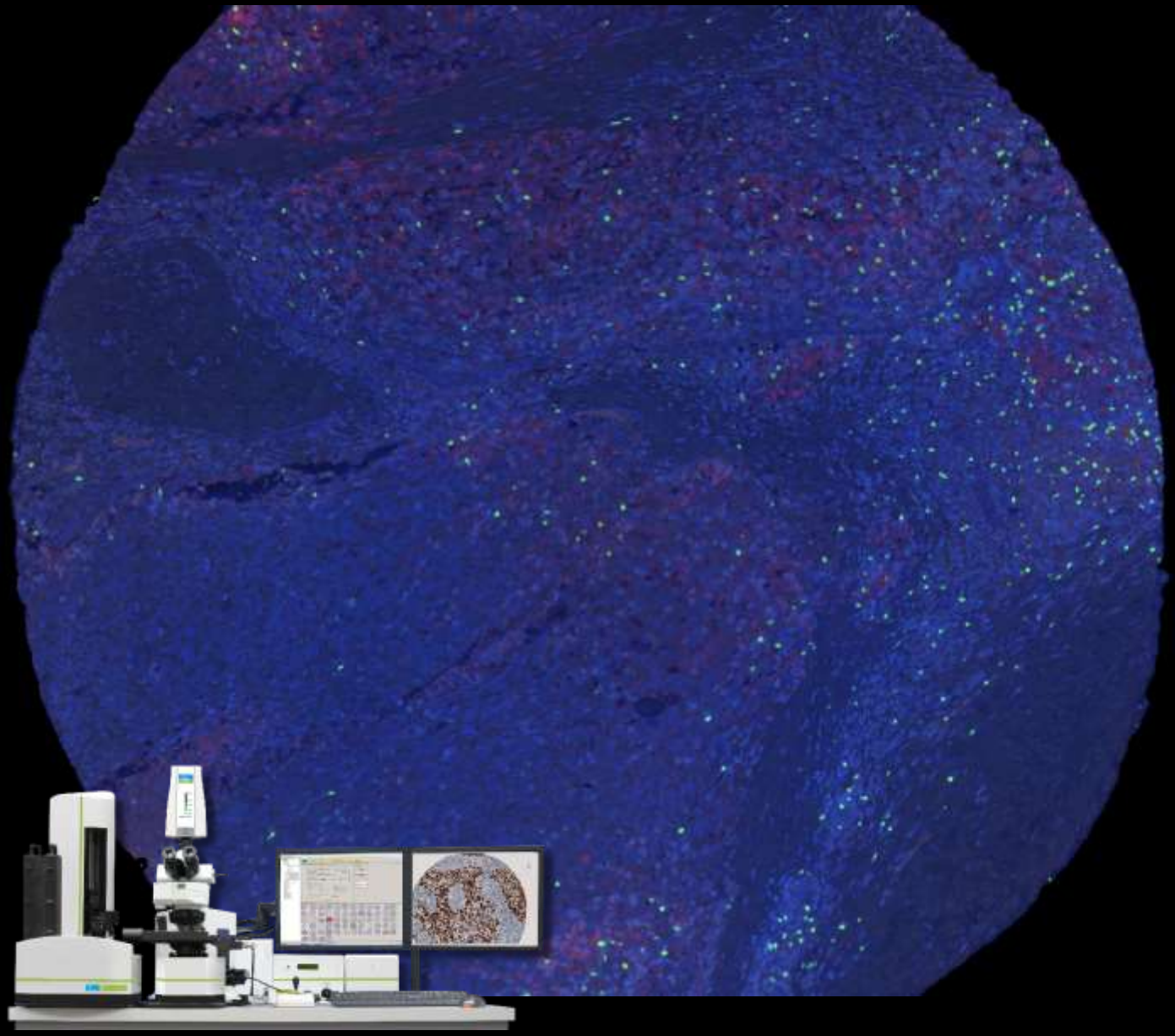
αVasopressin (rabbit)
αTyrosine Hydroxilase (rabbit)
αCRH (rabbit)

* Zsuzsanna E. Tóth & Éva Mezey (2007). **Simultaneous Visualization of Multiple Antigens with Tyramide Signal Amplification using Antibodies from the same Species**. *Journal of Histochemistry and Cytochemistry*; 55 (6): 545-554

- **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° & 2° 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**

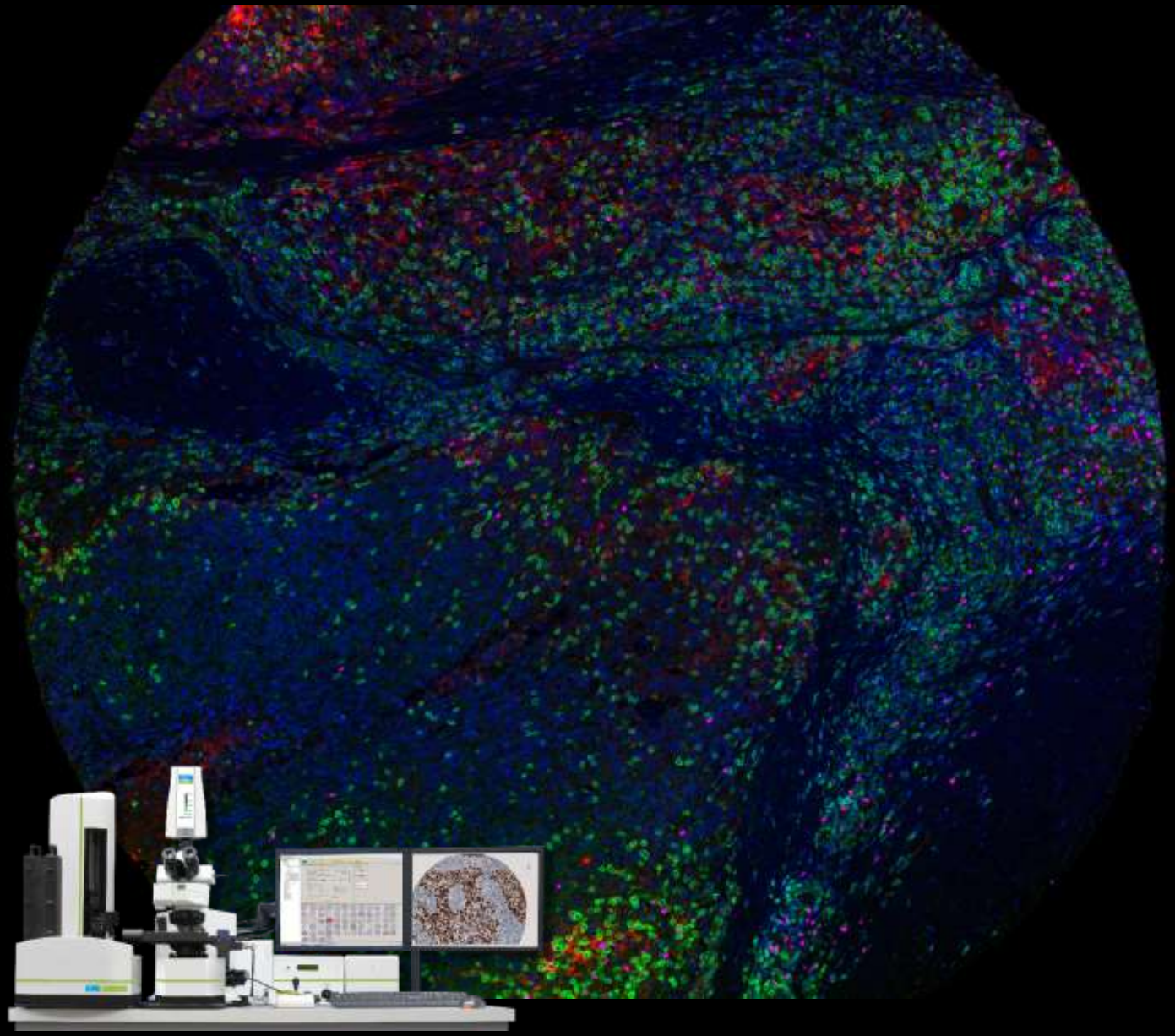
Research Use Only
Not for use in diagnostic procedures.

Melanoma TMA core: multiplexed labeling, conventional imaging



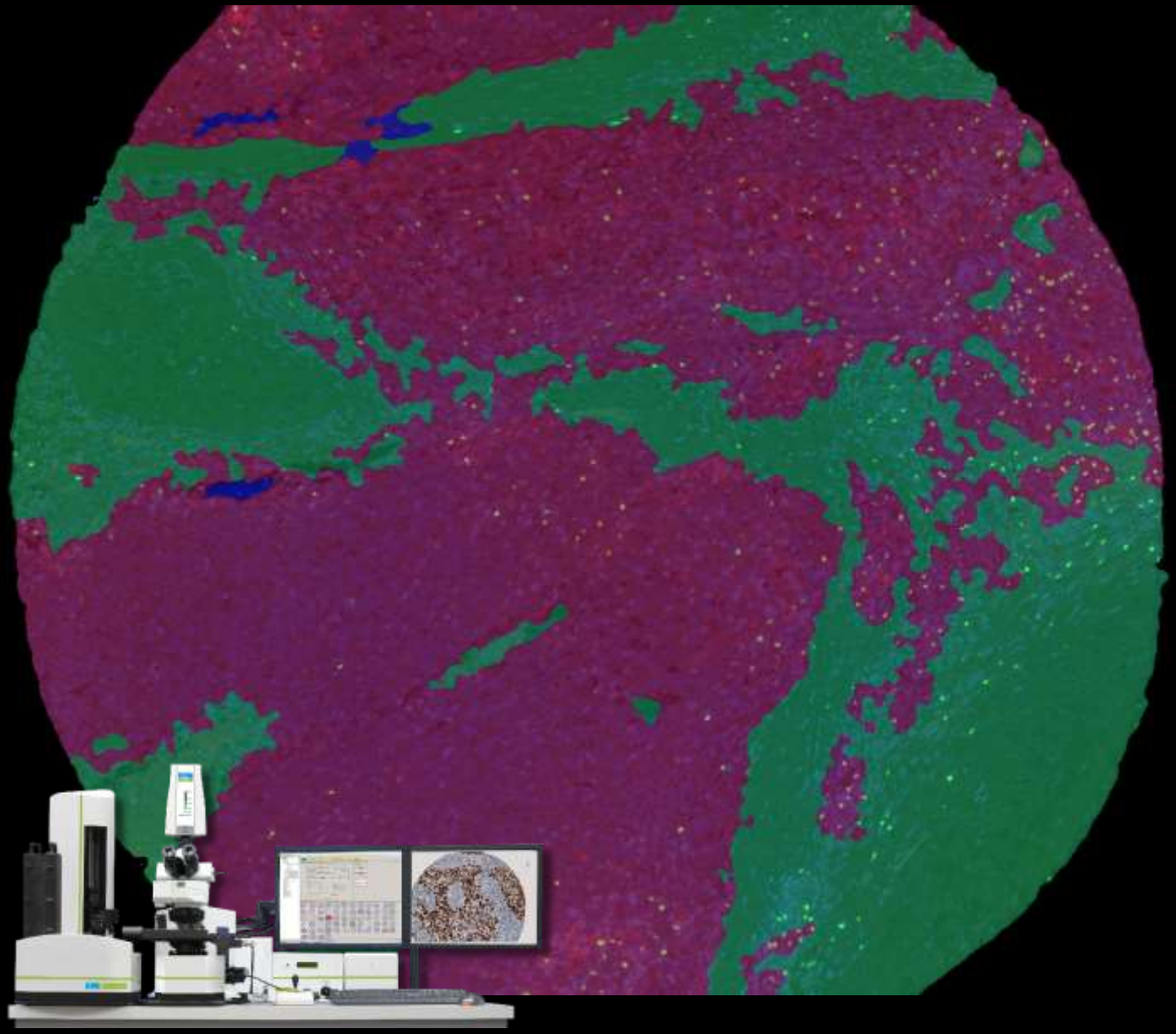
DAPI
CD8 – Cy3
CD34 – Cy5
FoxP3 – FITC
PD-L1 – coumarin
Autofluorescence

