

# *Tecnologia multispettrale per la caratterizzazione dell'immunofenotipo in situ*

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# Technological viability

## Challenges

- › Limited samples
- › Antibody cross reactivity
- › Autofluorescence in FFPE
- › Immunophenotyping

# Clinical relevance

SCIENTIFIC REPORTS

Clinical response

OPEN

**Multiplex immunohistochemistry accurately defines the immune context of metastatic melanoma**

2018

Received: 15 February 2018  
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Published online: 24 July 2018

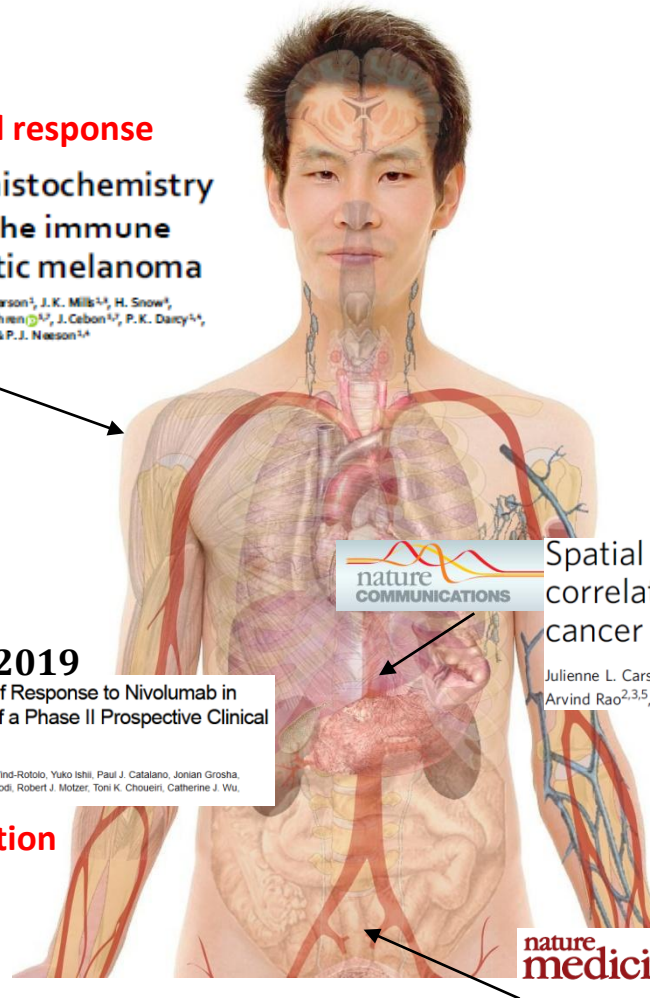
H. Halse<sup>1</sup>, A. J. Colebatch<sup>1,2</sup>, P. Petrone<sup>1</sup>, M. A. Henderson<sup>1</sup>, J. K. Mills<sup>1,3</sup>, H. Snow<sup>4</sup>, J. A. Westwood<sup>5</sup>, S. Sandhu<sup>1,6</sup>, J. M. Raleigh<sup>1</sup>, A. Bahren<sup>1,7</sup>, J. Cebron<sup>1,8</sup>, P. K. Darcy<sup>1,9</sup>, M. H. Karshaw<sup>1,9</sup>, G. A. McArthur<sup>1</sup>, D. E. Gyorko<sup>1,10</sup> & P. J. Neeson<sup>1,4</sup>

**Clinical Cancer Research** 2019

**irRECIST for the Evaluation of Candidate Biomarkers of Response to Nivolumab in Metastatic Clear Cell Renal Cell Carcinoma: Analysis of a Phase II Prospective Clinical Trial**

Jean-Christophe Pignon, Opeyemi A. Jegede, Sachet A. Shukla, David A Braun, Christine E. Horak, Megan Wind-Rotolo, Yuko Ishii, Paul J. Catalano, Jonian Grosha, Abdallah Flifel, Jesse S. Novak, Kathleen M. Mahoney, Gordon J. Freeman, Ariene H. Sharpe, F. Stephen Hodi, Robert J. Motzer, Toni K. Choueiri, Catherine J. Wu, Michael B. Atkins, David F. McDermott, and Sabina Signoretti

Atypical response explanation



nature COMMUNICATIONS

**Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer** 2017

Julienne L. Carstens<sup>1,\*</sup>, Pedro Correa de Sampaio<sup>1,\*</sup>, Dalu Yang<sup>2,3</sup>, Souptik Barua<sup>2,3</sup>, Huamin Wang<sup>4</sup>, Arvind Rao<sup>2,3,5</sup>, James P. Allison<sup>6</sup>, Valerie S. LeBleu<sup>1</sup> & Raghu Kalluri<sup>1,7,8</sup>

nature medicine

**Modulation of immune-therapies**

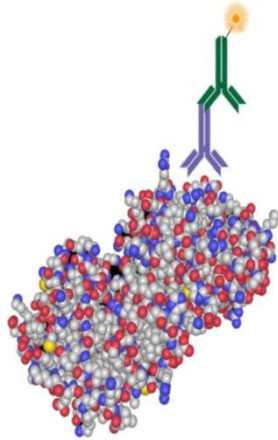
**VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer** 2017

Jianjun Gao<sup>1</sup>, John F Ward<sup>2</sup>, Curtis A Pettaway<sup>2</sup>, Lewis Z Shi<sup>1</sup>,

