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Department of Translational Molecular Pathology (TMP)
Translational Molecular Pathology Immunoprofiling Laboratory (TMP-IL) Moonshot's Platform
Division of Pathology and Lab Medicine
The University of Texas MD Anderson Cancer Center

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Multiplex Immunofluorescence (mIF) Analytical Validation

Version 1.0

1. ***Analyte(s):***
   Panel Vectra 9C (**CD34, CD3, CD8, CD45RO, FOXP3, PD1, PD-L1, CD68**)

2. ***Technical Platform(s):***
   This procedure describes an automated system for staining multiplex immunofluorescence paraffin sections using the Bond RX by Leica Biosystems and its Research Detection System 2. The Bond RX instrument enables small volumes of reagent (as little as 150 µl per slide) to be uniformly applied over the tissue sections on a slide and has continuous batch processing, allowing for independent start and finish times for each batch of 10 slides.

3. ***Summary of Reagents, Controls, and Calibrators (see for details staining SOP):***
   Optimized reagents:
   - Bond Research Detection System 2 (Leica Biosystems, DS9777) (enhance staining quality)
   - Detection Buffer (1X TBS)
   - OPAL 7-COLOR AUTOMATION IHC KIT (AKOYA BIOSCIENCES, NEL821001KT):
     - OPAL PKI Blocking Buffer
     - Opal Polymer HRP Mouse + Rabbit
     - 1X Amplification Diluent
     - Opal 520 Reagent
     - Opal 540 Reagent
     - Opal 570 Reagent
     - Opal 620 Reagent
     - Opal 650 Reagent
     - Opal 690 Reagent
     - Spectral DAPI solution 1X
   - OPAL POLARIS 780 REAGENT PACK (AKOYA BIOSCIENCES, FP1501001KT):
     - Opal Polaris 780 Reagent (780R)
     - Opal TSA-DIG 780 (780D)
   - OPAL POLARIS 480 REAGENT PACK (AKOYA BIOSCIENCES, FP1500001KT):
     - Opal Polaris 480 Reagent
   - Bond Dewax Solution (Leica Biosystems, AR9222)
   - 100% alcohol
   - Bond Wash solution (Leica Biosystems, AR9590)
   - Bond Epitope retrieval solution ER1 Low pH (Leica Biosystems, AR9961) or ER2 High pH (Leica Biosystems, AR9640)

- TBS Buffer (Santa Cruz Biotechnology Inc., SC-362186)

Panel 9C Antibodies

Antibody	PD1	CD68	FOXP3	CD45RO	CD34	CD8	PD-L1	CD3 ε
Clone	EPR4877(2)	PG-M1	D2W8E	UCHL1	EP373Y	C8/144B	E1L3N	D7A6E
Vendor	Abcam	Dako	Cell Signaling Technology	Leica Biosystems	Abcam	Thermo Scientific	Cell Signaling Technology	Cell Signaling Technology
Catalog #	AB137132	M087601-2	98377S	PA0146	AB81289	MS-457s	13684S	85061
(+) Control Tissue	Tonsil							
(-) Control Tissue	Tonsil (only Primary antibody without Opal Polymer-HRP Ms + Rb and TSA-Dye, DAPI) Tonsil (only Opal Polymer-HRP Ms + Rb without Primary antibody and TSA-Dye, DAPI) Tonsil (only TSA-Dye without Primary antibody and Opal Polymer-HRP Ms + Rb, DAPI)							
Retrieval Method	High target	Low target	Low target	Low target	High target	Low target	Low Target	Low Target
Dilution	1:250	1:50	1:50	PURE	1:100	1:25	1:1500	1:100
TSA-Dye	620(1:100)	540(1:100)	650(1:200)	570(1:100)	480 1:100	520(1:100)	690(1:100)	780 R (1:25) 780 D(1:100)
Detection Kit	Opal Polymer HRP Ms + Rb							

4. ***Quality control parameters for specimens:***
   Quality Control (QC) in histopathology involves external and internal components for improving the standards of technical work and reporting. All tissue specimens collected for this analytical validation came from the Tissue Bank at MD Anderson. Hematoxylin and eosin (H&E)-stained sections from the bone marrow clots were scanned in the Aperio™ digital pathology scanner system and were revised by a specialist Hematopathology (Dr. Bueso Ramos) to confirm the diagnosis of the specimens supported by clinical information. Three consecutive sections, four micros thickness, were obtained from each clot paraffin block to run the samples at three different time points.

5. Analytical Performance characteristics:

Multiple Immunofluorescence (mIF) Panel 9C		
(i)	Parameters	CD34, CD3, CD8, CD45RO, FOXP3, PD1, PD-L1, CD68 number of cells per 5 areas (931µm x 698µm 0.65mm^2 each) using VectraPolaris Inform v.3.0 software on tonsil controls.
(ii)	Accuracy	20/20 (100%) is the ratio of tonsil control tissues expressing positive marker.
(iii)	Precision	Inter-pathologist scoring concordance using Inform 2.6.0 image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in different compartments on tonsil control tissues
(iv)	Analytical sensitivity	Human Formalin-Fixed and Paraffin-Embedded (FFPE) marker expression on tonsil: Positive in 10/10 attempts
(v)	Reportable range of assay results for assay system	By Inform 2.6.0 software range observed in tonsils tissue is 0-5,000 for each cell marker.
(vi)	Establishment of appropriate quality control & improvement procedures	Lot variation analysis of antibodies and Opal kits; calibration of equipment; 10 run of tonsil control tissue in 10 days inter-technologist (100% concordance); external validation is not available

6. Analytical Data and Analyses

The entire bone marrow clot from each sample was selected using regions of interest (ROIs, Figure 1), and quantitative analysis of each marker was performed using image analysis software. The individual cells (defined by nuclei [DAPI] staining) identified by the cell segmentation tool are subjected to the phenotyping pattern recognition learning algorithm tool to characterize the marker expression as well as co-localization of the various other markers by the image analysis software (InForm, Akoya Biosciences). Positive and negative (auto-

florescence) controls were included during the analysis to obtain uniform, specific, and accurate positive signals across all channels with a threshold between 10 to 30 counts of intensity without any auto-fluorescence signal interference. The individual cell marker report created by the InForm software was processed by Phenoptr" R package version 0.2.9 and phenoptrReports version 0.2.10 (Akoya Biosciences, Kent S Johnson, 2022) to create a final data report expressing the results as absolute numbers, percentage of cell marker by total cells (Table below) and number of cells/mm2 from each cell marker.