Melanoma TMA core: spectrally separated, autofluorescence removed



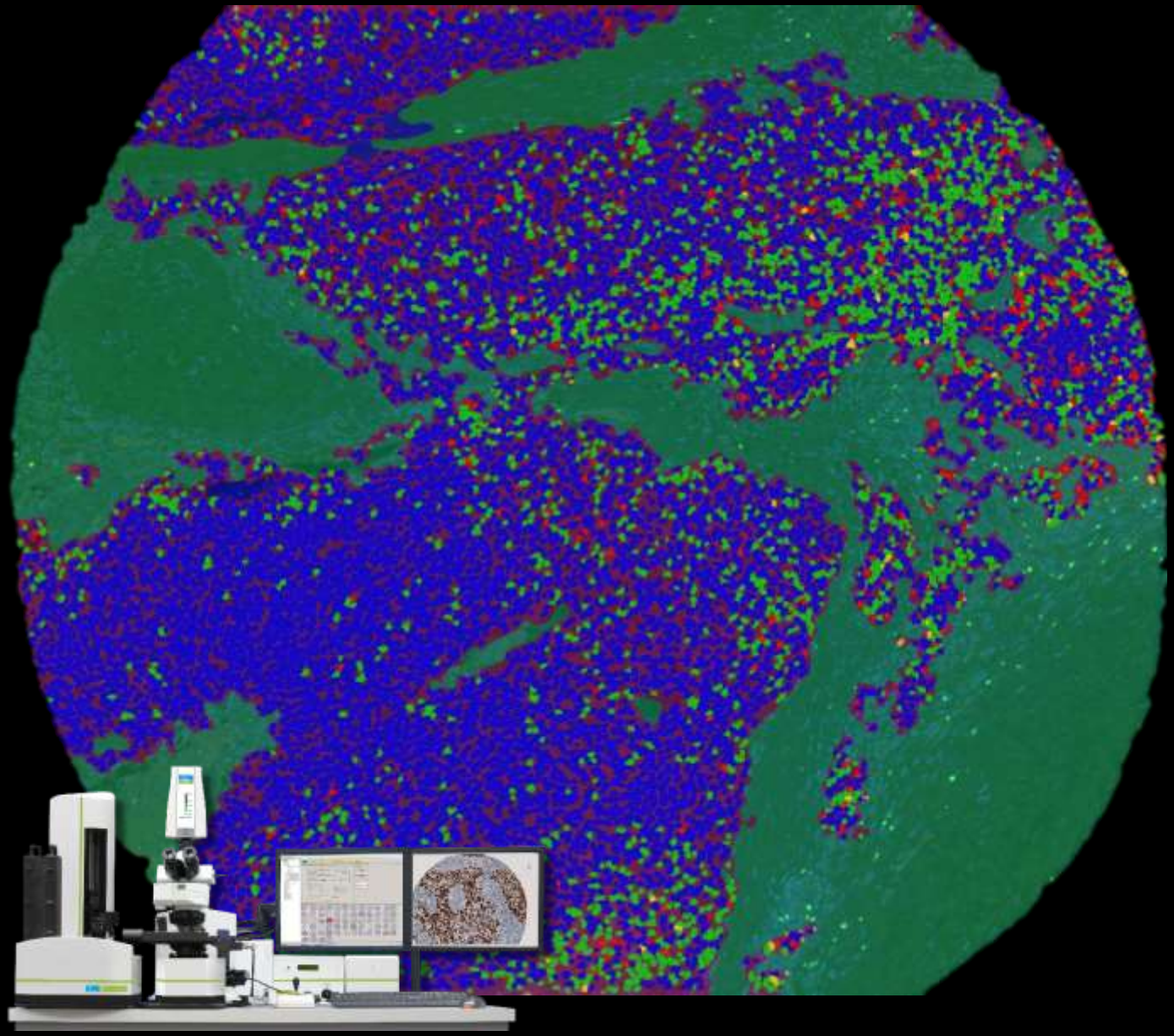
Unmixed composite:
DAPI – blue
CD8 – green
CD34 – yellow
FoxP3 – purple
PD-L1 – red