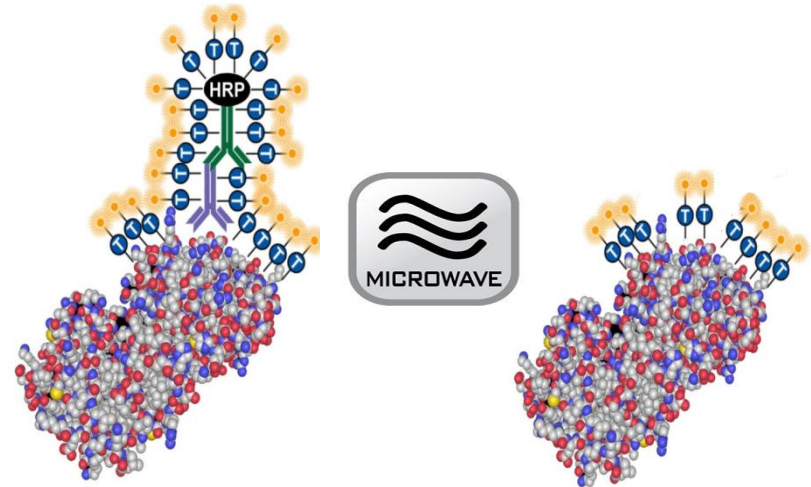


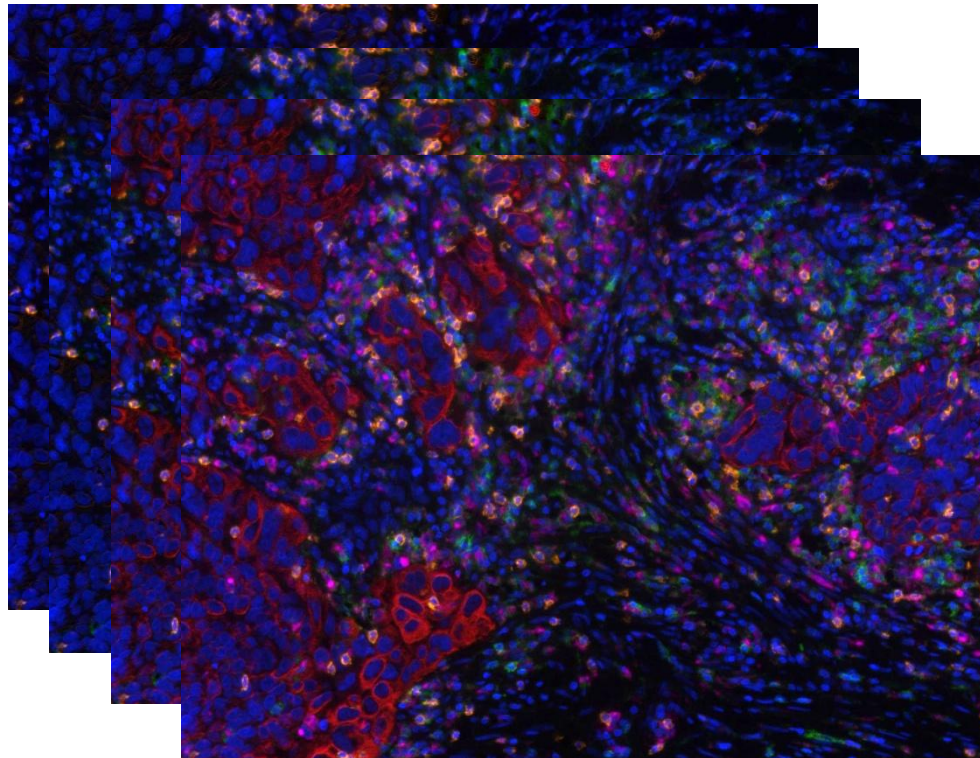
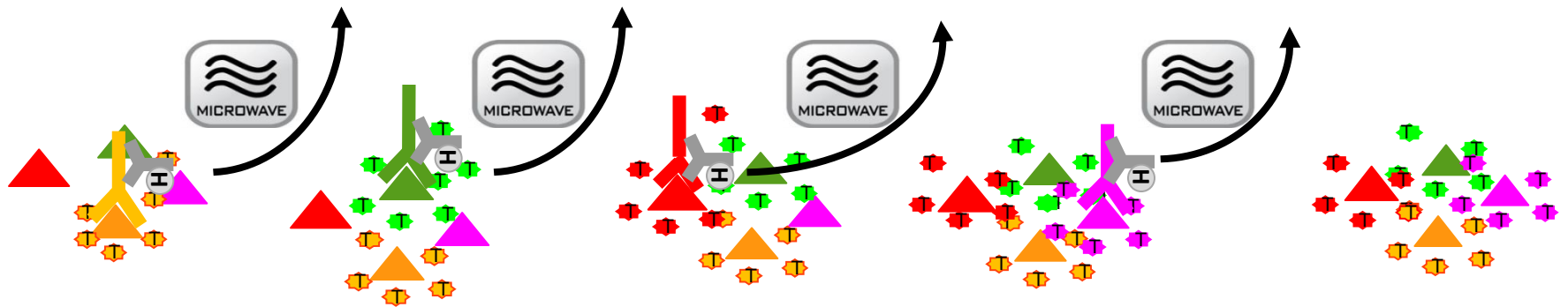
# Technological reliability

*Standard Detection*



*TSA Detection*





**CRO**  
AVIANO

Centro di Riferimento Oncologico di Aviano  
Istituto di Ricovero e Cura a Carattere Scientifico