Spearman's rank correlation test was used to measure the strength of the linear relationship between cell markers that were measured in different mIF staining batches. The coefficient of variation (CV) was calculated for each cell marker across weeks to determine if there was a batch effect. The statistical software program R version 4.0.1 (2020-06-06) was used to perform the computations for all analyses.

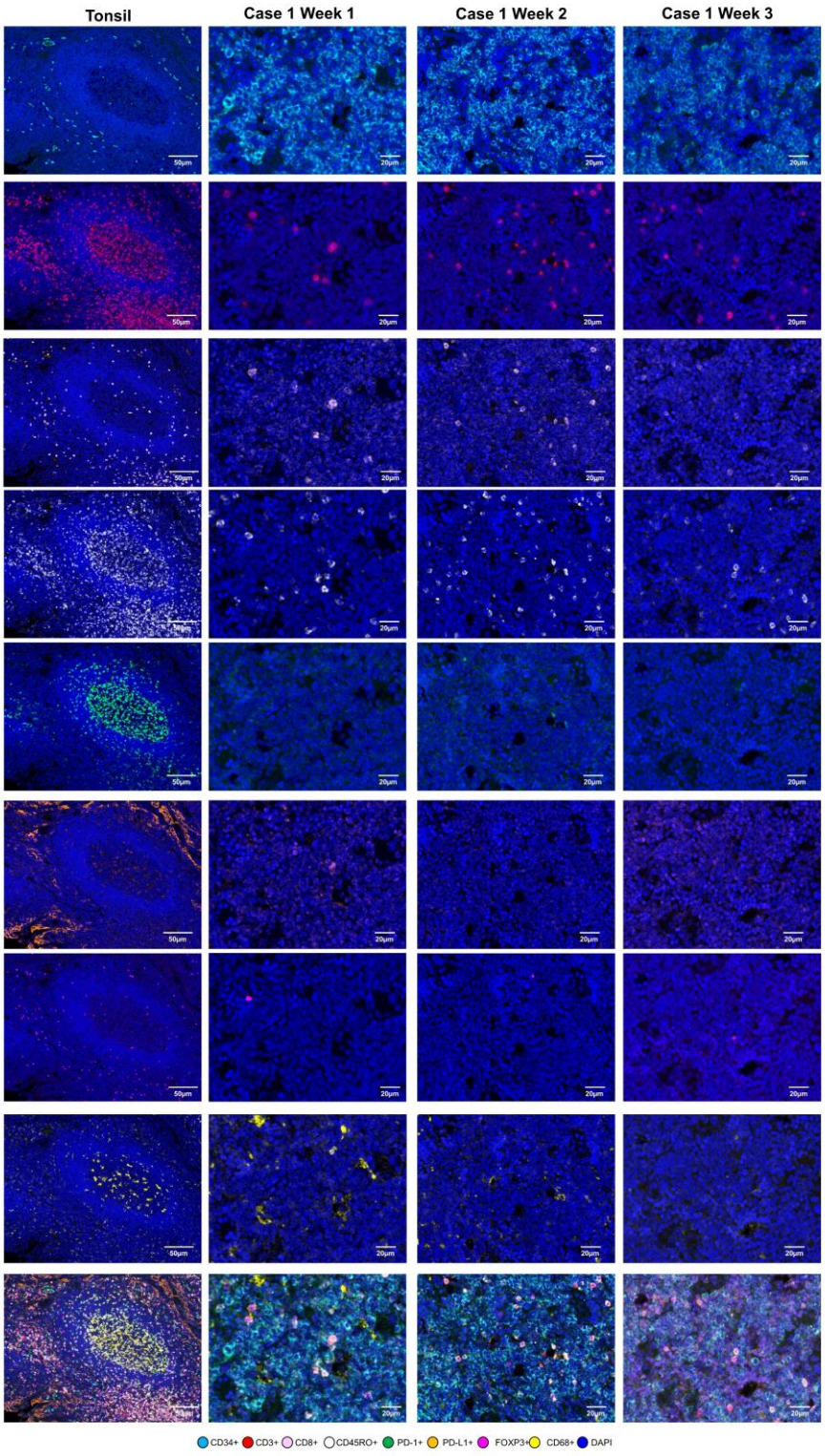
		Multiplex staining Panel 9C (CD34, CD3, CD8, CD45RO, FOXP3, PD-1, PD-L1, CD68)								
Date	**sample**	**Results (Percentage of cells by the total number of cells)**								
		CD34 %	**CD3%**	**CD8%**	**CD45RO% %**	**FOXP3%**	**PD-1%**	**PD-L1%**	**CD68%**	**Total cells**
Week 1	BM-AML-1	0.58	0.01	0.01	0.01	0.00	0.00	0.02	0.01	22067.00
	BM-AML-2	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.02	1080.00
	BM-AML-3	0.01	0.00	0.00	0.04	0.00	0.00	0.02	0.01	1035.00
	BM-AML-4	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	459.00
	BM-AML-5	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	3496.00
	BM-AML-6	0.01	0.02	0.02	0.02	0.00	0.00	0.00	0.00	4542.00
	BM-AML-7	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	227.00
	BM-AML-8	0.00	0.01	0.02	0.03	0.00	0.02	0.01	0.01	3913.00
	BM-AML-9	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.01	5777.00
	BM-AML-10	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.00	833.00
Week 2	BM-AML-1	0.86	0.02	0.01	0.02	0.00	0.00	0.01	0.00	21515.00
	BM-AML-2	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.02	1117.00
	BM-AML-3	0.01	0.00	0.00	0.03	0.00	0.00	0.01	0.01	1053.00
	BM-AML-4	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	289.00
	BM-AML-5	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	3311.00
	BM-AML-6	0.02	0.03	0.02	0.01	0.00	0.00	0.00	0.00	4778.00
	BM-AML-7	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	280.00
	BM-AML-8	0.00	0.00	0.02	0.02	0.00	0.02	0.00	0.01	4328.00
	BM-AML-9	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.01	5685.00
	BM-AML-10	0.00	0.01	0.01	0.02	0.00	0.00	0.00	0.00	940.00
Week 3	BM-AML-1	0.49	0.02	0.01	0.01	0.00	0.00	0.00	0.01	21457.00
	BM-AML-2	0.03	0.01	0.00	0.00	0.00	0.00	0.01	0.02	1015.00
	BM-AML-3	0.01	0.00	0.00	0.03	0.01	0.00	0.01	0.01	808.00
	BM-AML-4	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	277.00
	BM-AML-5	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	3051.00
	BM-AML-6	0.00	0.02	0.02	0.02	0.00	0.00	0.00	0.00	4354.00
	BM-AML-7	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	547.00
	BM-AML-8	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.01	4397.00
	BM-AML-9	0.00	0.01	0.01	0.03	0.00	0.00	0.00	0.00	5499.00