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

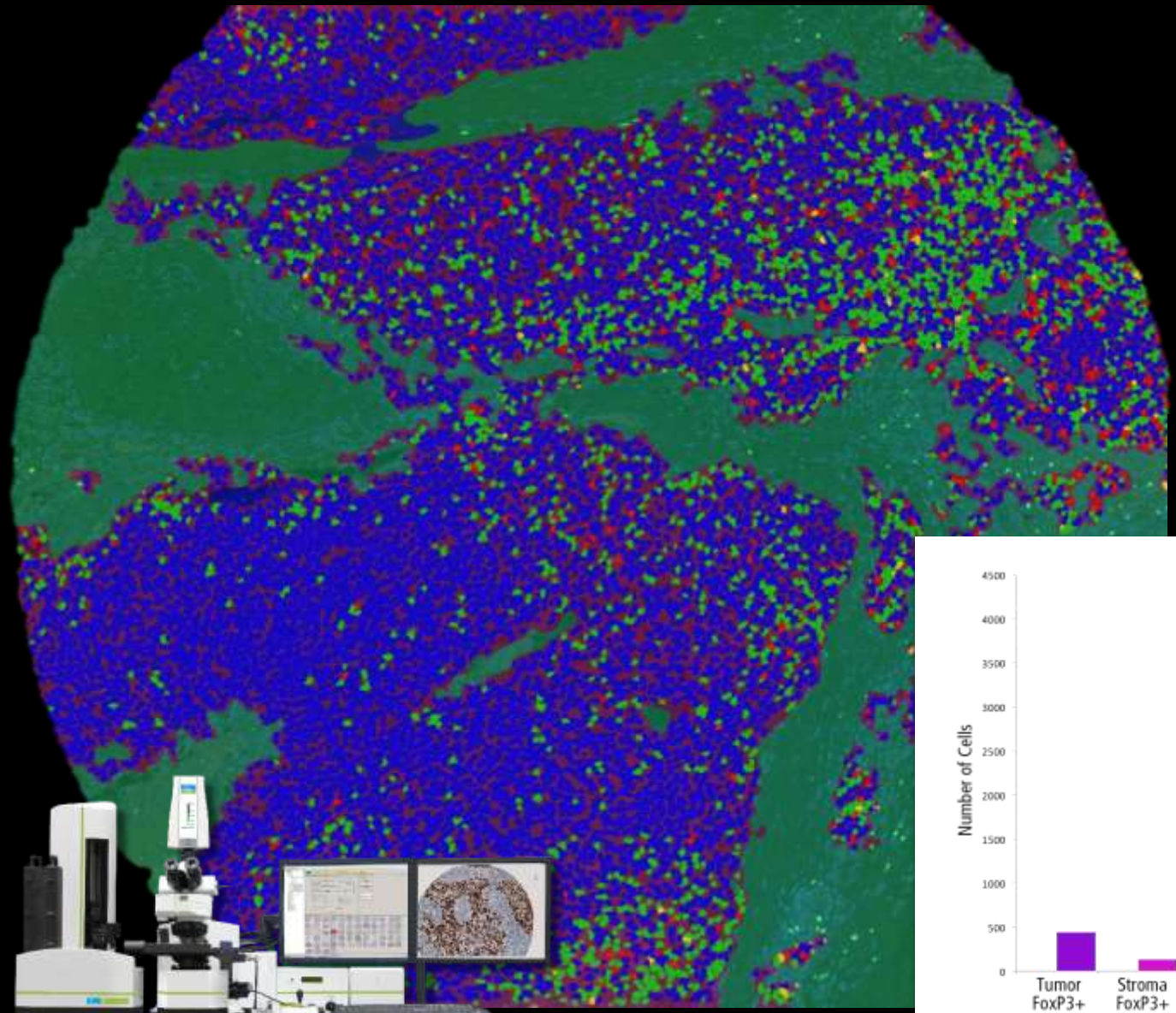
Blue – CD8-/FOXP3-

Green – CD8+/FOXP3-

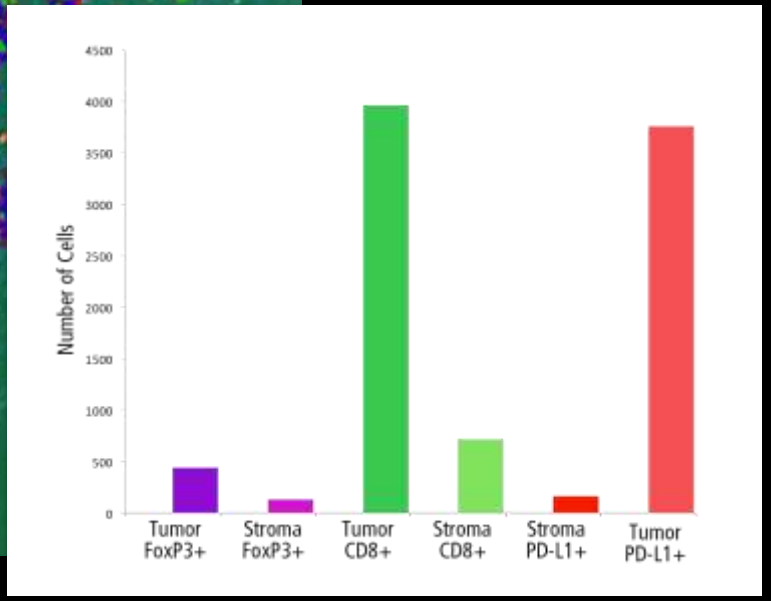
Red – CD8-/FOXP3+

Yellow – CD8+/FOXP3+

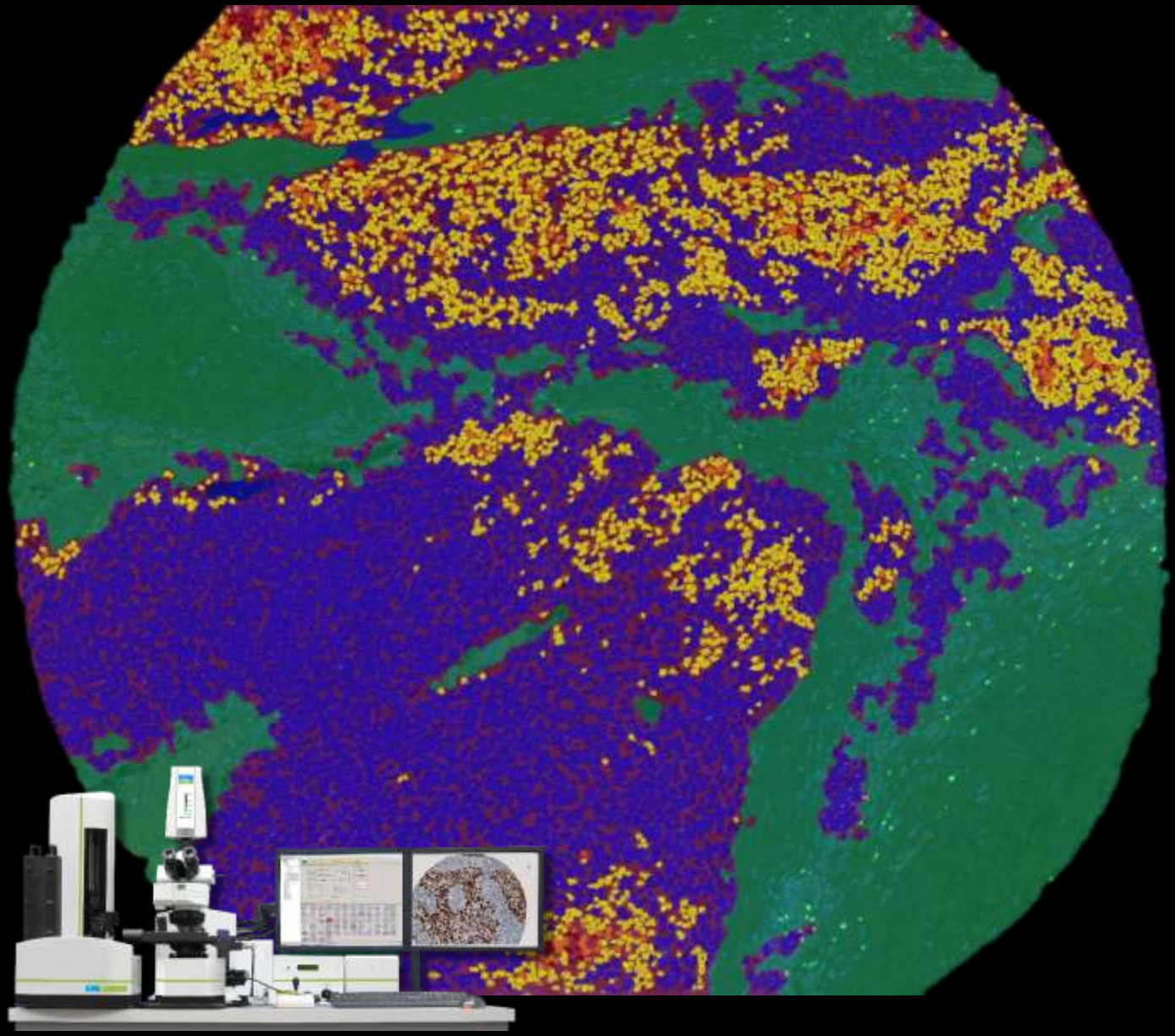
Melanoma TMA core with multiplexed immunofluorescence staining



Compartment-specific cell counts



Melanoma TMA core with multiplexed immunofluorescence staining

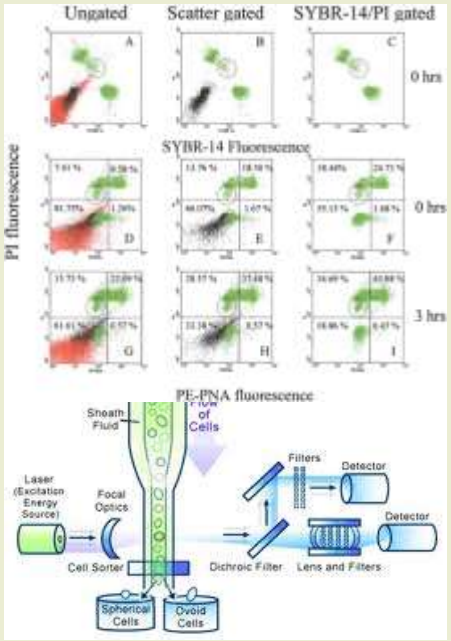


Per-cell intensity quantitation

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

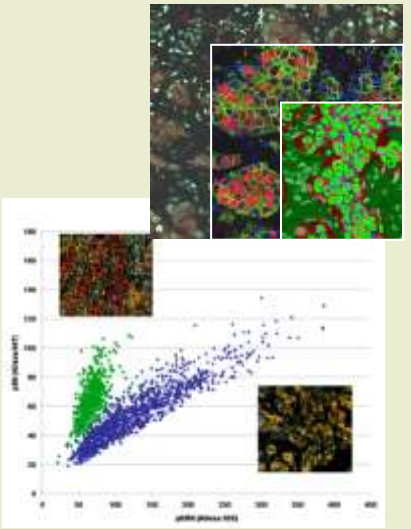
- Multiple markers (up to 20) from individual cells measured one at a time
- Requires homogenous samples
- Blood and other fluids
- Disassociated tissue samples

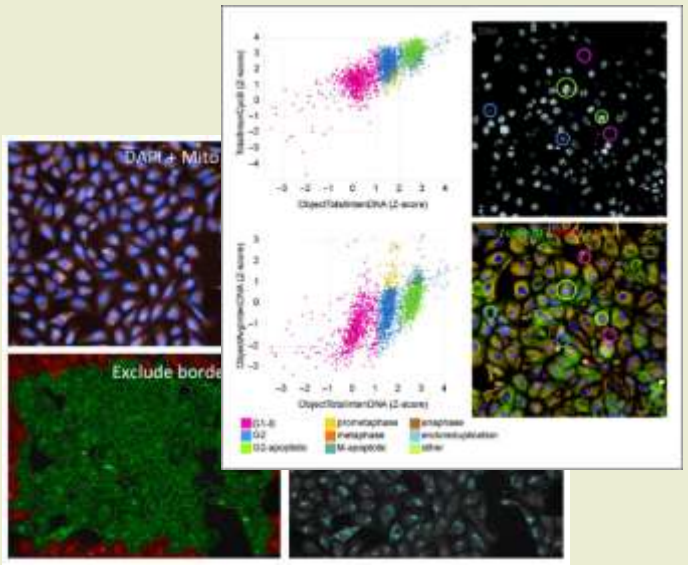
No context

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

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Not for use in diagnostic procedures.

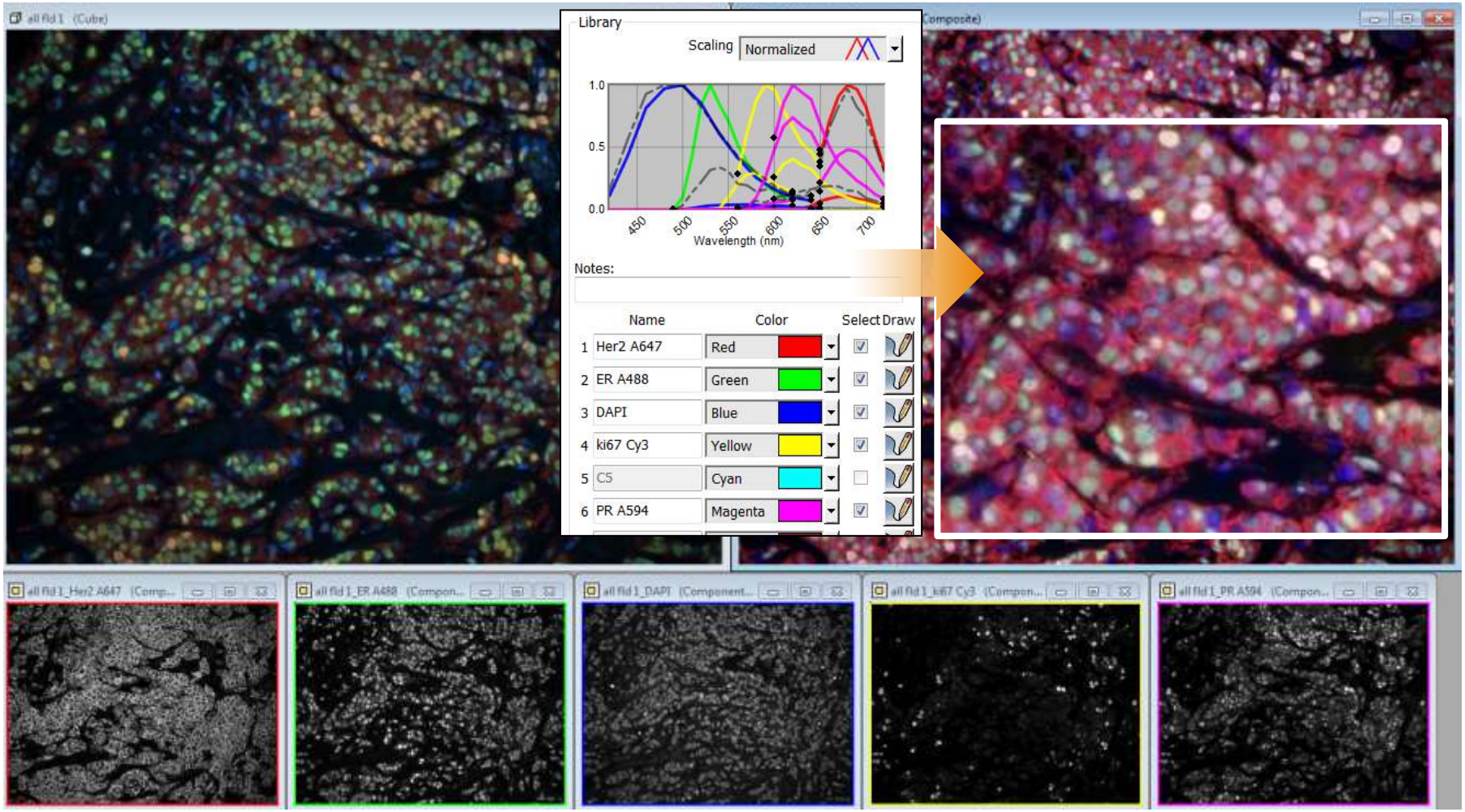
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Case Studies