# Multispectral Quantitative Pathology System



# Technological reliability

720nm



440nm

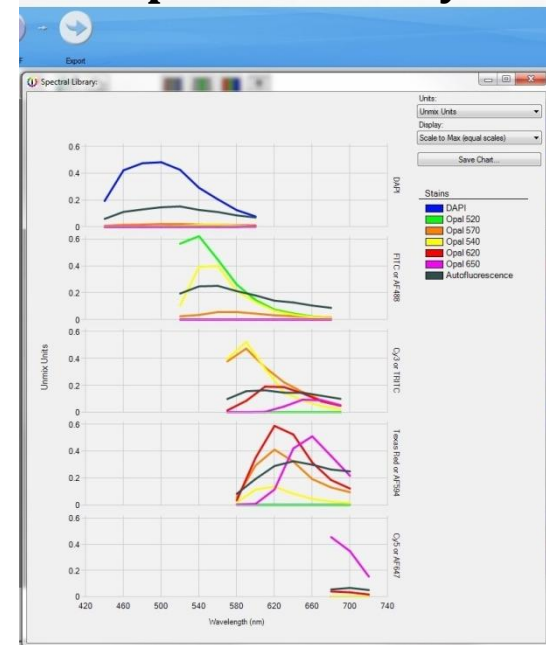


Multispectral camera

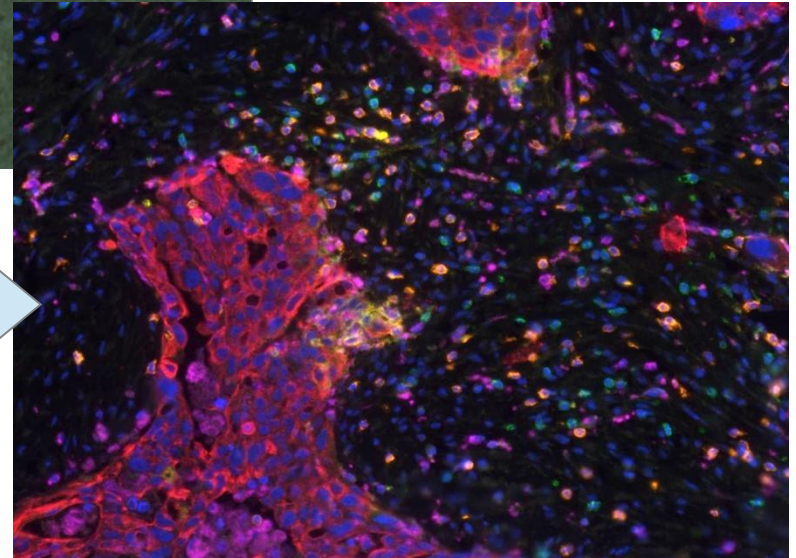
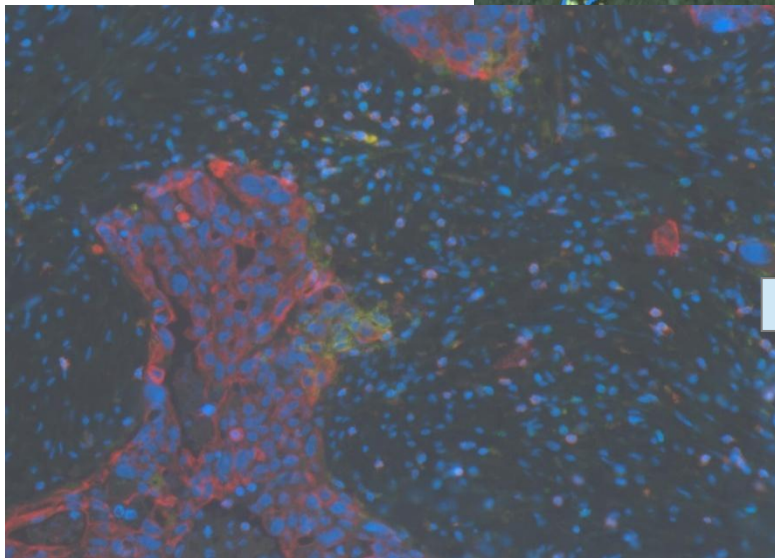
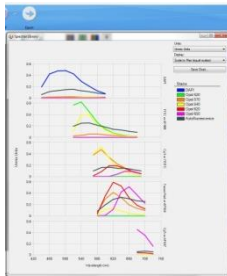
Filters



Spectral library

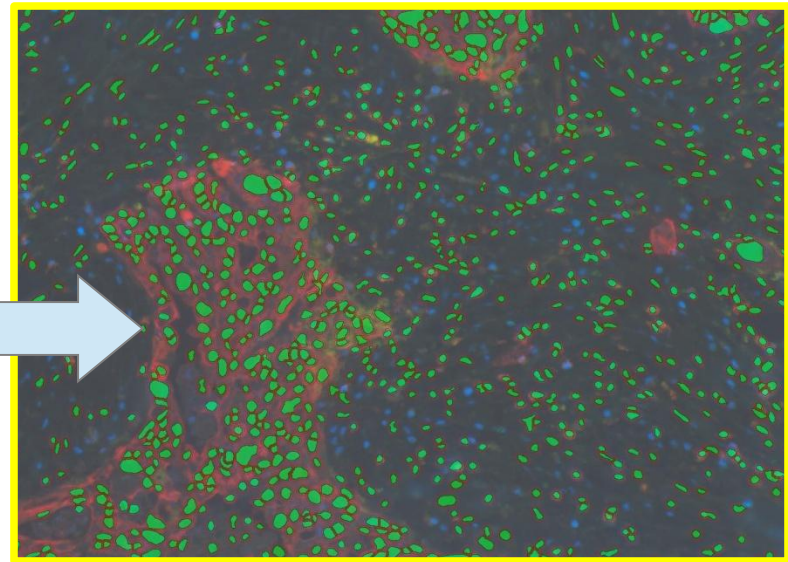
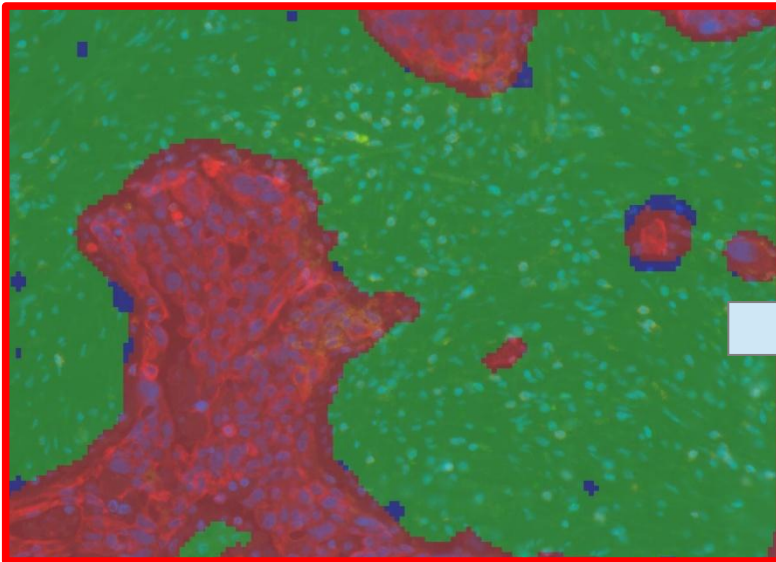