Note: Bone marrow **CLOTS**, BM; AML, Acute Myeloid Leukemia. Sample10 from the third week was lose during the staining procedure

RESULTS:

Week 1 Panel 1 Run 1 08/23/2022	Results Multiplex IF
TONSIL (Positive control)	+ (99%)
Cases (Acute Myeloid Leukemia, AML)	+
Week 2 Panel 1 Run 2 08/30/2022	Results
TONSIL (Positive control)	+ (99%)
Cases (Acute Myeloid Leukemia, AML)	+
Week 3 Panel 1 Run 3 09/06/2022	Results
TONSIL (Positive control)	+ (99%)
Cases (Acute Myeloid Leukemia, AML)	+

Figure 1. Representative image of reactive human tonsil (control staining from the first time point) and one representative ROI from sample BM-AML-1 across the different time points. Images generated by the Inform image analysis software.



Analytical reproducibility

8.1 ***Data Summary***

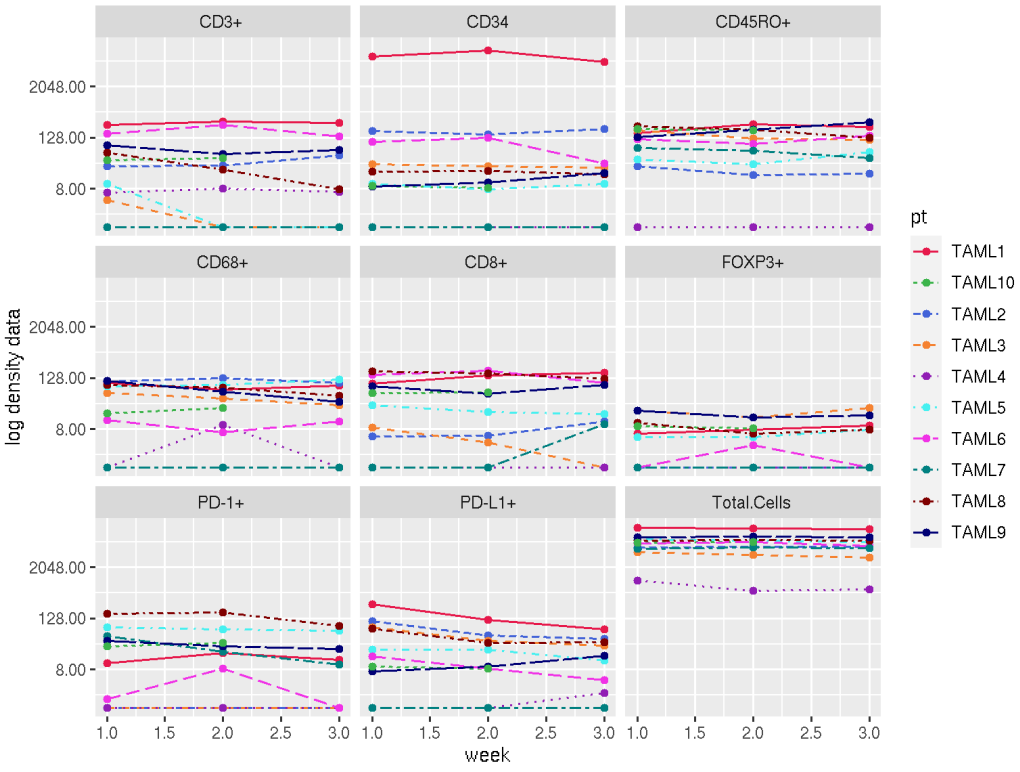
1. Data for new mIF panel (Panel 9C) are obtained from 10 patients (patient number=10)
2. Each patient is measured in 3 weeks
3. The following 8 markers are measured on panel 9C: CD34, CD3, CD8, CD45RO, FOXP3, PD1, PD-L1, CD68
4. Percent data of each marker are calculated by dividing the number of total cells on each panel.
5. Density and percent data are used for further analysis.

8.2 ***Trellis plot by patient***

8.2.1 Log2 transformed density data

For each patient, we generate trellis showing log2 transformed density data in three weeks, as shown below. With trellis plot, it is easy to visualize the consistency between three weeks for each marker in each patient. We observe good consistency between weeks.

