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The table below lists the nature of initial results produced (primary output), QC steps and analyses, and eventual output.

Assay type	Primary assay outputs	Pre-processing/Normalization /QC	Initial analyses	Derived outputs data
MICSSS	Up to 10 parameter multiplexed IHC images	Image adjustments, white balance, estimate stain vectors, pathologist QC review	Single cell segmentation and multiplexed population identification	Quantification and spatial distribution of defined immune populations in tissues

MICSSS MULTIPLEX IMMUNOHISTOCHEMISTRY	
(i) accuracy	<p>MICSSS has analogous accuracy compared with standard IHC chromogen procedures because it follows the same steps. Accuracy is highly dependent on each antibody clone used for marker detection, and Ab specificity should be determined based on review of literature as well as use of control tissues with known antigen expression. Based on tonsils and use of common markers CD3, CD8, CD20, CD68, accuracy is expected to be 100%, as shown in section 2F. These positive controls guide interpretation of accuracy in other tissues.</p> <p>Some common reagents such as CD45RO and CD4, while widely used in the literature, are judged not sufficiently reproducible even in single-plex IHC and are therefore not included in the MICSSS protocol.</p>
(ii) precision	<p>In contrast with all other commercial multiplex IHC offerings, MICSSS uses dedicated antigen retrieval prior to each marker to optimize the staining protocol with chromogen to the antibody used in that step. Precision is dependent on the validation of antibodies used, as is the case for any IHC application. Scoring of individual markers is done by two independent investigators or pathologists by evaluating expected type of staining (lymphocyte, myeloid cell, membranous, cytoplasmic, etc..).</p>

(iii) analytical sensitivity	Sensitivity was tested with permutation of staining order described below in section 2E. There was no significant difference in sensitivity compared to single-plex IHC for all lineage markers tested and analyzed regardless of order of staining and iterations. These sensitivity tests have not been as extensively performed for inducible markers such as PD-L1, and we therefore recommend prioritization of PD-L1 in the order of staining to make it as comparable as possible to standard single-plex IHC tests for PD-L1.
(iv) analytical specificity including interfering substances	There was no sterical hindrance observed in MICSSS even with the use of several markers staining the same cell (CD2, CD3, CD8, Ki-67...). Specificity is defined above in accuracy and precision. Artifacts related to tissues (folds, length of time since cut from block, nicotine or melanin deposits forming dark stained areas) may impact analyses and these should be recorded when tissues are analyzed.
(vi) reference intervals (normal values) with controls and calibrators	Values of background staining vs. specific signal staining are optimized based on ranges expected with single-plex IHC, and are determined with titrations of each reagent used as well as other experimental conditions such as incubation time and pH. The goal is to achieve minimal to no staining in tissues expected to be negative, while having the brightest staining for the marker of interest. Calibrators include the use of reference tissues described below (tonsils, lymph nodes, placenta, tumors).
(vii) standardization, harmonization, reproducibility and ruggedness of analytical performance if the assay is to be performed in multiple laboratories	MICSSS protocol was successfully transferred to several external labs studying pancreatic tissues for diabetes markers, inflammatory bowel disease, or NK cell biology. After training a series of at least 6 fellows with the method according to SOP, the method was judged to work similarly regardless of operator. In addition, the use of control and calibrator tissues allows us to quantify reproducibility across time with the same operator, as described below.  Ongoing work for comparisons with other multiplex IHC approaches such as Perkin-Elmer's VECTRA are underway and will be updated as they become available.
(viii) establishment of appropriate quality control and improvement procedures	All steps follow established SOPs. Implementation of reference tissues made from known biological samples with expected positive and negative staining for individual markers including CD8, CD3, PD-L1, etc. These mash-up references are included either as whole tissues or in the form of tissue microarray in each staining procedure, as reference and harmonization tool.

<p>(ix) any other performance characteristics required for assay performance</p>	<p>While the protocol was optimized using manual settings, implementation of automation was successful using a Leica Bond autostainer.</p> <p>The scanner used after each staining step is important to harmonize and uses built-in calibrators.</p> <p>Once image are acquired, analytical tools for cell segmentation, image analysis and facilitated visualization of high-dimensional data can be done with a variety of software. Current implementation includes QuPath for segmentation and AI training of cell detection and annotation, but other tools such as HistoCAT and Halo are currently being assessed.</p> <p>Analysis of coexpression of markers requires registration of images and quantification on an individual cell level, with the eventual goal of creating flow cytometry like datasets for unbiased clustering algorithms developed for CYTOF.</p>
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# 1. Purpose of assay