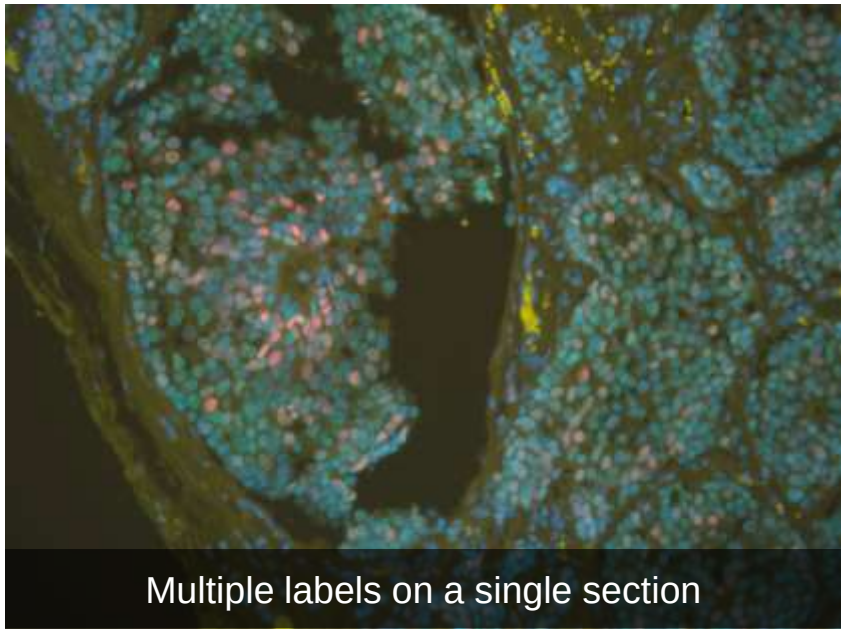
Application to Breast Cancer ER/PR/Her2/ki67

Collaborator: Dr. Kent Osborne, Director of Baylor Cancer Institute, Houston, TX

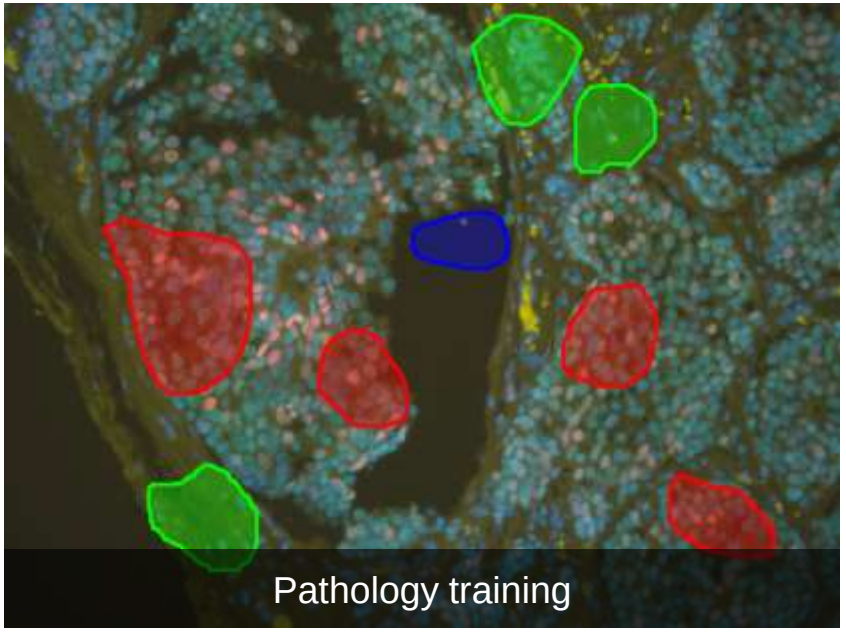


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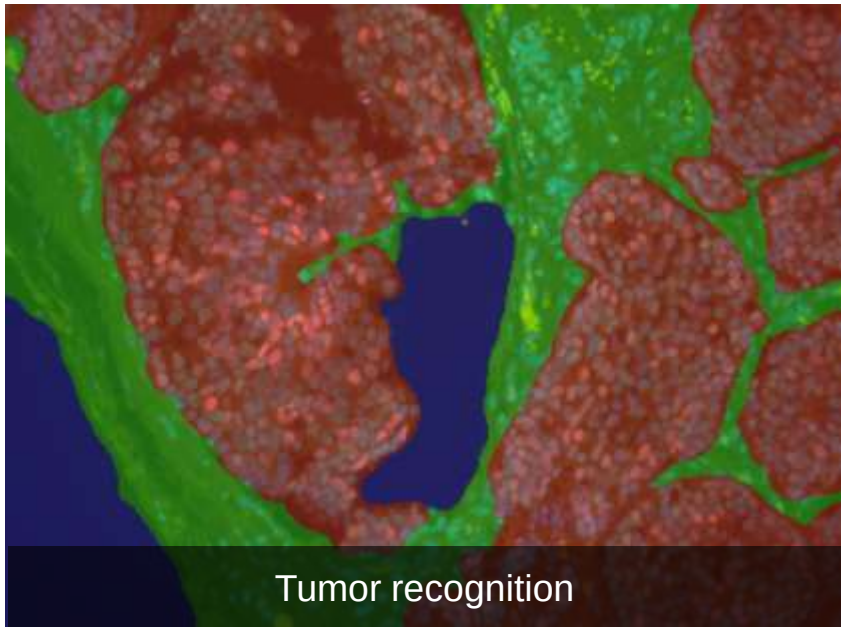
Application to ER/PR/Her2/ki67