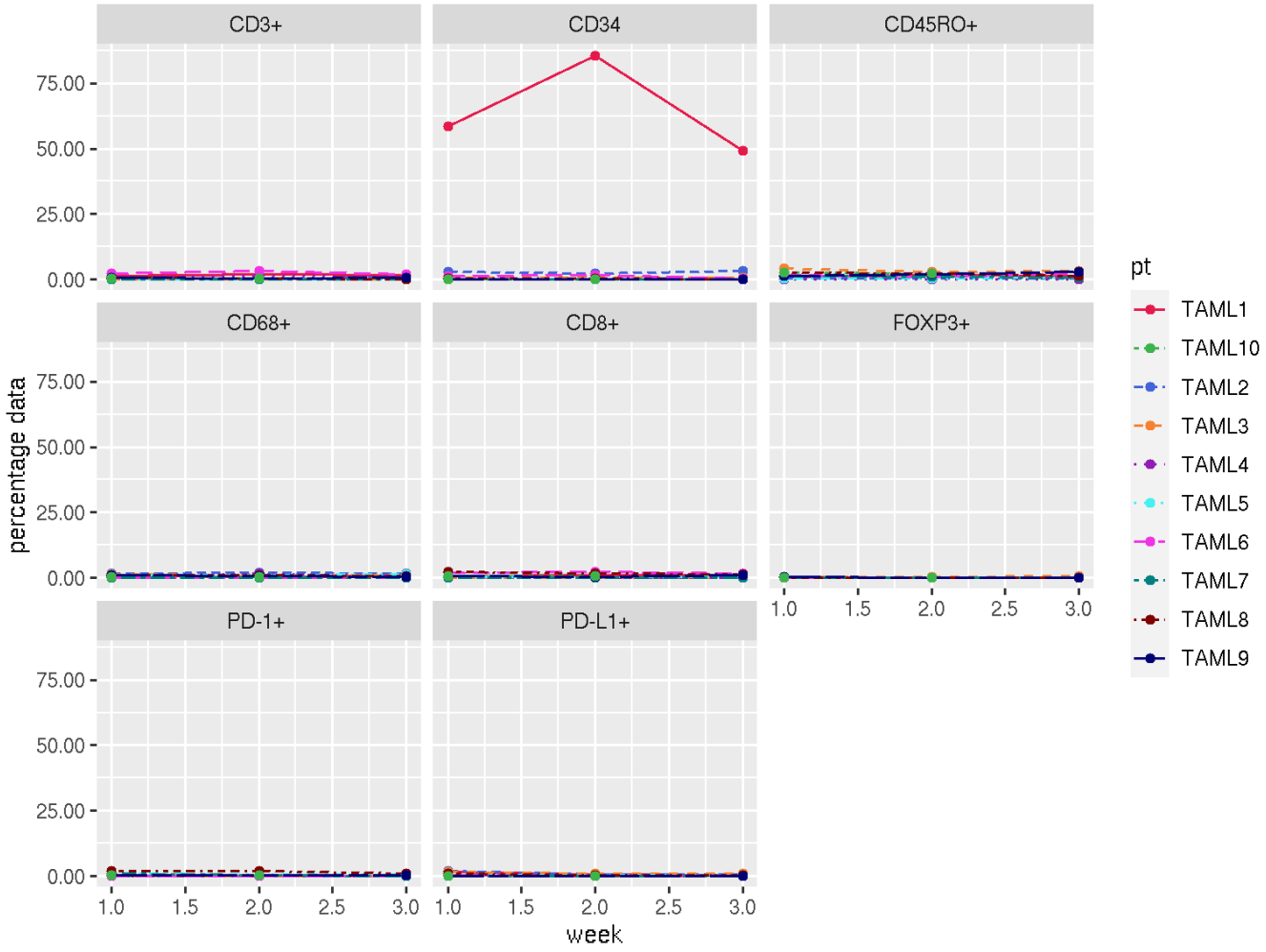
NOTE: In addition, we observe TAML1 (Acute Myeloid Leukemia, AML – case 1) has much higher CD34 than other patients/markers consistent with the image visualization of this particular case and the clinicopathologic report showing that this patient has an increased number of blast cells CD34+. This is an outlier with peculiar clinical conditions. We also observed high variability of the absolute number of cells, densities, and percentages of markers across the samples reflecting the variability of expression of these markers across this type of tumor.