The strongest rationale for understanding the immune microenvironment at the tumor site comes from recent successes of cancer immunotherapy. Favorable outcomes of immune checkpoint blockade have been in part attributed to factors in the tumor immune microenvironment (TIME)<sup>1</sup>, including density and organization of local lymphocytic or myeloid cell infiltration in different areas of the tumor, and expression of suppressive molecules on cell subsets within the TIME<sup>2</sup>. Several studies have established that high lymphocyte or myeloid infiltration in tumors is prognostic of progression-free or overall survival<sup>2-8</sup>. Pathologists around the world are now validating the use of immune marker assessment, or “Immunoscore”, to complement standard staging in routine clinical cancer settings<sup>9</sup>. These efforts are bound to eventually expand and include comprehensive assessment of the nature and distribution of a multitude of immune markers in cancer tissues, to dissect immunoregulatory mechanisms and predict therapeutic outcomes.

To address the clinical need for high dimensional analysis of tissues for *in situ* characterization of immune infiltrates, the Gnjatic and Merad labs developed MICSSS (for multiplex immunohistochemical chromogenic staining on a single slide), a multiplexed chromogen-based IHC staining assay independent of proprietary reagents or equipment that could be readily integrated in routine clinical pathology<sup>10</sup>. This new technique is performed on formalin-fixed paraffin embedded (FFPE) tissue using iterative cycles of staining, revelation, scanning and bleaching of labile chromogenic substrate, for up to 10 markers from a single tissue slide (see Figure 1). MICSSS can be applied to various tumor tissues and can easily be implemented to most existing IHC protocols with primary Ab staining, secondary Ab detection and antigen retrieval conditions, thus retaining previously established specificity and sensitivity parameters. In contrast to popular fluorescence-based multiplex instruments such as Perkin-Elmer’s VECTRA, MICSSS uses sensitive chromogen (no autofluorescence) and generates full-slide images of each marker. MICSSS can therefore characterize many parameters including colocalization of multiple markers on single cells while preserving tissue antigenicity and architecture. Importantly, it is much less expensive than other current multiplex IHC commercial solutions and offers flexibility in choice of marker panels for staining.

We have implemented this sample-sparing method on biopsies or surgical resections to assess the TIME, namely with a core panel of markers consisting of immune cells (CD3 and CD8 T cells, FOXP3 Tregs, CD19 B cells, CD68 macrophages, HLA-DR, DC-LAMP mature DCs), as well as immune checkpoint expression (PD-1, PD-L1). Given controversies for PD-L1 staining, we validated PD-L1 clone E1L3N from Cell Signaling Technologies as an appropriate consensus that matches most closely FDA-approved reagents<sup>11,12</sup>. The goal is to assess not only immunocompetence of tissues at baseline, but also potential changes by treatment whenever possible, in order to obtain a comprehensive phenotypic characterization, organization and functionality assessment of samples in relation to clinical parameters and treatment outcome<sup>13-15</sup>.

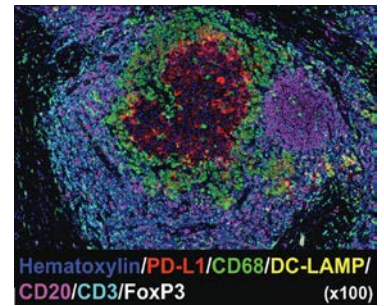


Fig. 1. MICSSS analysis of a single melanoma section slide post ipilimumab treatment, in pseudocolors, showing the complexity of the tumor immune microenvironment.