# Technological reliability





# Technological reliability



# Technological reliability



The screenshot displays the Inform 2.4 software interface. The main window shows a histological image with segmented cells and a histogram of results. The left sidebar contains settings for 'Score IHC or IF Settings (research use only)'. The 'First Marker Settings' are configured for 'Opal 540' with a compartment of 'Nuclei' and a threshold of 5.00. The 'Second Marker Settings' are configured for 'Opal 520' with a compartment of 'Nuclei' and a threshold of 60.00. The 'Histogram Results (Percent)' are shown as follows:

Category	Percentage
Single Positive 1	1.78 %
Double Positive	2.37 %
Double Negative	11.72 %
Single Positive 2	84.12 %

# Technological viability

## Challenges

- Limited samples
- Antibody cross reactivity
- Autofluorescence in FFPE
- Immunophenotyping

Simultaneous identification  
up to 6 antigens

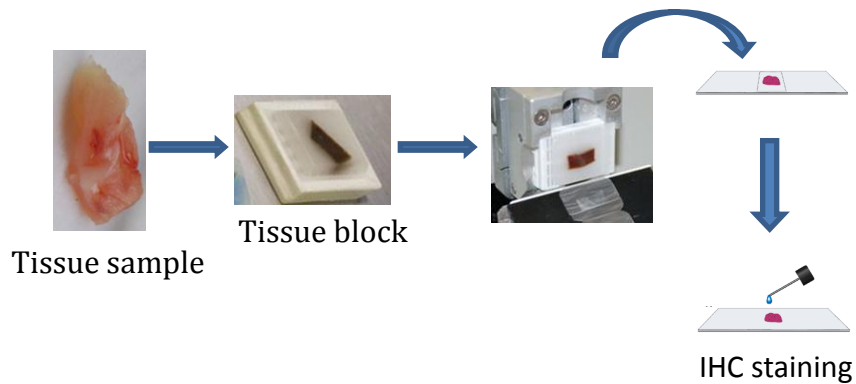
Autofluorescence subtraction

Tissue contextualization

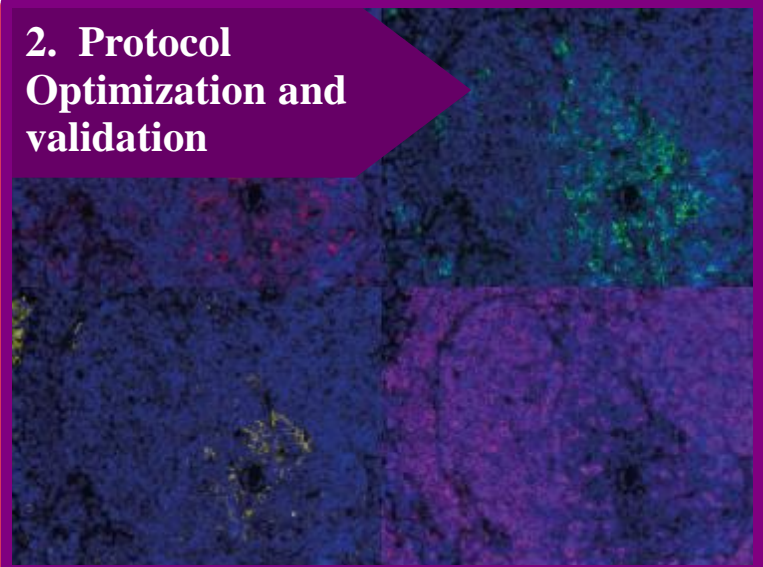
Quantification

# Technological validation

## 1. Sample processing

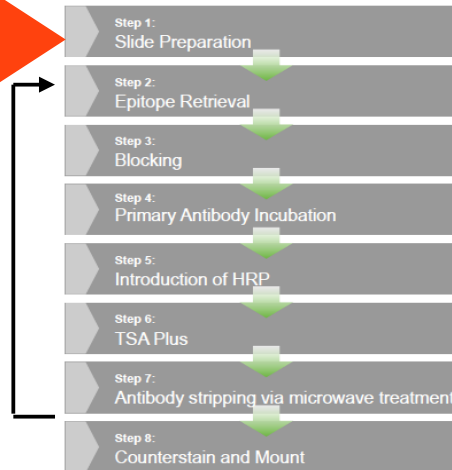


## 2. Protocol Optimization and validation

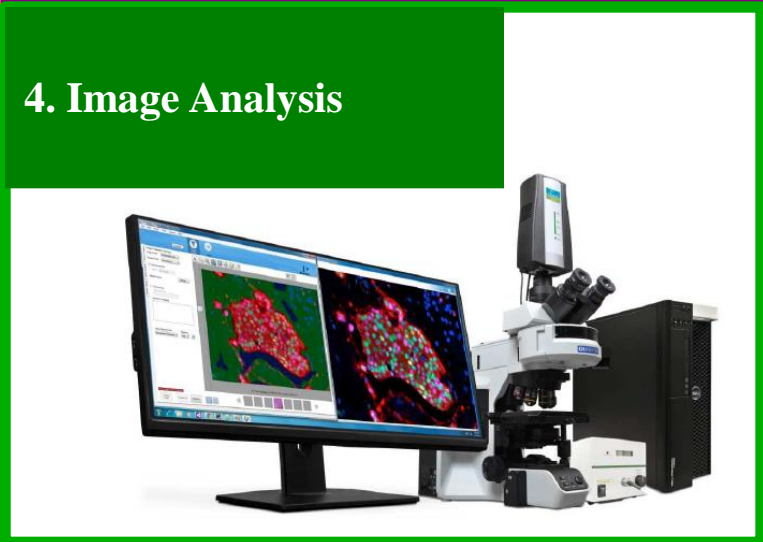


## 3. mIHC protocol development

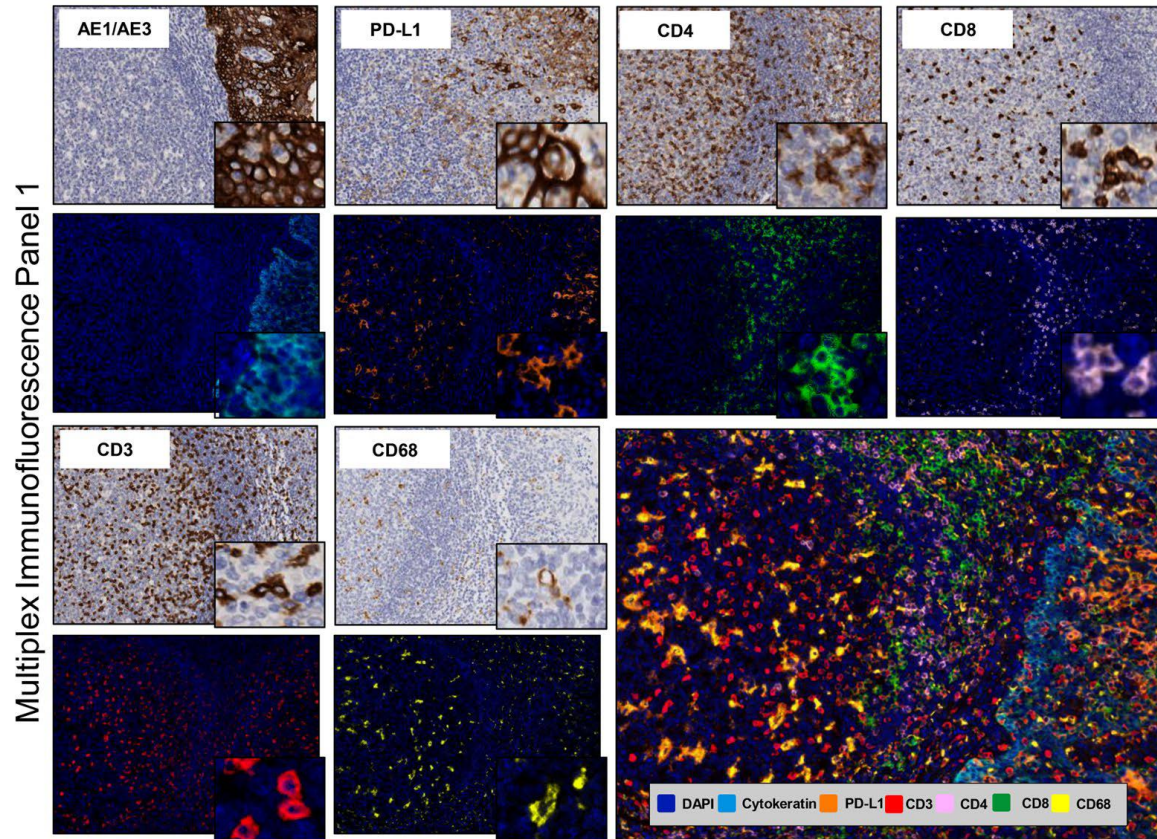
CD4 (OPAL52)  
CD8 (OPAL57)  
PD-L1 (OPAL54)  
pan Cks (OPAL650)  
CD68 (OPAL650)