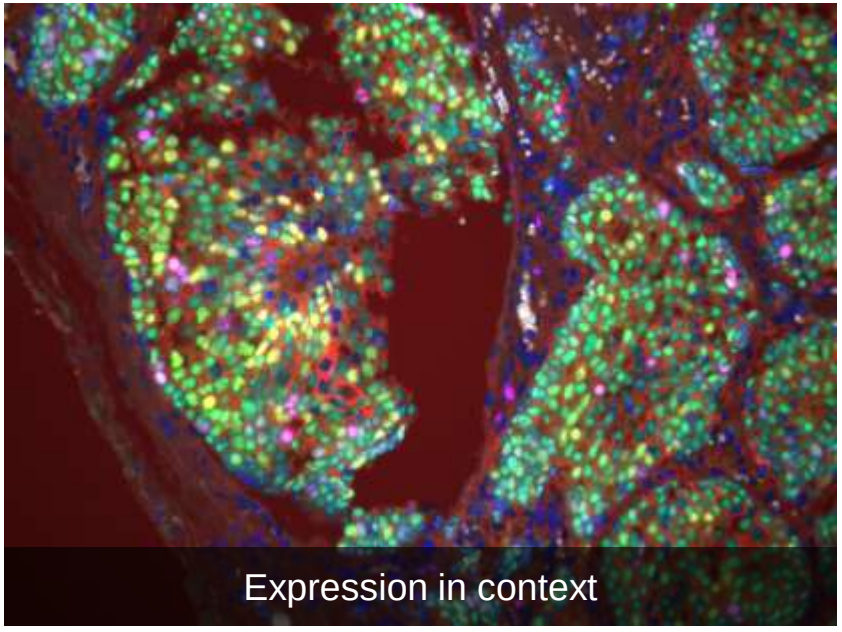
Multiple labels on a single section



Pathology training

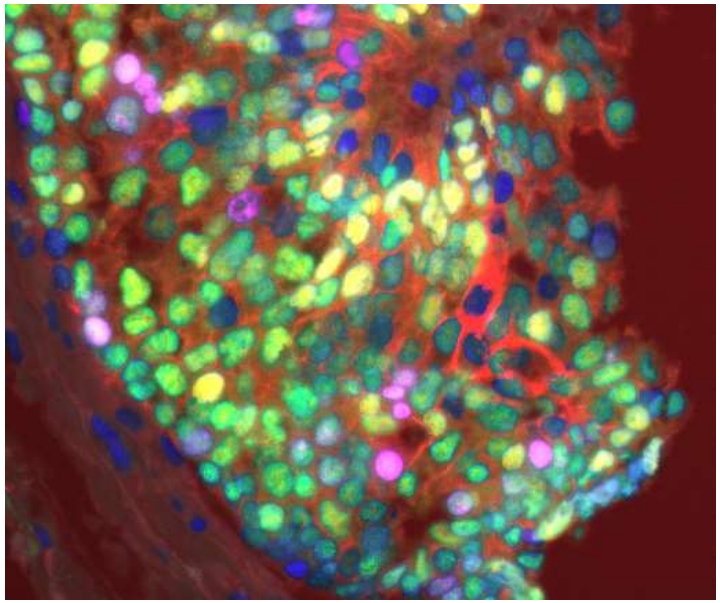


Tumor recognition

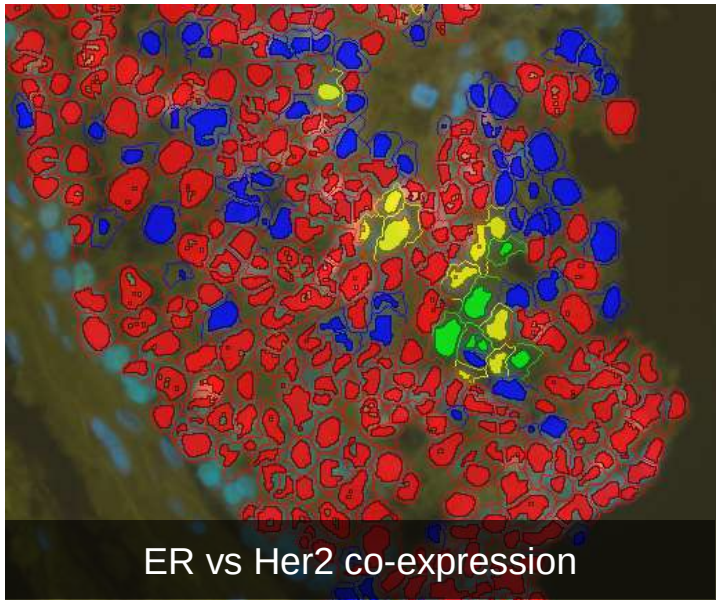


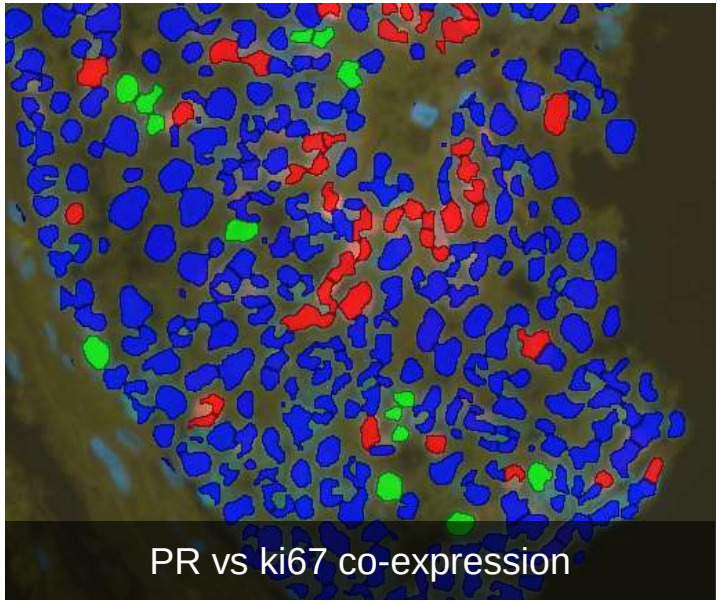
Expression in context

Application to ER/PR/Her2/ki67: measuring positivities



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





ER vs Her2

Single Positive 1	Double Positive
68.94 %	0.83 %
Double Negative	**Single Positive 2**
29.92 %	0.32 %

PR vs ki67

Single Positive 1	Double Positive
4.38 %	0.00 %
Double Negative	**Single Positive 2**
93.15 %	2.47 %

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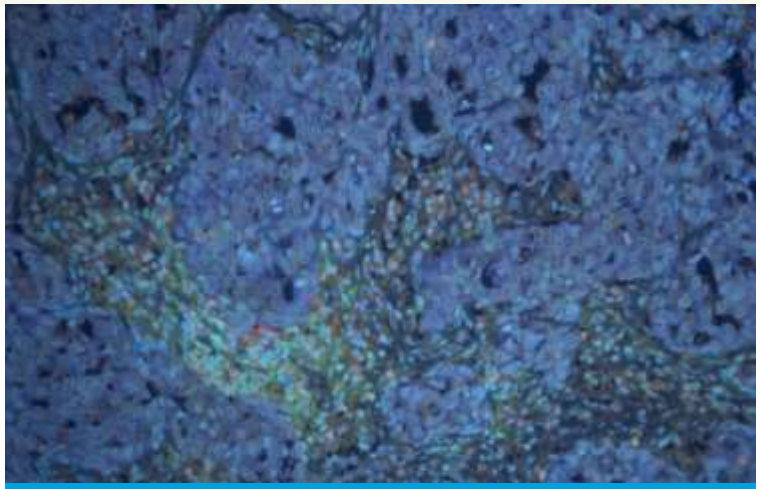
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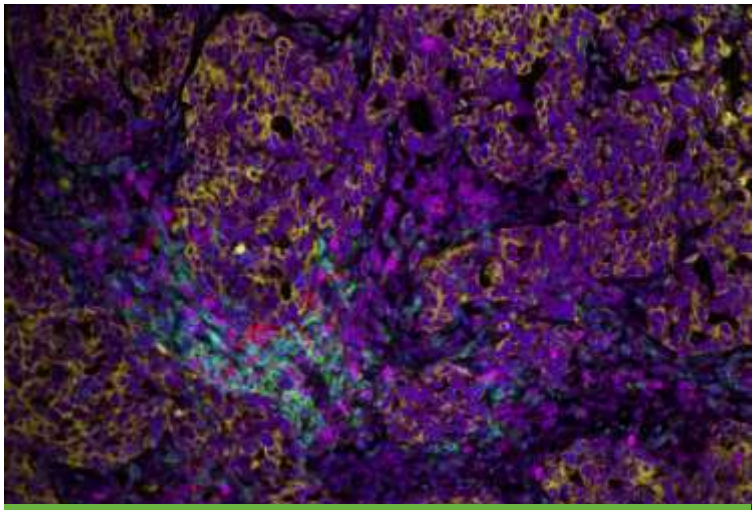
Breast Cancer Example
CD4 / CD8 / CD20 / cytokeratin / DAPI

Helper T cell (T_H), Cytotoxic T cell (T_C) and B Cell Panel

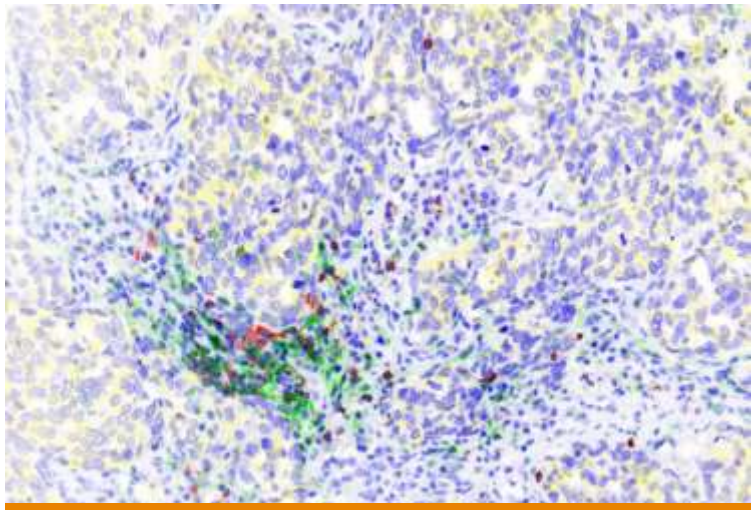
T_H, T_C & B cell panel in breast cancer



As seen by microscope



Spectrally unmixed

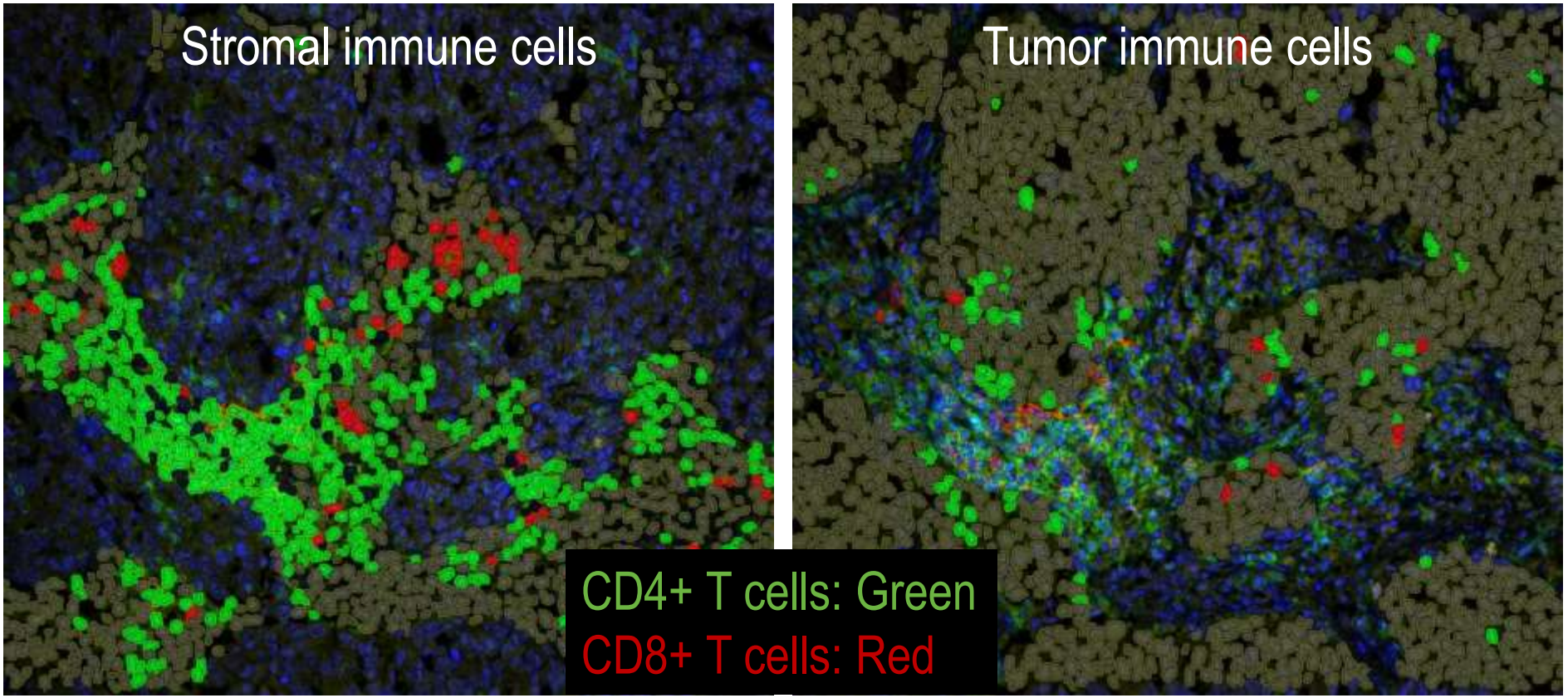


Simulated IHC view

CD4, CD8, CD20, CK, DAPI

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T_H, T_C and B cell panel in breast cancer



Two views of the same sample

Showing both stromal and tumor immune cell distributions

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Not for use in diagnostic procedures.