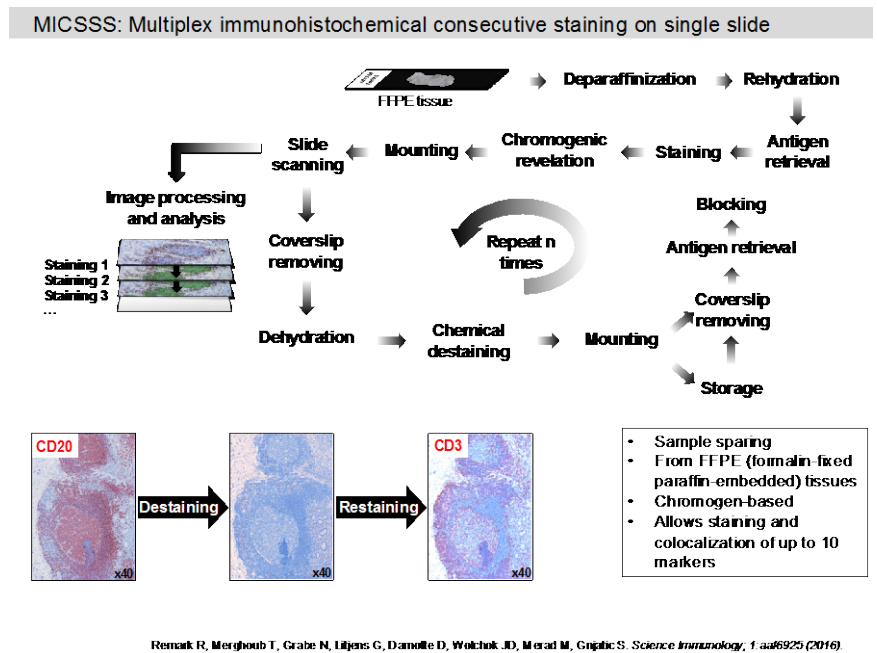


Figure 2. MICSSS protocol schema

Overview of protocol is shown in Figure 2. Analytical validation is based on our publication in *Science Immunology*<sup>10</sup>.

## 2. Assays performance characteristics based on Mt Sinai CIMAC's internal validation

### 2A. General materials and methods for assay characterization and validation.

One of the main advantages of using MICSSS over other multiplex immunohistochemistry methods is the ability to use a variety of primary antibodies without the need for a pre-defined panel based on compatible markers and reagents. Indeed, because of the iterative nature of MICSSS and the use of tailored antigen retrieval and staining conditions for each marker, any of the antibodies in the table below (Table 1) could potentially be included in a mixed-and-match manner and used consecutively until tissue degradation may start occurring (after 10 cycles). Still, despite the long list of reagents already tested below, an inherent limitation of IHC is the availability of appropriate antibody reagents and we are actively pursuing efforts to validate new useful reagents.

Primary antibody validation was performed using reference normal tissues, including tonsils, placenta, testis, as well as a variety of deidentified tumor tissues. These were obtained from the Mount Sinai biorepository and will be described further in sections below. As a general rule, antibodies are first tested as recommended by manufacturer, but are then additionally validated for activity at different pH, concentration, and incubation time. These tests and titrations are done to optimize maximal detection of positive signal while retaining negative or low signal in negative populations for each marker. The table below lists the optimal conditions determined for each Ab clone tested.

**Table 1** is divided into two tiers, with antibodies in the first part (Tier 1/1pre) representing those that have been most extensively used and therefore validated in our hands. They correspond to markers most frequently used for characterizing immune infiltration in tissues, and cover a majority of cell subsets. Tier 2 are antibodies that appear to have good specificity and sensitivity in more limited tests, and that are typically used for specialized questions of myeloid cell biology or expression of tumor antigens. The majority of markers for MICSSS correspond to lineage markers, constitutively expressed in defined subsets of lymphocytes or tumor cells. However, some markers (PD-1, PD-L1, Foxp3, CD31) with weak or inducible expression, generally resulting in lower signal, should be prioritized in the staining order, as iterative staining steps could influence interpretation due to signal intensity as described below.

Tier	Marker	Cat#	Company	Clone	Species	Isotype	Antigen retrieval	Time for AR	Dilution	Incubation
1	CD3	790-4341	Ventana	2GV6	Rabbit	IgG	pH9	30 min	RTU	2h @RT
1	CD8	M7103	Dako	C8/144b	Mouse	IgG1	pH9	30 min	1/100	1h @RT
1	CD20cy	M0755	Dako	L26	Mouse	IgG2a	pH6	30 min	1/250	1h @RT
1	CD68	M0814	Dako	KP1	Mouse	IgG1	pH6	30 min	1/100	1h @RT
1	CD66b	555723	BD Pharmingen	G10F5	Mouse	IgM	pH9	30 min	1/600	1h @RT
1	Ki-67	790-4286	Ventana	30-9	Rabbit	IgG	pH9	30 min	RTU	1h @RT
1	HLA-DR	ab20181	Abcam	TAL1B5	Mouse	IgG1	pH6	30 min	1/500	1h @RT
1	DC-LAMP	DDX0191P	Novus biologicals	1010E1.01	Rat	IgG2a	pH9	30 min	1/80	1h @RT
1	CD138	M7228	Dako	MI15	Mouse	IgG1	pH6	30 min	1/100	1h @RT
1	HLA-ABC (class I)	ab70328	Abcam	EMR8-5	Mouse	IgG1	pH6	30 min	1/200	1h @RT
1	Pan-cytokeratins	M3515	Dako	AE1/AE3	Mouse	IgG1	pH6	30 min	1/50	1h @RT
1 Pre	FoxP3	ab20034	Abcam	236A/E7	Mouse	IgG1	pH6	30 min	1/80	2h @RT
1 Pre	PD-1	ab52587	Abcam	NAT105	Mouse	IgG1	pH6	30 min	1/50	1h @RT
1 Pre	PD-L1	13684S	CST (Cell Signaling)	E1L3N	Rabbit	IgG	pH9	30 min	1/100	1h @RT
2	CD1a	M3571	Dako	O10	Mouse	IgG1	pH6	30 min	1/50	1h @RT