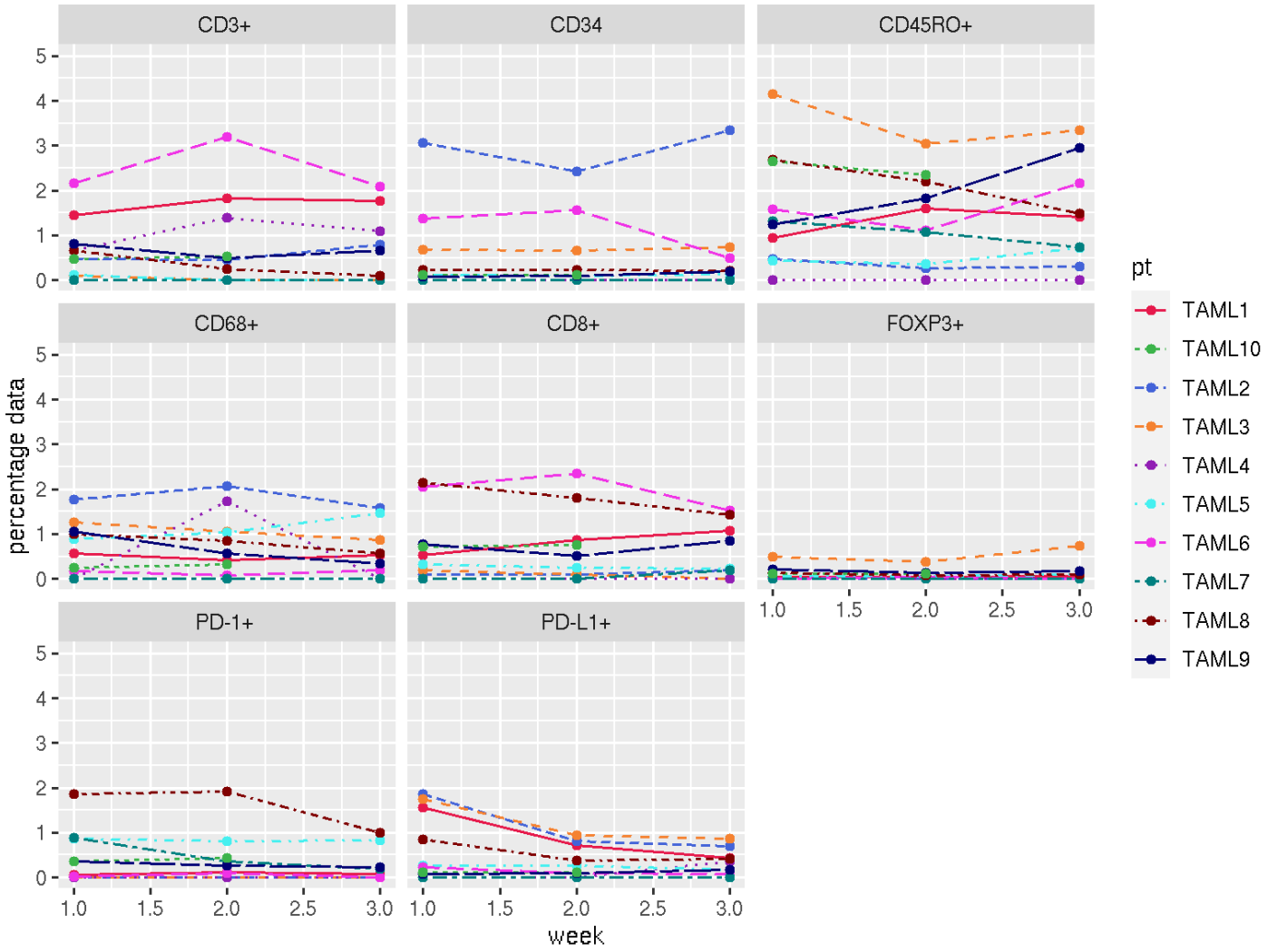
8.2.2 Percent data

Similarly, the trellis plot for percent data is shown below after normalizing the data according to the percentage of cell markers by the total number of cells detected.

NOTE: To avoid dominating effect from TAML1 CD34 data, we will exclude the outlier TAML1 CD34 from further analysis.



Trellis plot for percent data without TAML1 CD34.