## 4. Image Analysis



# Technological validation

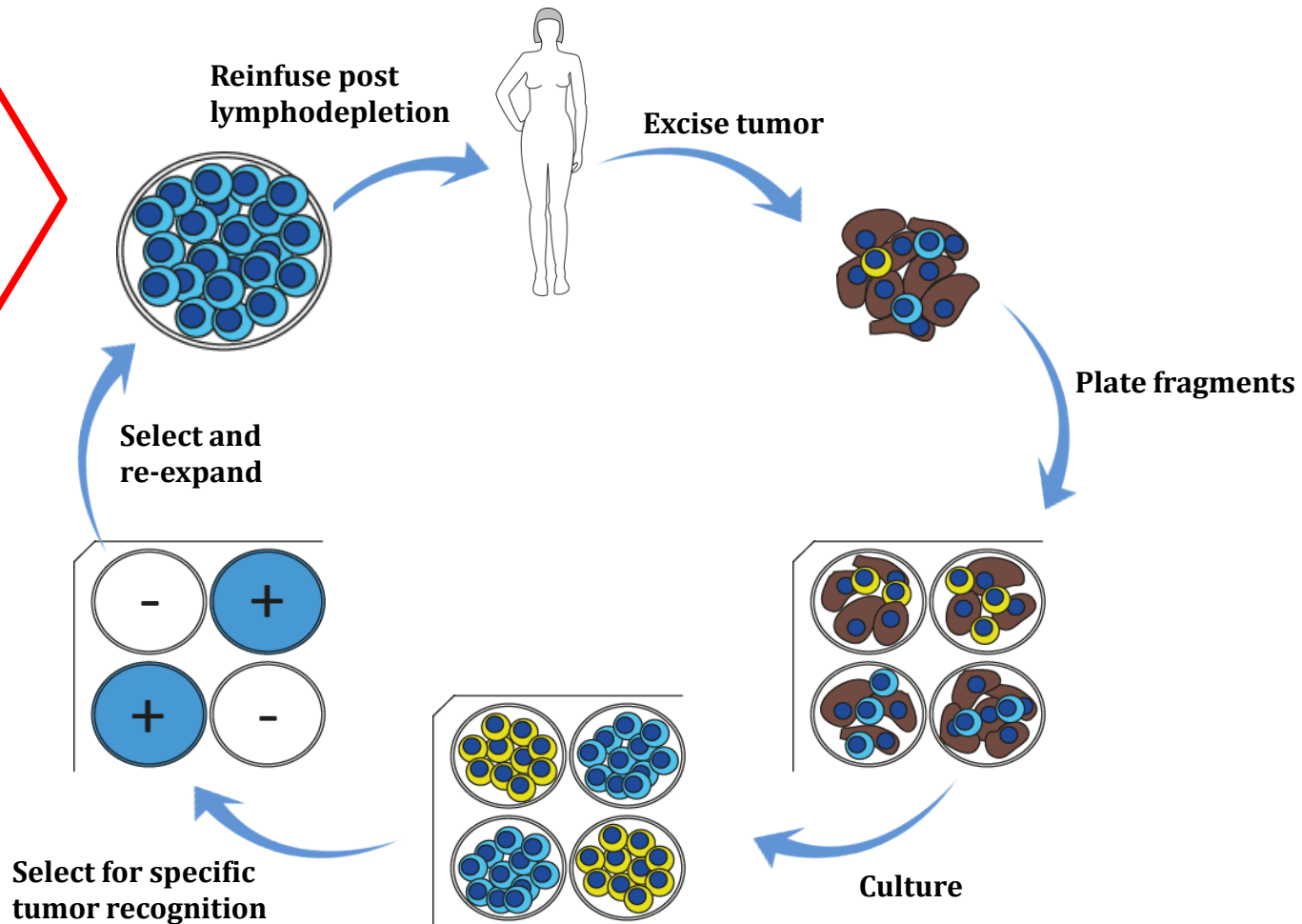


**“Validation of multiplex immunofluorescence panels using multispectral microscopy for immune-profiling of formalin-fixed and paraffin-embedded human tumor tissues”**  
Parra ER et al. 2017 Scientific Reports