2	CD2	M7309	Dako	AB75	Mouse	IgG1	pH9	30 min	1/40	1h @RT
2	T-bet	760-4598	Ventana	MRQ46	Rabbit	IgG1	pH9	30 min	RTU	1h @RT
2	CD103	ab129202	Abcam	EPR4166 (2)	Rabbit	IgG	pH9	30 min	1/500	1h @RT
2	CD1c	ab156708	Abcam	2F4	Mouse	IgG1	pH9	30 min	1/150	1h @RT
2	Langerin	392M	Dako	12D6	Mouse	IgG2b	pH6	30 min	1/50	1h @RT
2	CD206	64693	Abcam	polyclonal	Rabbit	IgG	pH6	30 min	1/500	1h @RT
2	Podoplanin	760-4395	Ventana (Cell Marque)	D2-40	Mouse	IgG1	pH9	30 min	RTU	1h @RT
2	Granzyme B	M7235	Dako	GrB-7	Mouse	IgG2a	pH9	45 min	1/50	1h @RT
2	CD163	NB110-59935	Novus biologicals	10D6	Mouse	IgG1	pH6	30 min	1/50	1h @RT
2	CK19	ab52625	Abcam	EP1580Y	Rabbit	IgG	pH9	30 min	1/400	1h @RT
2	CD21	M0784	Dako	1F8	Mouse	IgG1	(TRS) pH6	30 min	1/25	1h @RT
2	NYESO1	N2038-200ul	Sigma	E978	Mouse	IgG1	pH9	30 min	1/300	2h @RT
2	Mage-A1	sc-20033	Santa Cruz	MA454	Mouse	IgG1	pH9	30 min	1/50	3h @RT
2	P53	M7001	Dako	DO-7	Mouse	IgG2b	pH9	30 min	1/50	1h @RT
2	MELAN-A	M7196	Dako	A103	Mouse	IgG1	pH9	30 min	1/50	1h @RT
2	SURVIVIN	M3624	Dako	12C4	Mouse	IgG2a	pH6	45 min	1/100	1h @RT
2	PNAd	553863 (?)	BD Pharmingen	MECA-39 (79?)	Rat	IgM	pH6	30 min	1/50	1h @RT
2	IgA	A0262	Dako	polyclonal	Rabbit	IgG	pH9	30 min	1/100	1h @RT
2	ERG	M7314	Dako	EP111	Rabbit	IgG	pH9	30 min	1/50	1h @RT
2	CTLA-4	sc-376016	Santa Cruz	F-8	Mouse	IgG1		30 min	1/50	1h @RT
2	Mage-A3/6	collaboration	LICR	M3H67	Mouse	IgG1	pH6	30 min	1/1000	3h @RT
2	VISTA	64953	Cell Signaling Technology	(D1L2G) XP	Rabbit	IgG	pH6	30 min	1/50	O.N
2	TLR7	ADI-CSA-824-E	Enzo lifesciences	polyclonal	Rabbit	IgG	pH6	30 min	1/50	1h @RT
2	TLR8	ALX-804-376-C100	Enzo lifesciences	44C143	Mouse	IgG1	pH6	30 min	1/100	1h @RT

Additional reagents are currently under validation for NK cells and transcription factors, and the table will be updated as we gather information about specificity and experimental conditions.

Secondary and blocking reagents are listed in the **Table 2** below, and used as per manufacturer's recommendation with a titration performed with each new lot to match sensitivity of previous lot and to ensure minimal background reactivity.