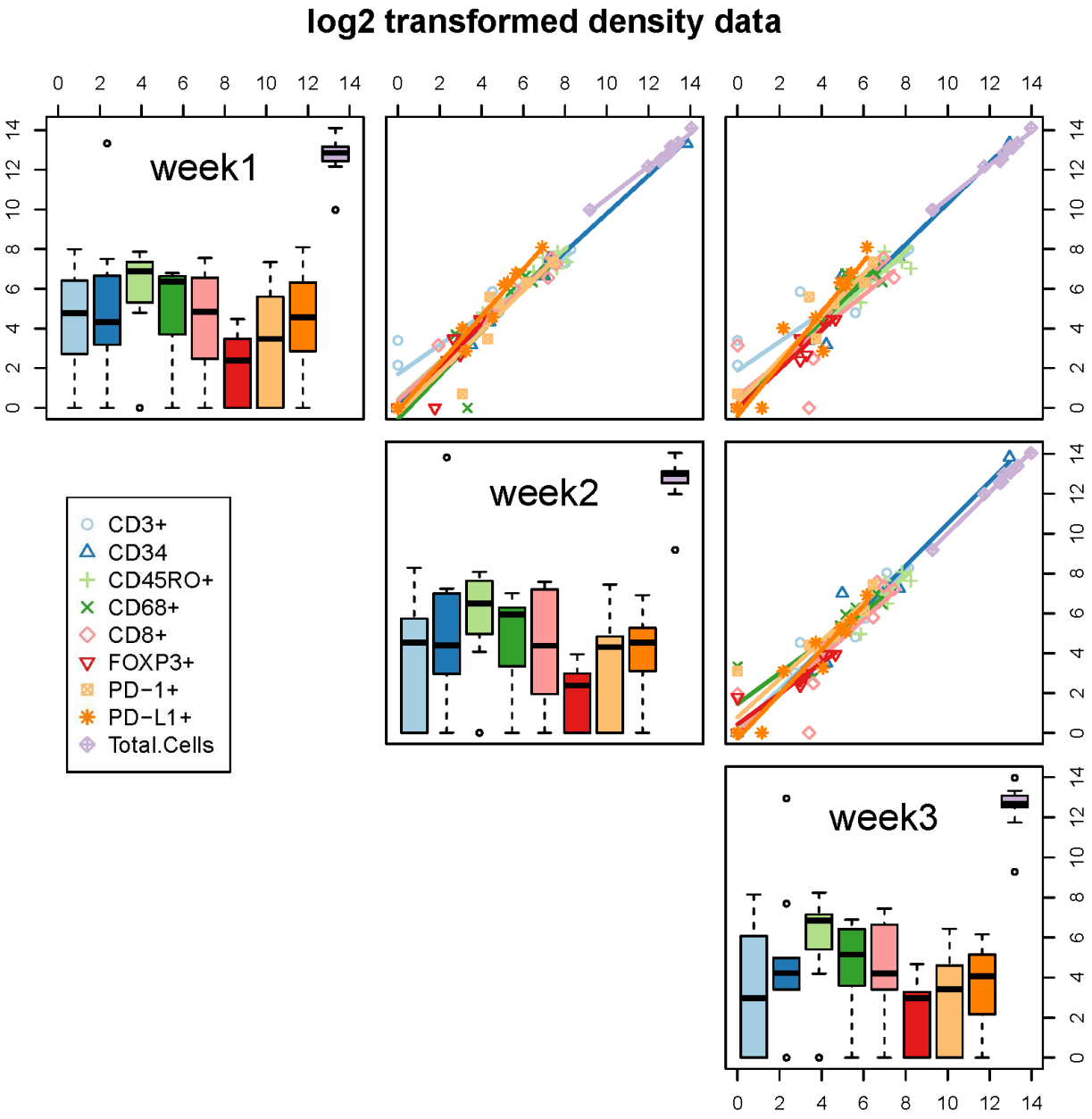


8.2.3 Correlation within each patient

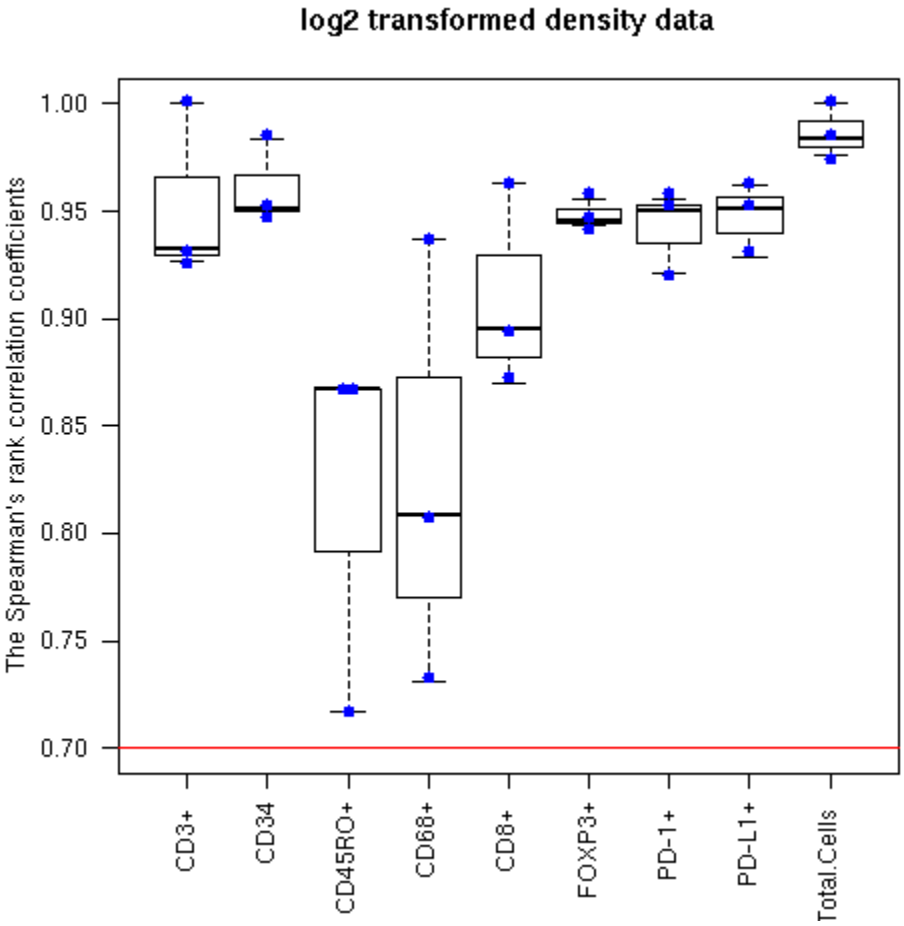
We assessed correlation for each marker across all patients among three weeks.

8.2.3.1 Log2 transformed density data

1. We labeled log2 transformed density data with different colors representing markers, as shown below. Boxplots for each week are shown on a diagonal; scatter plots for each pair of weeks are shown with linear regression lines labeled for each marker. We can see that the measurements across weeks are consistent. Still, there are variations of the markers across the samples, as we expected, related to the characteristics of this tumor type.