# Clinical utility

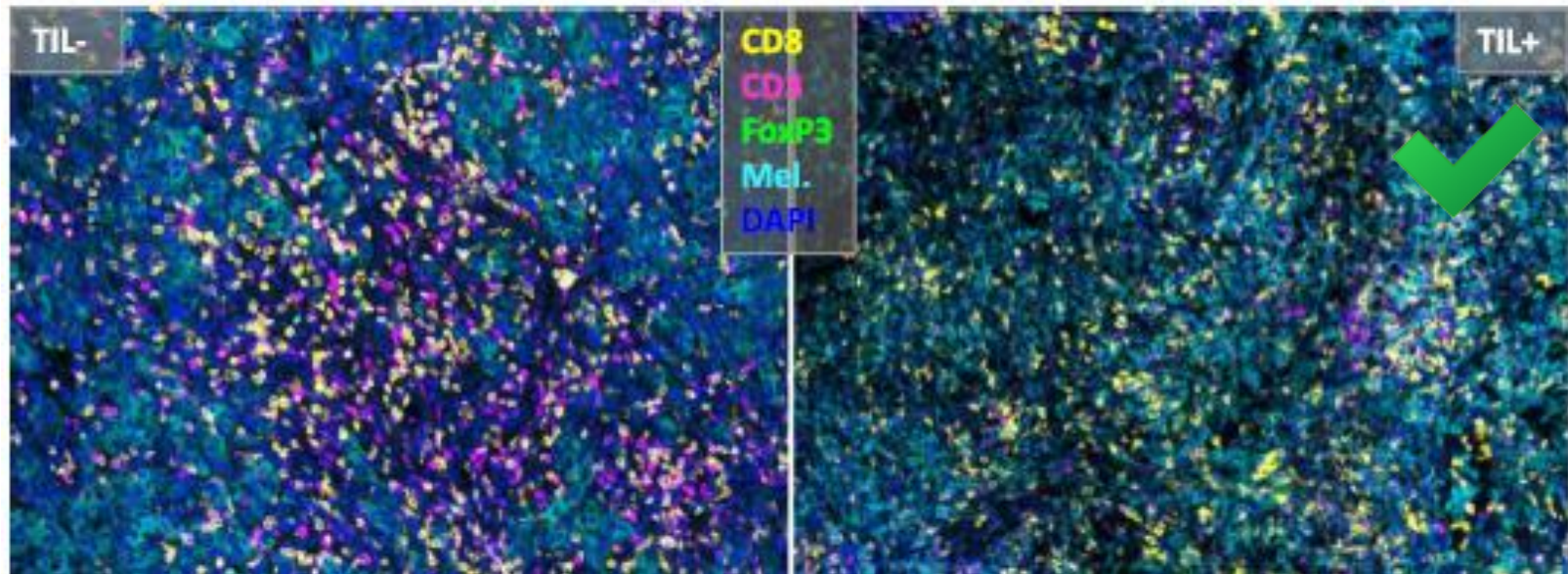
## Adaptive T cell therapy in melanoma

25-45%  
Inability to  
produce or  
expand active  
TIL against  
the tumor



# Clinical utility

## Adaptive T cell therapy in melanoma



**“Multispectral imaging of formalin-fixed tissue predicts ability to generate tumorinfiltrating lymphocytes from melanoma” Feng Z et al. 2015 Journal of Immunotherapy of Cancer**

# Clinical viability

## Opal Cancer Immunology IHC Panels

Opal 7 Tumor Infiltrating Lymphocyte Kit* (CD4, CD8, CD20, FOXP3, CD45RO, panCK)	
Opal 7 Solid Tumor Immunology Kit* (CD4, CD8, CD20, FOXP3, CD68, panCK)	
Opal 7 Immunology Discovery Kit* (CD4, CD8, CD68, +3 open channels)	
Opal 4 Lymphocyte Kit (CD4, CD8, CD20)	

**RESEARCH  
USE ONLY**





# mIHC vs FACS and IHC standard

## FACS:

Immunophenotyping  
Quantification

**! Tissue context is lost**

## Immunohistochemistry:

Tissue context

**! 2 markers**  
**! No immunophenotyping**

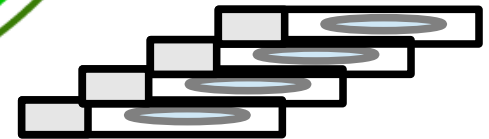
## Multiplex IHC

Immunophenotyping  
Quantification

Tissue context

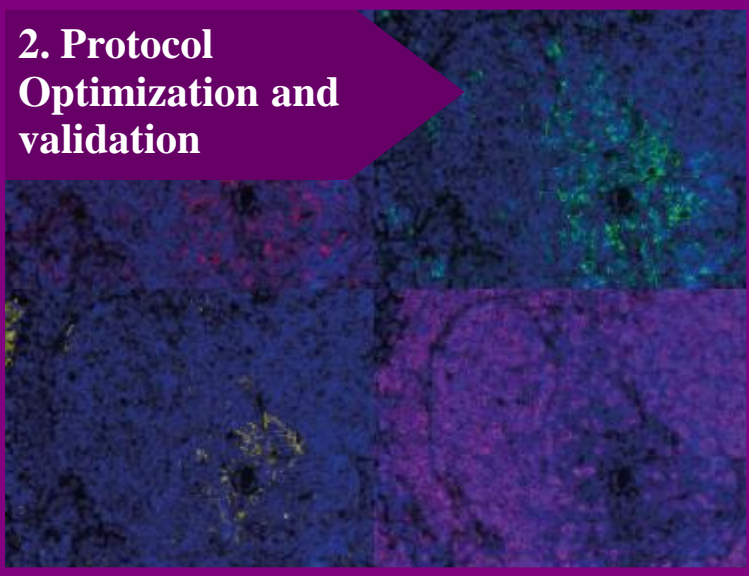


# Considerations



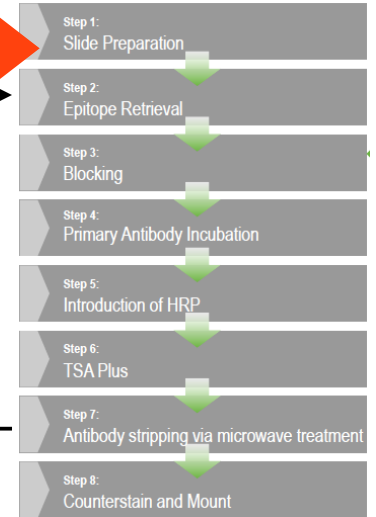
# Considerations

## 2. Protocol Optimization and validation



## 3. mIHC protocol development

CD4  
(OPAL52)  
CD8  
(OPAL57)  
PD-L1  
(OPAL54)  
pan Cks  
(OPAL650)  
CD68  
(OPAL650)



Which/how many markers?

Validated antibodies/protocols?

Multiplex protocol

Validated protocols?