**Table 2**

Reagent Name	Vendor	Cat#	Dilution	Comment
Target Retrieval solution, pH 9.0, conc (x10), 500 mL	Agilent	S236784-2	1:10 in water	Tris/EDTA, pH9, epitope retrieval solution needs to be diluted 1:10 in distilled water following the manufacturer's procedures
Dako TRS, Citrate pH 6 conc (x10), 500ml	Agilent	S236984-2	1:10 in water	Citrate Buffer, pH6, epitope retrieval solution needs to be diluted in distilled water following the manufacturer's procedures
Hydrogen Peroxide 3%	(Duane Reade)		ready-to-use	
Prot Block, Serum Free, 110 mL	Agilent	X090930-2	ready-to-use	0.25% casein in PBS, stabilizing protein 0.015 mol/L sodium azide. Ready-to-use following manufacturer's procedures
Dako Antibody Diluent	Agilent	S080983-2	ready-to-use	Tris-HCl buffer containing stabilizing protein 0.015mol/L sodium azide. Ready-to-use following manufacturer's procedures
EnVision+/HRP, polymer anti-Mouse, 110 mL	Agilent	K400111-2	ready-to-use	Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent.
EnVision+/HRP, polymer anti-Rabbit, 110 mL	Agilent	K400311-2	ready-to-use	Peroxidase labelled polymer conjugated to goat anti-rabbit immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent.
ImmPRESS REAGENT KIT anti-RAT (mouse adsorbed)	Vector	MP-7444	ready-to-use	Peroxidase) Polymer Anti-Rat IgG (mouse adsorbed) Reagent (made in goat, ready-to-use)
Normal Mouse Serum	Jackson Immuno Research	015-000-120	1:20 in Tris Buffered Saline (pH 7.4)	Serum was titrated 1:10 and 1:20, and using 1:20 was determined to cause less background. Suggested working dilution was 5%(v/v) solution (1:20 dilution from rehydrated volume)
Normal rabbit serum	Jackson Immuno Research	011-000-120	1:20 in Tris Buffered Saline (pH 7.4)	Serum was titrated 1:10 and 1:20, and using 1:20 was determined to cause less background. Suggested working dilution was 5%(v/v) solution (1:20 dilution from rehydrated volume)
Goat anti-rat whole serum	Jackson Immuno Research	112-001-001	1:20 in Tris Buffered Saline (pH 7.4)	Serum was titrated 1:10 and 1:20, and using 1:20 was determined to cause less background
Affini Pure Fab Fragment Donkey anti-mouse IgG (H+L) 1mg	Jackson Immuno Research	715-007-003	1:50 in Tris Buffered Saline (pH 7.4)	Suggested working conc 20-40 ug/ml. Serum was titrated 1:100 and 1:50, and using 1:50 was determined to cause less background.
Affini Pure Fab Fragment Donkey anti-rabbit IgG (H+L) 1mg	Jackson Immuno Research	711-007-003	1:50 in Tris Buffered Saline (pH 7.4)	Suggested working conc 20-40 ug/ml. Serum was titrated 1:100 and 1:50, and using 1:50 was determined to cause less background.
Affini Pure Fab Fragment Donkey anti-rat IgG (H+L) 1mg	Jackson Immuno Research	712-007-003	1:50 in Tris Buffered Saline (pH 7.4)	Suggested working conc 20-40 ug/ml. Serum was titrated 1:100 and 1:50, and using 1:50 was determined to cause less background.

## 2B. Reagents controls and calibrators

The reagents listed in Table 1 are primarily markers of immune cells, with some also expected to react with tumor cells. Therefore, calibration and controls of antibodies against these markers comprise the following tissues: tonsils or lymph nodes (expected to react with a majority of lymphoid and myeloid markers in an organized fashion, with germinal centers corresponding to CD20 B cells, CD138 plasma cells, CD3 and CD8 T cells, and interstitial CD68 macrophages), placenta (notably for PD-L1 expression as well as some cancer-testis antigens such as NY-ESO-1), and a variety of tumor tissues (for cytokeratins, tumor antigens, but also infiltration by immune cells such as CD66b neutrophils, T cells, B cells, macrophages). The ideal calibrator for new reagents is to use recombinant cell lines that express or not the marker of interest, or cells engineered to knock-down the marker if constitutively expressed. Melanoma cell lines SK-MEL-19 and SK-MEL-37 for example are used as positive controls for cancer-antigen expression as confirmed by RT-PCR expression of transcripts and protein expression by intracellular flow cytometry. These lines are also useful negative controls for HLA reagents,

because of genetic loss of alleles in some of them. These cell lines can grow rapidly and be assembled in gel as cell pellets that are then formalin fixed and paraffin embedded to match conditions used for tissues. We work with the biorepository to ensure a steady supply of these reference biospecimens, so that the same tissues can be used over the course of an entire study as a stable control.