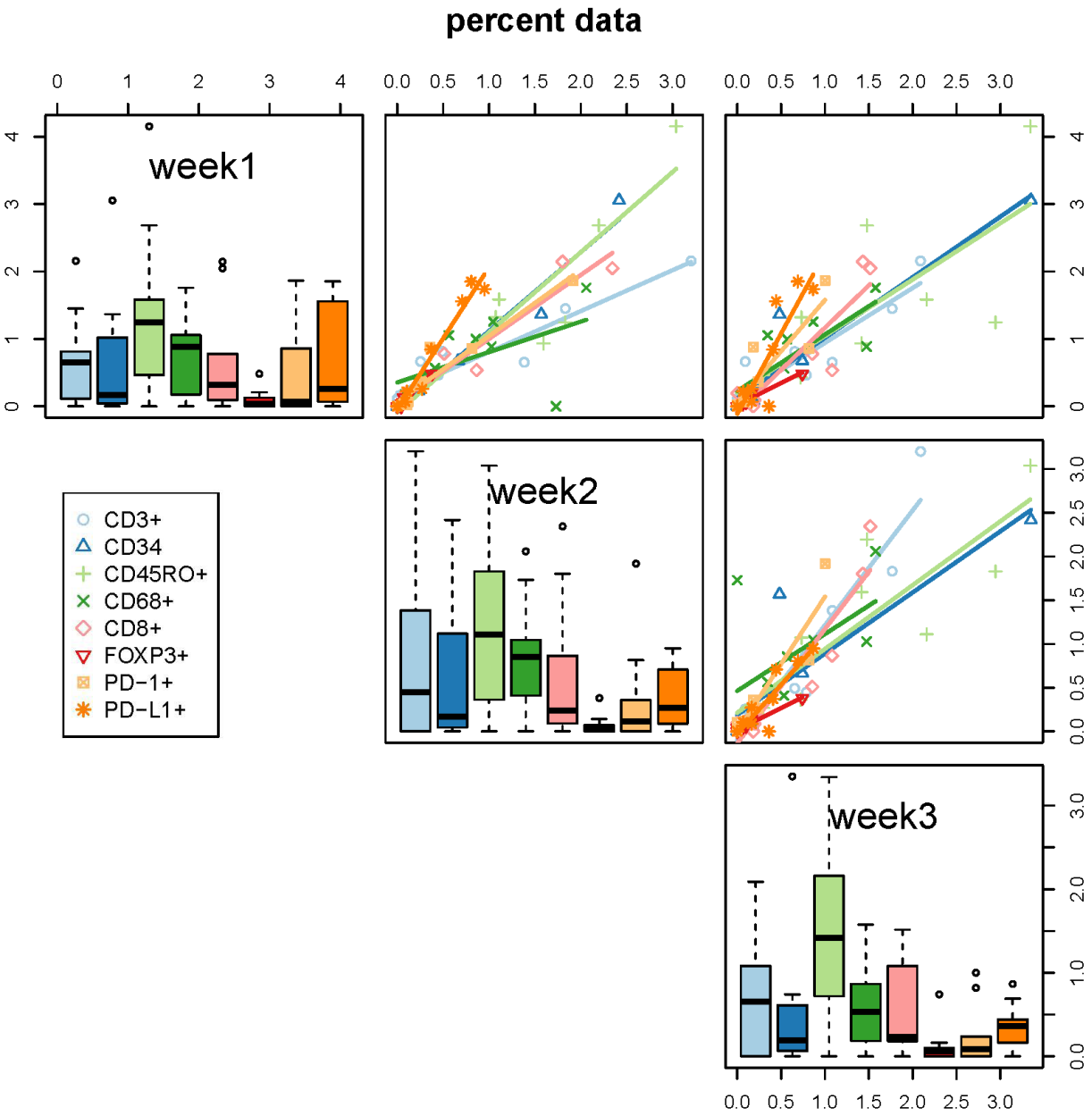


2. We calculated the Spearman's rank correlation coefficients for pair of weeks for each marker. We generated boxplot to show the correlation coefficients for all markers. We observe that all markers have minimum correlation coefficients > 0.7.

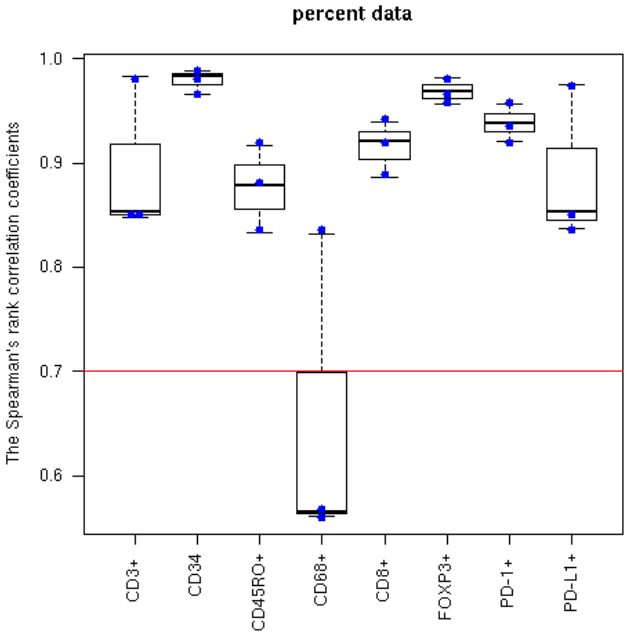


8.2.3.2 Percent data

1. Similar plot is generated for percent data.



2. The following boxplot shows the correlation coefficients for all markers with percent data. All markers except CD68+ have minimum correlation coefficients > 0.7.

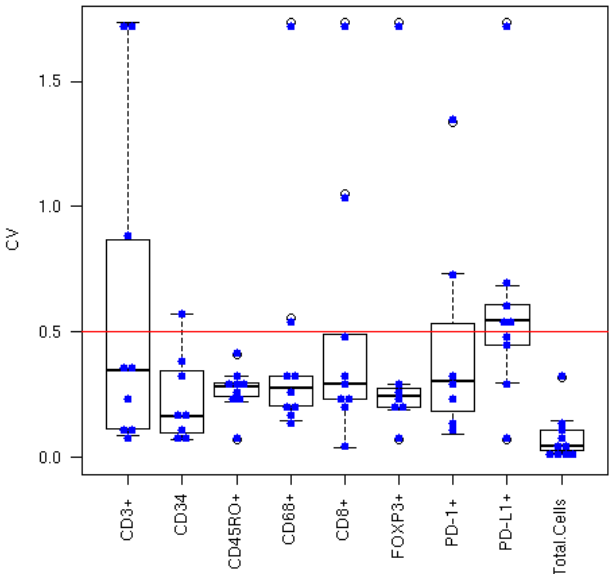


8.4 Coefficient of variation (CV)

We calculated CV within of each patient (3 values from 3 weeks) for each marker.