T_H, T_C and B cell panel in breast cancer

Quantitative image analysis - Results

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

Green
ER+ cases

Blue
Her2+ cases

Red
triple negative cases

	Avg. CD8+ Dist. to Tumor Edge (μ)		Average Cell Density (per sq mm)			
			CD4+		CD8+	
Case ID	Within Tumor	Within Stroma	Within Tumor	Within Stroma	Within Tumor	Within Stroma
BRER-01-6H	57.1	29.1	0	16	8	4
BRER-02-3G	8.2	32.6	0	24	10	140
BRER-03-2P	20.7	20.5	8	36	16	92
BRER-04-1N	18.2	34.2	0	20	8	56
BRHR-03-2C	N/A	18.8	0	0	0	8
BRHR-04-2J	14.4	40.2	0	144	63	688
BRHR-09-2B	57.4	52.2	0	236	7	468
BRHR-10-2C	N/A	29.0	0	4	0	636
BRTN-21-2J	19.6	26.3	8	276	159	440
BRTN-22-2L	17.8	24.3	0	4	47	1168
BRTN-23-3L	19.0	46.2	12	80	53	424
BRTN-24-2H	N/A	23.9	8	28	34	184

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Hoyt, et al, 2014

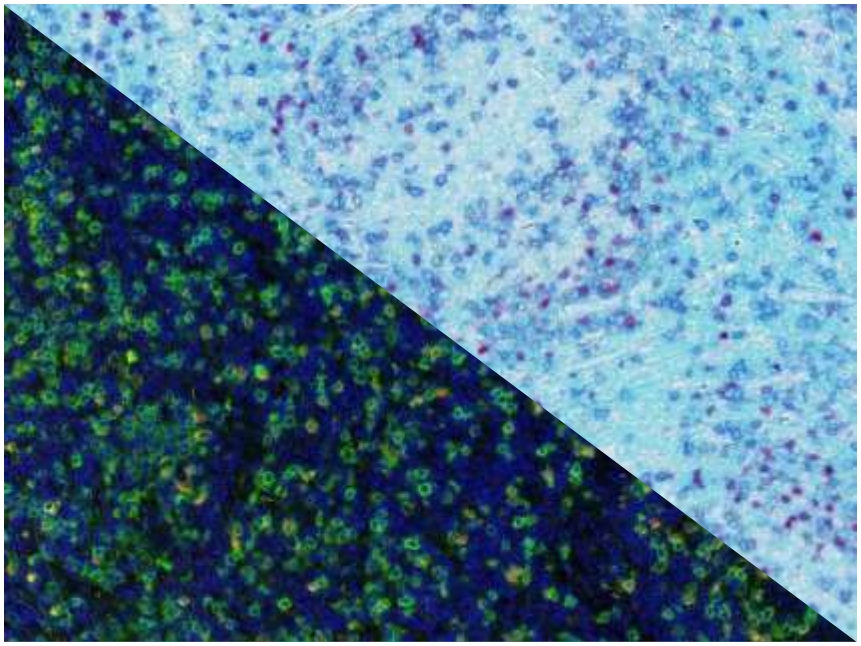
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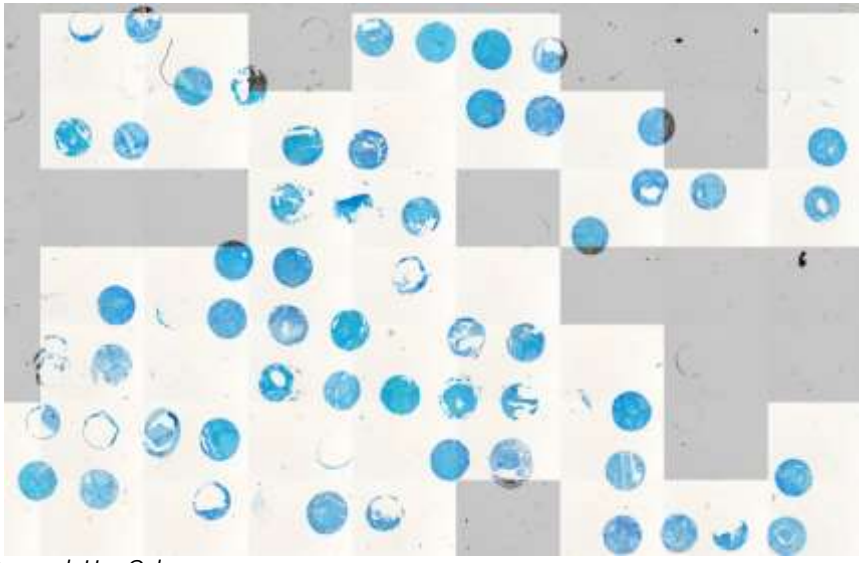
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Quantitation of activated (CD69) and regulatory (FOXP3) T cells in follicular lymphoma

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

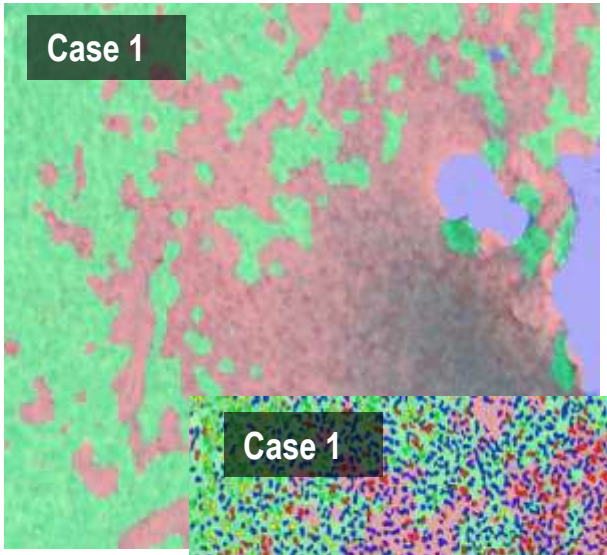
CD3: all T cells
CD3+/FOXP3+: “Tregs”
CD3+/CD69+: Tacts

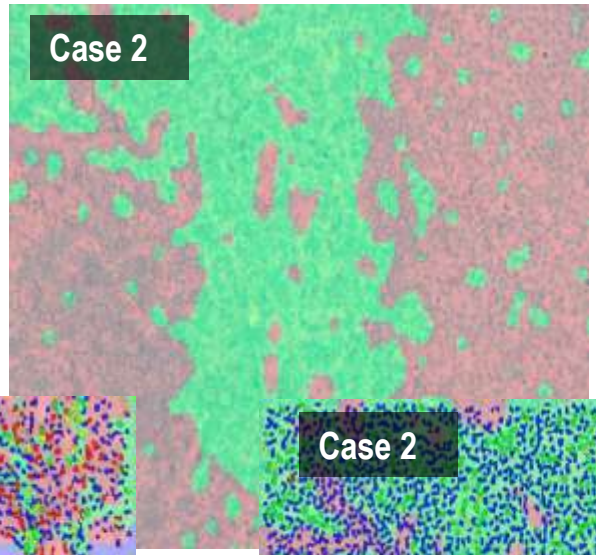
Richard Byers, U Manchester
Lilli Nelson, U Manchester
Chris van der Loos, AMC Amsterdam
James Mansfield, PerkinElmer

Looking for CD3+/FOXP3+ and CD3+/CD69+ cells

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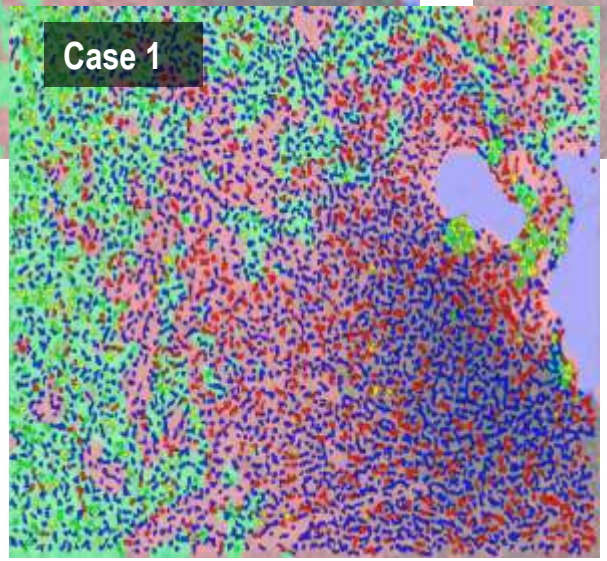
Two cases: different outcomes

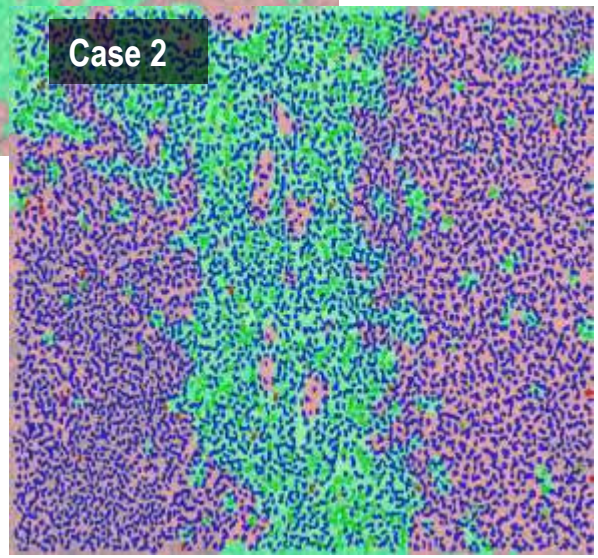




Cases :

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





Sample 1

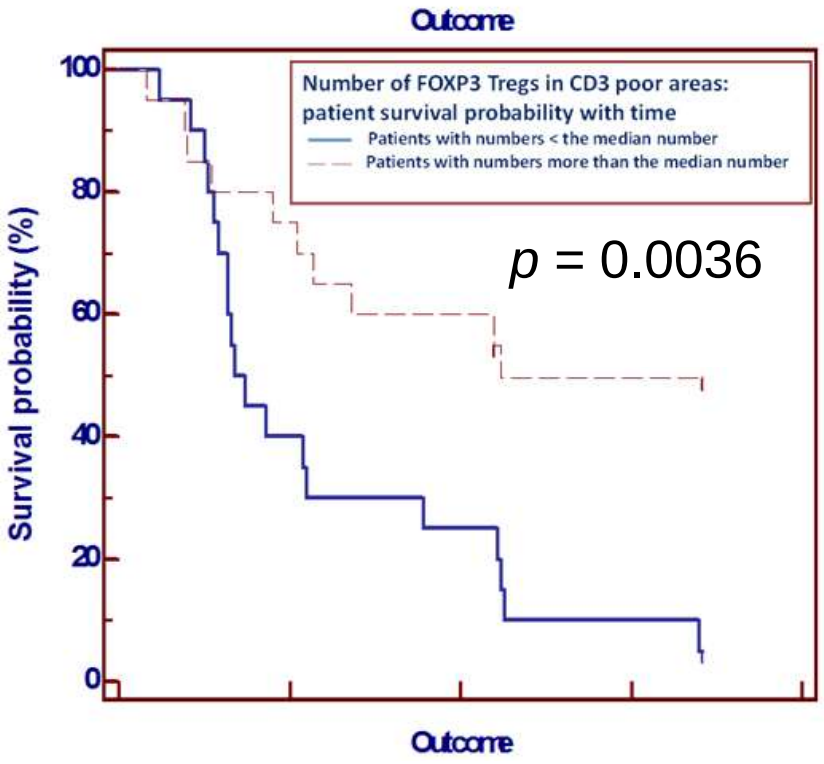
Extra-follicular cells:1473
CD3+/FOXP3-: 13.9%
CD3+/FOXP3+: 12.3%
Survival: 171+ months