We are implementing a QC system for antibody staining and specificity based on the reference tissues above, which control for every step of staining from primary antibody to secondary to chromogen revelation. Whenever possible, we include these tissues along with each staining steps when a batch of specimens are analyzed, though our ultimate goal (work in progress) is to construct mini-tissue microarrays (TMAs) that represent a mash-up of all these control tissues and cell lines that could be directly placed on each individual slide of tests biospecimens, so that each slide includes an internal control.

**Table 3** below summarizes the QC and validation steps implemented at all steps of the MICSSS assay and analysis pipeline, from processing and staining, to data acquisition, to data processing and analysis.

**Table 3**

Sample processing and staining	MICSSS Data acquisition	Data processing and analysis
Strict SOPs and detailed sample processing logs	Daily instrument optimization and performance evaluation (stainer, scanner)	Visual control of staining quality before and after acquisition by pathologist
Optimized antibody staining conditions	Stain up to 30 tissues simultaneously to minimize potential batch effects	Annotation of areas and quantification of individual cells by pathologist
Control tissue reference standards (tonsils, placenta, tumor)	Acquire images for up to 30 tissues at a time with automated slide scanner	Semi-automated analysis pipeline includes metrics for staining intensity and aspect

## 2C. Validation of detection accuracy relative to single-plex IHC

The advantage of MICSSS is that it follows the same protocol steps as single-plex IHC, so that results in the first staining cycle are identical and no comparison is necessary because there is no difference in methodology. The only distinction in MICSSS vs. single-plex IHC is that MICSSS requires the use of a labile chromogen that can be removed by de-staining steps in ethanol. The preferred dye is AEC, which is widely used in single-plex IHC as well and provides similar sensitivity to DAB<sup>16</sup>. While DAB is often preferred in single-plex IHC due to its longevity, AEC is adequate for the purpose of MICSSS.

The next important question is whether detection accuracy of MICSSS still holds true after a series of iterative cycles of staining and destaining, and this is addressed in the sections below. An important step in the implementation of MICSSS is the use of a blocking reagent to prevent any spillover reactivity from one staining cycle to the next. **Figure 3** shows how blocking efficiently prevents immediate restaining of tissues.

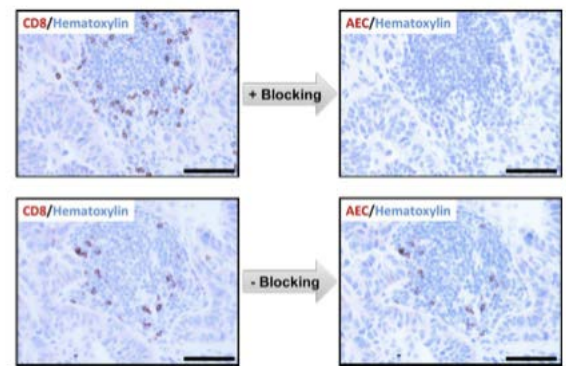


Figure 3. Efficiency of blocking method. Two adjacent 5  $\mu$ m paraffin-embedded tumor tissue sections were stained with anti-CD8 Ab and scanned (left panels). Stained tissue slides were destained before being exposed (upper right panel) or not exposed (lower right panel) to blocking reagents and revealed with AEC. Scale bar, 50  $\mu$ m.

## 2D. Validation of specificity of detection regardless of species of primary antibody or sterical hindrance

MICSSS allows the use of primary antibodies from the same species without issues of carry-over reactivity from cycle to cycle. In **Figure 4**, a colon cancer tissue was stained with CD2, CD3, CD8, CD20, DC-LAMP, MAGE-A3, Ki-67, and Foxp3 and counterstained with hematoxylin. Images were acquired after each staining and the AEC chromogen was replaced digitally with an artificial color to facilitate interpretation in overlaid images. It should be noted that cells stained for CD2, CD3, Foxp3 and/or CD8 were entirely distinct from cell stained with CD20 or DC-LAMP, as expected from these distinct T cell, B cell, and DC cell lineages respectively. Importantly, several primary Ab reagents were mouse IgG1 (CD2, CD8, MAGE-A, Foxp3), and yet they all showed distinctive staining patterns: CD8 was only coexpressed on a portion of CD2 T cells, MAGE-A was negative in this tumor, and Foxp3 was only found in some CD2/CD3 positive cells but CD8 negative. Finally, it should also be noted that individual cells could be stained with multiple markers (CD2, CD3, CD8; or CD2/CD3/Foxp3/Ki-67) without evidence of sterical hindrance.