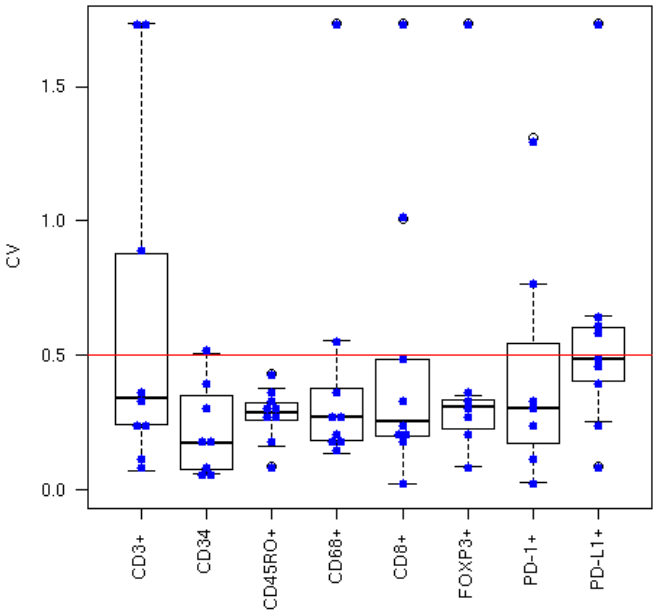
8.4.1 Log2 transformed density data

The following figure shows CVs calculated in for density data. We observe that all markers except PD-L1+ have median CV < 0.5. PD-L1+ has median CV = 0.55



8.4.2 Percent data

The following figure shows CVs calculated in for percent data. All markers have median CV < 0.5.



8.5 Summary

Combining results from CVs and correlation study, we observe that:

1. Most markers have relatively high correlation across patients (median > 0.7 except percent data of CD68+)
2. Most markers have reasonable CV (median < 0.5 except density data of PD-L1+).
3. We observed different variations of marker expression across the samples related to the characteristics of this tumor type.

Multiplex Immunofluorescence and Image Analysis Laboratory:

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_____ Date_03/03/2023_____

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_____ Date__2/28/2023__

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____ _____ Date__2/28/2023_____

Addendum

Critical pre-analytic variables

Although the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) has developed guidelines for handling tissues for IHC staining with some specific markers such as ER, PR and HER2 detection in breast cancer patients, these guidelines are not available for other surgical specimens or biomarkers and still less for mIF assays. In our experience, a good pre-analytic standardization is an important step to handing samples, and it is a modifiable factor to a proper assessment of the different biomarker's studies with mIF. Some recommendations, as tissue fixation quickly as possible after resection (less than 20 minutes as a prudent guideline to follow), recommended overall sample dimensions (1.5x1.5x0.4 cm as maximum for a good fixation), in an adequate volume of fixative (10-20 times the volume of the tissues for immersion fixation) and adequate time (6-18 hours for biopsy specimens and 24-72 hours for standard samples), can be the difference between higher or poorer quality of mIF staining (see MD Anderson SOPs). Another pre-analytical variable to be considered is the storage condition of FFPE blocks or prepared slides. However, limited studies addressing storage conditions of FFPE blocks and prepared slides tissue have been done. In our experience this is a critical point to consider in this assay. It is known that time and temperature are common factors to cause protein tissues degradation and loss of antigenicity in FFPE storage blocks and slides. The oxidation process of the tissue is a key factor involved in the protein tissues degradation and loss of antigenicity of different markers. Due to these facts, paraffin coating blocks before and after their use is a good practice to try to avoid any type of tissue antigenicity degradation. If it is not possible to obtain freshly cut sections for mIF, storage slides using vacuum sealed desiccator, paraffin coating, colder conditions (-4/−18°), as well as complete removal of water (presence of water both endogenously and exogenously plays a central role in loss of antigenicity) from those is highly recommended for their preservation. However, the optimal storage of unstained sections remains poorly defined, making freshly cut sections or sections stored for less than two months as the ideal to use to for mIF.

All the antibodies included in the panel were previously validated using conventional chromogenic immunohistochemistry (IHC) and in some cases using western blot technique. For the tumour associated immune cell expression by IHC, human tonsil FFPE tissues with and without primary antibody were used as positive and negative controls for staining as a first step of the validation (1).

Similarly, each marker was tested and validated for IF in same tissues as IHC (1,2). As performed with the IHC staining, the correct titration in the single IF slides were chosen carefully to obtain a uniform, specific, and correct signal across all channel to produce a well-balanced staining pattern during the multiplex staining. The correct signal from all the fluorophores, defined between 10 to 30 counts of intensity, needs to be maintained through the image to obtained good balance with all the stained markers. Once each target was optimized in single IF slides, multiplexed assay is used to generate multiple staining slides and each signal from the different marker was checked again to capture not only the correct signal expression but a similar threshold of intensity following the already guidelines publish in the literature to avoid "blocking" or an "umbrella effect", (1,2,3,4). To detect possible variations in staining and optimal separation of the signal, positive and negative (auto- florescence) controls are included during each run of staining to make sure, especial with the internal positive control, that all the antibodies are working together (4). Auto -fluorescence controls with an expected spectral of 488 nm will be able to accurate remove the auto -fluorescence from all the label signals during the analysis process (4).

References

1. Parra ER, Jiang M, Solis L, Mino B, Laberiano C, Hernandez S, et al. Procedural Requirements and Recommendations for Multiplex Immunofluorescence Tyramide Signal Amplification Assays to Support Translational Oncology Studies. Cancers (Basel). 2020;12(2).
2. Lazcano R, Rojas F, Laberiano C, Hernandez S, Parra ER. Pathology Quality Control for Multiplex Immunofluorescence and Image Analysis Assessment in Longitudinal Studies. Front Mol Biosci. 2021;8:661222.
3. Parra ER, Zhai J, Tamegnon A, Zhou N, Pandurengan RK, Barreto C, et al. Identification of distinct immune landscapes using an automated nine-color multiplex immunofluorescence staining panel and image analysis in paraffin tumor tissues. Sci Rep. 2021;11(1):4530.

4. Taube JM, Roman K, Engle EL, Wang C, Ballesteros-Merino C, Jensen SM, et al. Multi-institutional TSA-amplified Multiplexed Immunofluorescence Reproducibility Evaluation (MITRE) Study. J Immunother Cancer. 2021;9(7).