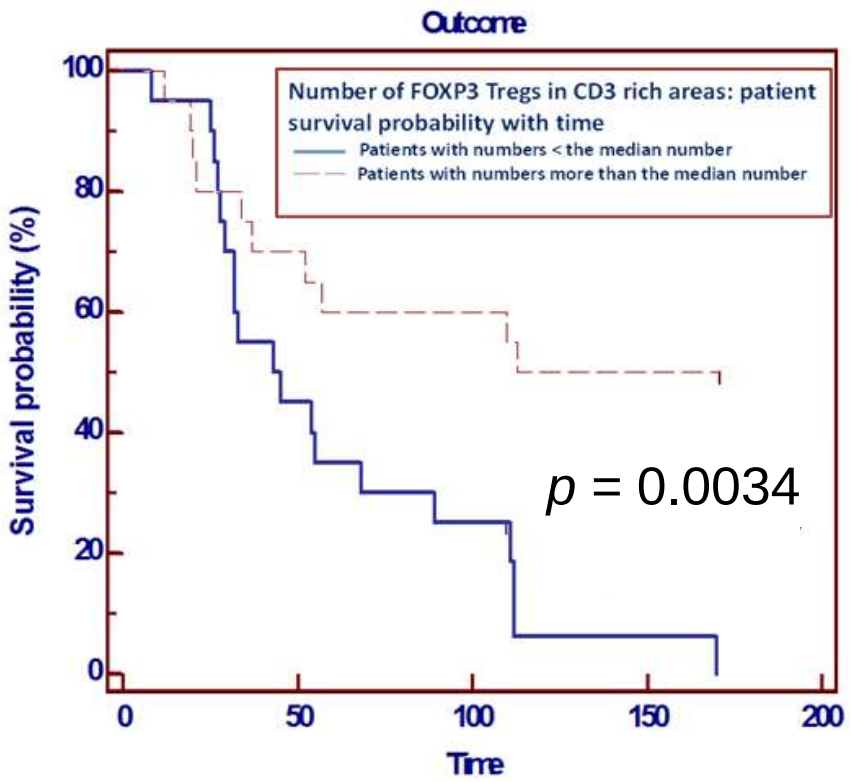
Sample 2

Extra-follicular cells: 1917
CD3+/FOXP3-: 19.66%
CD3+/FOXP3+: 0.66%
Survival: 54 months

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Kaplan-Meier plots show significant differences with Tregs





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

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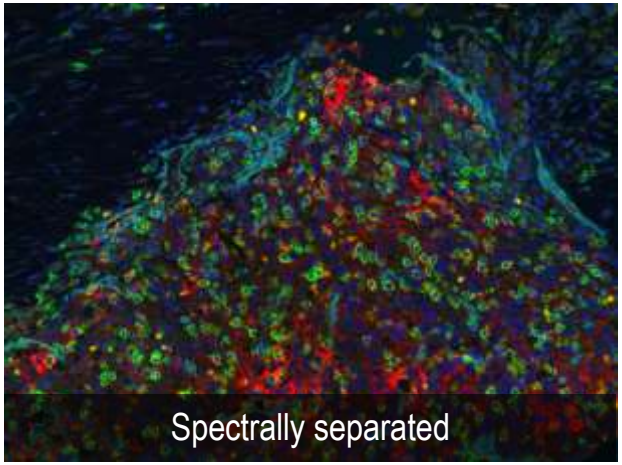
Melanoma Example
PD-L1, FOXP3, CD8, CD34, DAPI

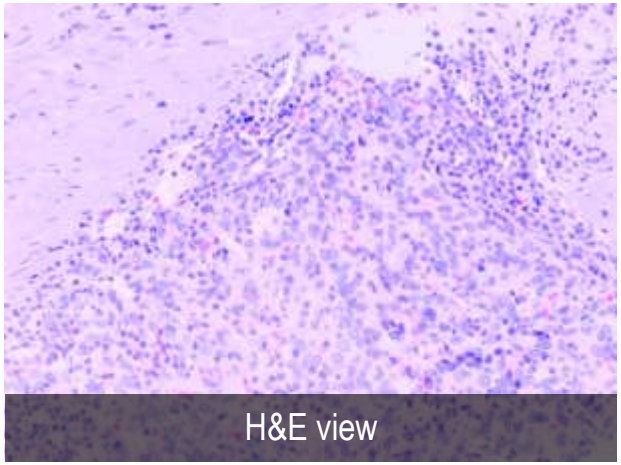
Melanoma samples courtesy Bernie Fox, Providence Cancer Center, Oregon
PD-L1 Antibody (Clone E1L3N) courtesy Cell Signaling Technology, Inc.

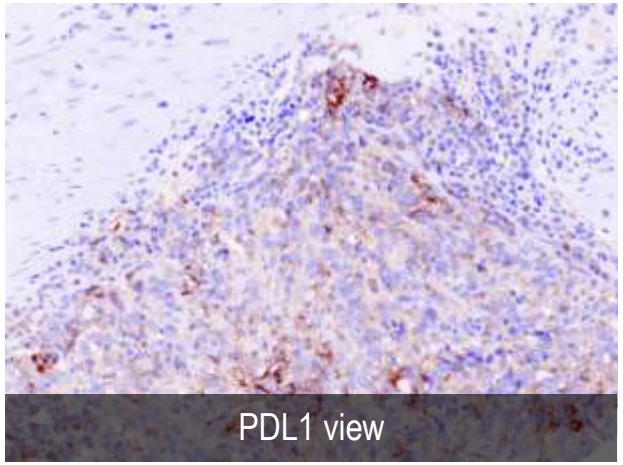
Multiplexed marker analysis of melanoma TMA core

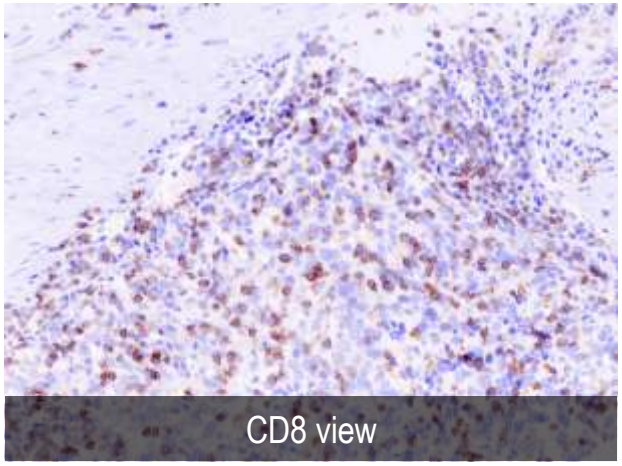
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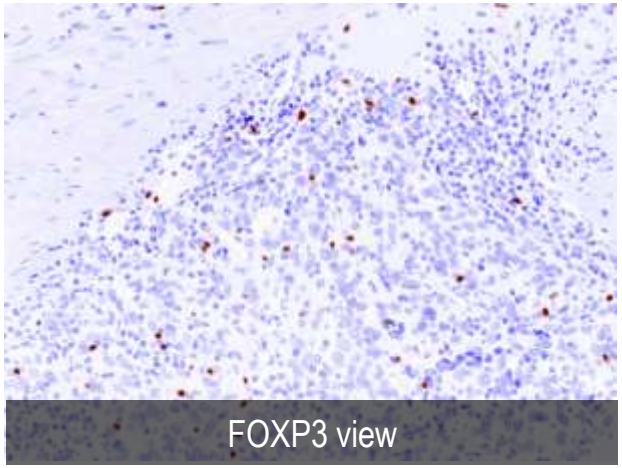
PD-L1 / Cy5	CD34 / coumarin	CD8 / FITC	FOXP3 / Cy3	DAPI

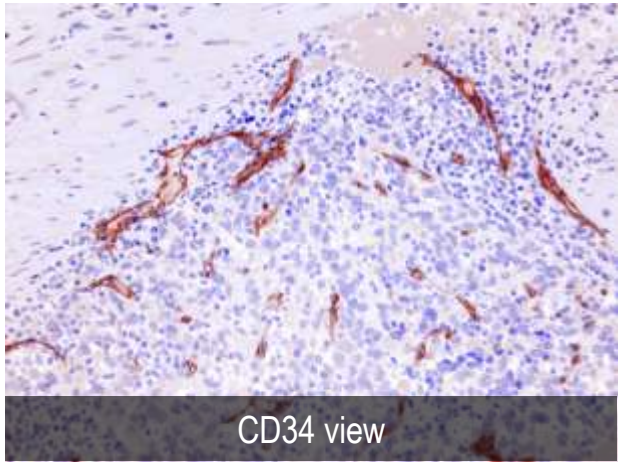






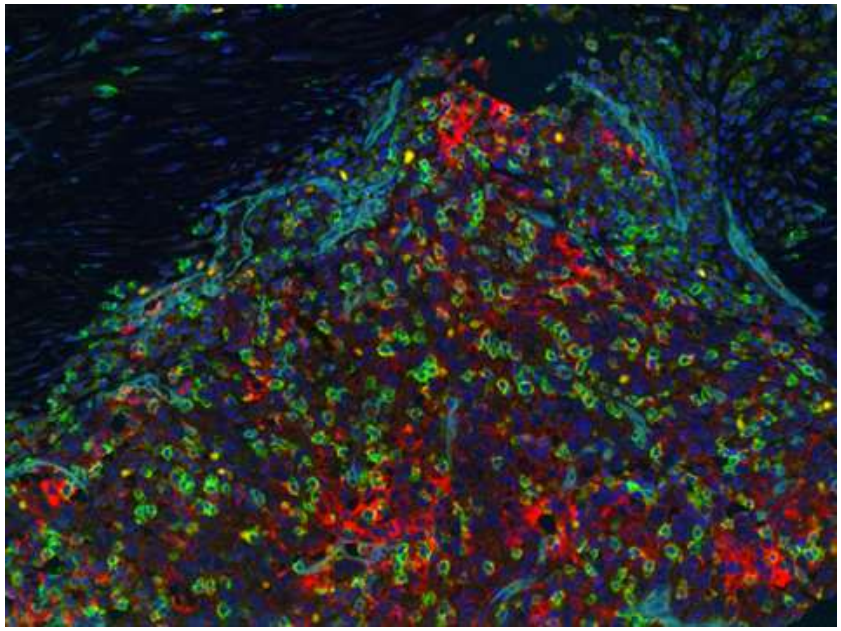






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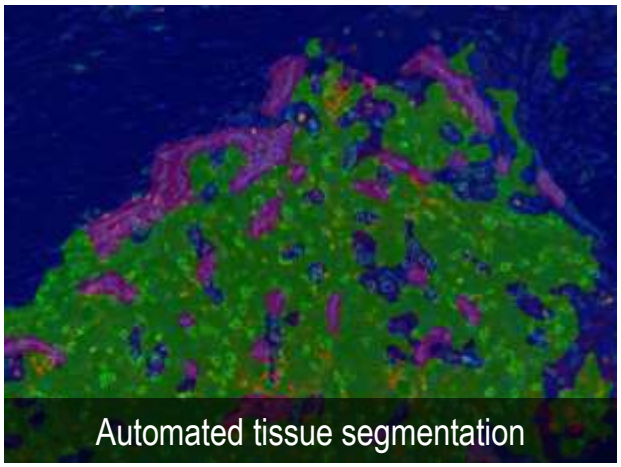
Multiplexed four marker analysis of melanoma TMA core