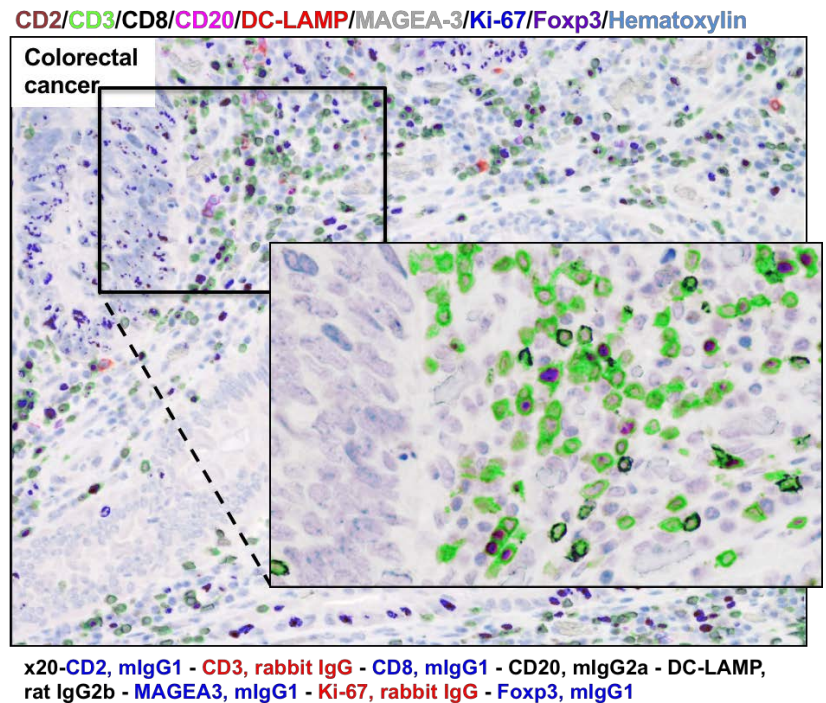


Figure 4. Staining of a colorectal tissue with 8 markers in the order indicated on the top, using primary antibodies of various origin as indicated on the bottom. Colors corresponding to AEC staining were artificially replaced after image color deconvolution, to facilitate visualization after overlaying.

Therefore, the use of MICSSS is considered to be flexible in the type of antibodies used for primary and secondary staining, which should retain their specificity in a similar fashion to what would be expected in single-plex IHC.

## 2E. Validation of sensitivity of detection regardless of staining order

To ensure that sensitivity of marker detection is not affected negatively by the iterative steps of staining and destaining, we used consecutive slides from the same tumor block to quantify the frequency of a series of immune markers while using them in different staining order for each slide. In **Figure 5**, a series of markers with a variety of expected densities were tested in different permutations. As an example, CD3 was tested in one slide first in the order of staining (0 prior destaining), then second in order of staining in an adjacent slide, and so on up to 8<sup>th</sup> in the order, i.e., after 7 prior destainings from other markers. Other markers were used along this process and also quantified after each cycle of staining. As shown in **Figure 5**, permutation of staining order did not change the overall detection sensitivity for individual markers. Therefore, it is possible to use these markers in any order for MICSSS without loss of sensitivity or specificity. It should be noted however that these analyses were performed using lineage markers with strong expected expression and detection. It is therefore possible that weaker stainings, in particular those where intensity of staining may be critical such as PD-L1, may be riskier to use after a series of staining and destaining cycles. We recommend that MICSSS, like most methods based on enzyme-linked amplification of signal, should be analyzed as a dichotomous yes/no quantification on a single cell level, rather than a continuous h-scoring based on signal intensity. Because of the multiple cycles of destaining, it would not be fair to make comparison of signal intensity without consideration for order of staining. Therefore, assays that depend on weak signal scoring such as Her2 or PD-L1 are recommended to be performed simultaneously as the first step in MICSSS, so that they follow the same protocol as a standard single-plex IHC and could be used for interslide comparisons.