# Pre analytical phase

## 1. Tissue processing

Pre - analytical  
phase

Analytical  
and  
Post analytical  
phases

# Post analytical phase

## 4. Image Analysis



# Post analytical considerations

17:48:33 PM

PerkinElmer

Prepare Images → Segment Tissue → Segment Cells → Score IHC or IF → Export

Tissue Category	Cell ID	Total Cells	Tissue Category Area (pixels)	Cell Density (per megapixel)	Cell X Position	Cell Y Position	Nucleus Area (pixels)	Nucleus Area (percent)	Nucleus Compactness	Nucleus Minor Axis	Nucleus Major Axis	Nucleus Axis Ratio	Nucleus Opal 520 Mean (Normalized Counts, Total Weighting)	Nucleus Opal 520 Max (Normalized Counts, Total Weighting)	Nucleus Opal 520 Std Dev (Normalized Counts, Total Weighting)	Nucleus Opal 520 Total (Normalized Counts, Total Weighting)	Nucleus DAPI Mean (Normalized Counts, Total Weighting)	Nucleus DAPI Max (Normalized Counts, Total Weighting)	Nucleus DAPI Std Dev (Normalized Counts, Total Weighting)	Nucleus DAPI Total (Normalized Counts, Total Weighting)
epitelo	all	457	260608	1754			57638	22.12 %					0.159	3.289	0.288	9149.851	9.891	27.685	2.382	570087.353
All	all	1847	14476...	1275.8			229449	88.04 %					0.338	17.731	0.495	77667.714	10.608	30.813	2.496	2434039.190
epitelo	1				886	9	121	0.05 %	0.72	11.01	14.01	1.27	0.000	0.000	0.000	0.000	10.713	16.420	2.348	1296.233
epitelo	2				866	9	163	0.06 %	0.70	14.42	14.43	1.00	0.002	0.191	0.016	0.275	8.482	14.782	2.055	1382.549
epitelo	3				925	9	98	0.04 %	0.42	11.00	16.00	1.45	0.014	0.306	0.055	1.343	6.831	9.973	1.201	669.438
epitelo	4				855	11	83	0.03 %	0.62	10.23	11.68	1.14	0.000	0.000	0.000	0.000	5.976	8.924	0.930	495.968
epitelo	5				1074	12	138	0.05 %	0.59	14.14	14.81	1.05	0.010	0.236	0.035	1.411	14.632	19.538	2.446	2019.227
epitelo	6				1113	14	99	0.04 %	0.74	11.08	11.98	1.08	0.002	0.108	0.014	0.238	11.314	17.848	2.534	1120.048
epitelo	7				795	14	121	0.05 %	0.72	12.01	13.01	1.08	0.001	0.095	0.011	0.177	8.793	14.636	1.986	1063.910
epitelo	8				1174	14	97	0.04 %	0.58	11.61	11.61	1.00	0.000	0.000	0.000	0.000	12.458	20.119	2.835	1208.464
epitelo	9				960	17	105	0.04 %	0.71	9.03	14.06	1.56	0.003	0.175	0.018	0.263	11.217	14.745	1.798	1177.743
epitelo	10				845	20	162	0.06 %	0.75	13.06	15.02	1.15	0.000	0.000	0.000	0.000	11.275	16.084	2.344	1826.597
epitelo	12				807	20	113	0.04 %	0.67	10.01	15.01	1.50	0.000	0.000	0.000	0.000	11.978	20.236	3.077	1353.483
epitelo	13				1166	20	133	0.05 %	0.67	12.00	15.01	1.25	0.003	0.159	0.020	0.438	13.643	20.928	3.273	1814.492
epitelo	14				1145	23	151	0.06 %	0.55	11.17	18.78	1.68	0.012	0.600	0.069	1.766	12.076	19.547	3.107	1823.477
epitelo	15				814	25	93	0.04 %	0.60	9.54	12.69	1.33	0.000	0.000	0.000	0.000	7.335	9.751	1.622	682.195
epitelo	16				830	25	166	0.06 %	0.62	14.47	15.15	1.05	0.001	0.049	0.005	0.092	7.772	13.162	1.717	1290.185
epitelo	18				1075	25	91	0.03 %	0.52	8.16	17.26	2.12	0.027	0.313	0.055	2.491	14.877	22.229	3.445	1353.841
epitelo	19				1101	30	75	0.03 %	0.82	9.01	10.01	1.11	0.009	0.213	0.030	0.643	9.846	14.850	1.985	738.487
epitelo	21				946	31	99	0.04 %	0.71	10.00	13.05	1.31	0.004	0.098	0.017	0.378	9.522	13.770	2.203	942.658
epitelo	23				824	30	110	0.04 %	0.53	10.50	16.37	1.56	0.000	0.023	0.002	0.223	7.724	15.150	2.513	849.598
epitelo	24				1171	34	92	0.04 %	0.72	8.02	14.02	1.75	0.005	0.130	0.019	0.459	10.657	14.099	2.038	980.479
epitelo	26				882	37	73	0.03 %	0.52	9.99	12.04	1.20	0.093	0.885	0.161	6.777	10.863	15.361	2.727	792.963
epitelo	28				811	38	106	0.04 %	0.58	10.16	14.32	1.41	0.000	0.000	0.000	0.000	14.174	20.312	2.818	1502.427
epitelo	31				1051	42	83	0.03 %	0.47	8.02	16.01	2.00	0.054	0.566	0.089	4.503	9.995	15.562	2.049	754.877
epitelo	32				841	42	118	0.05 %	0.70	10.02	15.01	1.50	0.000	0.000	0.000	0.000	7.672	12.373	1.472	905.305
epitelo	33				969	41	97	0.04 %	0.63	7.89	16.95	2.15	0.004	0.131	0.019	0.347	8.048	14.140	2.080	780.627
epitelo	34				866	39	183	0.07 %	0.32	12.33	23.62	1.92	0.101	3.278	0.395	18.407	8.835	19.193	3.238	1616.746

View Editor

Data Displayed: Cell Segmentation Data

Table Contents

Components:

- All Components
- Opal 520
- DAPI
- Opal 690
- Opal 540
- Autofluorescence

Tissue Categories:

- All Categories
- epitelo
- stroma
- altro

Component Stats:

- Min
- Mean
- Max
- Std Dev
- Total

Position Stats:

- X Position
- Y Position
- Process Region ID
- Distance from Process Region Edge (pixels)
- Category Region ID
- Distance from Tissue Category Edge (pixels)

TMA Core Info:

- Show Core ID

Show Slide Info

Shape Stats:

- Area (pixels)
- Area (percent)
- Compactness
- Minor Axis
- Major Axis
- Axis Ratio

# Acknowledgements

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