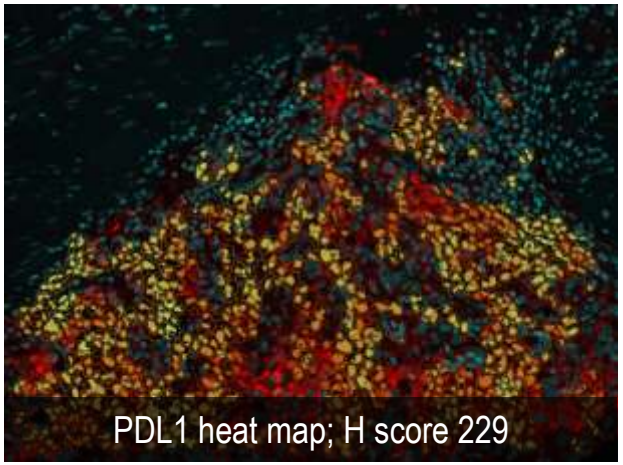
Green = tumor compartment

Blue = stroma and inflammation

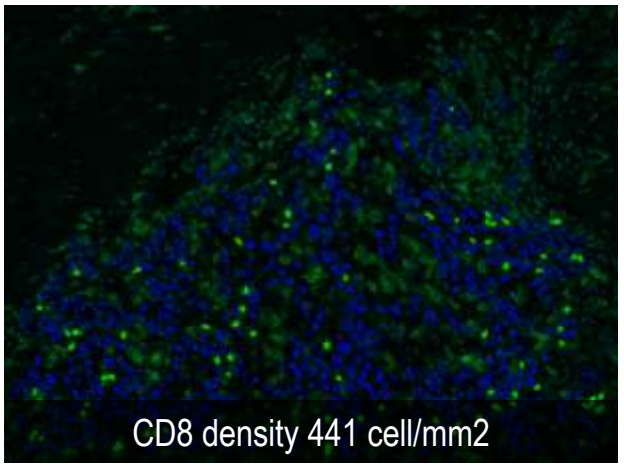
Purple = vessels



Automated tissue segmentation



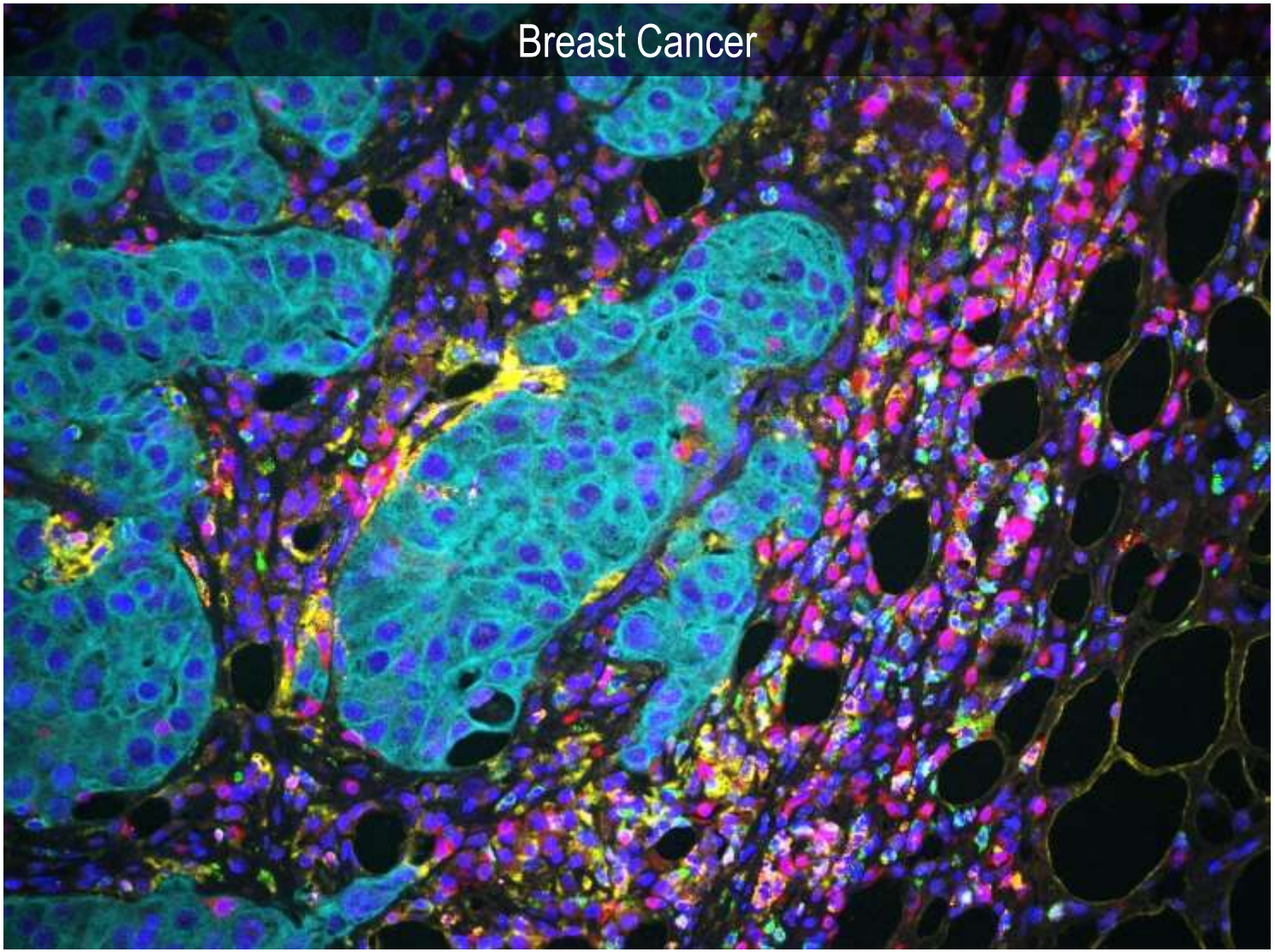
PDL1 heat map; H score 229



CD8 density 441 cell/mm2

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Our latest 6-plex assay



CD4 | CD8 | CD20 | PD-L1 | FOXP3 | AE1/AE3 | DAPI

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Example cancer immunology panels on Vectra

Breast / Ovary TILs:
CD4 / CD8 / CD20 / cytokeratin / DAPI

- Dr. Michael Feldman, University of Pennsylvania

Melanoma Tregs:
CD3 / CD8 / FOXP3 / S100 / hematoxylin

- Dr. David Rimm, Yale University

Tumor-draining lymph node and breast cancer: CD3 / CD8 / PD-1 / PD-L1 / cytokeratin / hematoxylin

- Dr. Peter Lee, City of Hope Research Center

B-cell lymphoma:
CD3 / CD10 / CD20 / CD21 / BcL2 / BcL6 / DAPI

- Dr. Michael Feldman, University of Pennsylvania

Follicular lymphoma:
CD4 / CD25 / FOXP3 / hematoxylin

- Dr. Richard Byers, University of Manchester, UK

Melanoma:
PD-L1 / FOXP3 / CD8 / CD34 / DAPI

- Dr. Bernie Fox, Providence Cancer Institute

Melanoma:
CD3 / CD4 / CD8 / dendritic cell markers

- Dr. Carl Figdor, University of Nijmegen

Melanoma:
CD3 / CD8 / PD-L1

- Drs. Pardoll, Topalian, Taube, Johns Hopkins

Breast cancer:
CD4 / CD8 / CD20 / PD-L1 / FOXP3 / AE1-AE3 / DAPI

- MD Anderson

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Quantitative Pathology Solutions



Contract discovery services









Labeling Strategies	Imaging Strategies	Image Analysis Strategies	Automation Strategies
Multicolor IHC and IF Opal™ Multiplexing Kits TSA Plus™ reagents	**Quantitative Multispectral Imaging** Vectra® Intelligent Imaging System Mantra™ Quantitative Pathology Imaging System	**Advanced Image Analysis Software** inForm® Tissue Finder™	**Whole slide and TMA imaging** Vectra® Intelligent Imaging System

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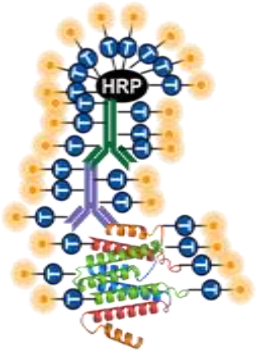
For each piece of the puzzle

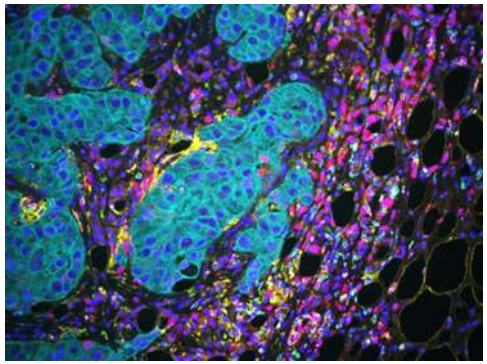
Thank you for your attention!

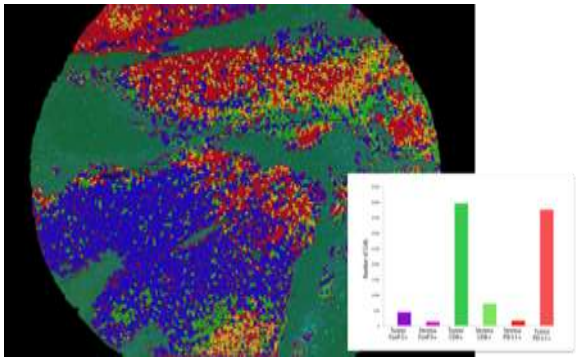
PerkinElmer offers:

- 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







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Are there any questions?

Thank you for your attention!



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