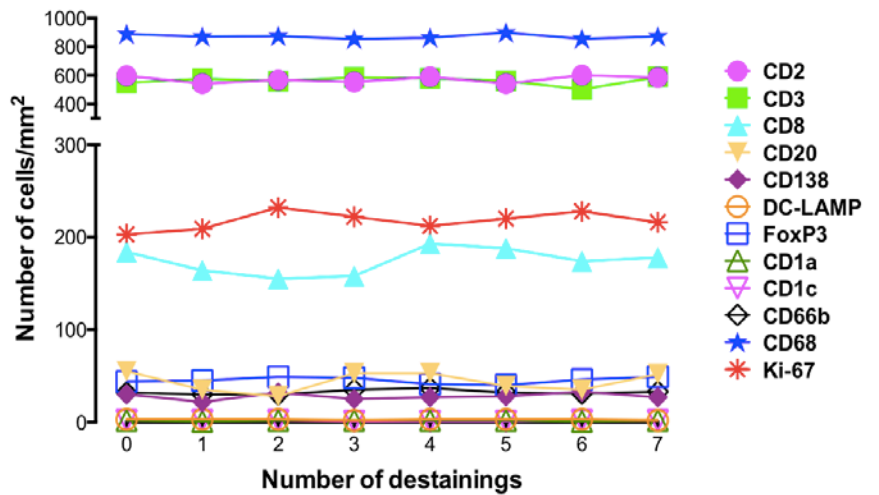


Fig. 5. Consecutive slides from a same tumor tissue block were stained with antibodies to markers indicated in the top left figure, but in different order for each slide (x-axis shows number of prior destainings for each marker). No loss of antigenicity was observed. Signal intensity was also similar regardless of staining order.

Despite these caveats, it should be noted that for constitutive markers such as CD3, CD20, etc., intensity of the chromogen signal was only marginally reduced after 7 cycles of destaining (**Figure 6**).

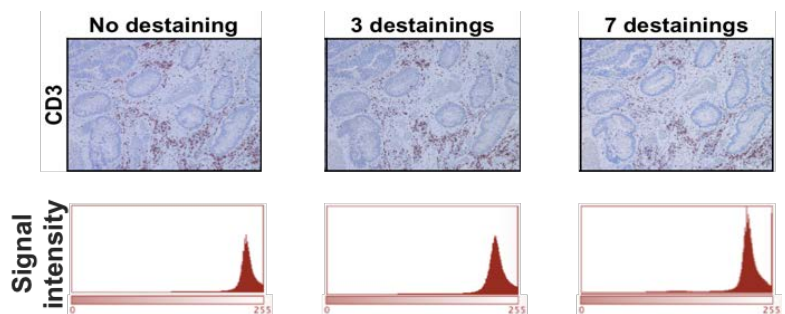


Figure 6. Stable signal intensity observed for CD3 after the first staining or after a series of prior destainings.

## 2F. Validation of intra- and inter-assay reproducibility

To evaluate overall inter- and intra- assay variability in reagents, sample processing and instrument performance, we employed reference tissues such as tonsils, lymph nodes, and placenta. The following tables quantify each marker for the expected cells stained, positivity, intensity, pattern by cell and zone, percentage, and level of background staining. For some markers such as CD66b neutrophils, tonsils are not adequate controls and these are validated on other tissues not shown in tables below. As described in section 2B, the goal is to include reference tissues for each marker in every single staining cycle, using a mash-up tissue mixture of normal, tumor, and lines in the form of a TMA. As this data becomes available, tables below will be updated.

Analyte	CD3										
Clone	2GV6										
FDA Class	FDA Class I IVD										
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage (reserved for quantitative antibodies)	Background Staining	
1/14/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a
2/3/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a
4/11/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a
1/29/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a
2/11/18	Hamamatsu Nanozoomer	Qupath	Tonsil #2	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	1+	immunostain distribution problem on the section

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

Analyte	CD20										
Clone	L26										
FDA Class	FDA Class I IVD										
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage (reserved for quantitative antibodies)	Background Staining	
11/16/17	Hamamatsu Nanozoomer	Qupath	Tonsil #1	B lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	immunostain distribution problem on the slide

2/28/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	B lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	immunostain distribution problem on the slide
5/4/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	B lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	minimal	n/a
1/12/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	B lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	minimal	n/a
2/28/18	Hamamatsu Nanozoomer	Qupath	Tonsil #2	B lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	immunostain distribution problem on the slide

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

CD8											
C8/144b											
FDA Class I IVD											
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining	
1/10/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a
5/1/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a
2/9/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	n/a	n/a	n/a	n/a	n/a	n/a	3+	all background staining including lymphoid zones and epithelium
2/5/18	Hamamatsu Nanozoomer	Qupath	Tonsil #2	T lymphocytes	positive	3+	membranous	T cell distribution in lymphoid zones and intraepithelial	n/a	none	n/a

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

The assay performed on 2/9/18 did not work as expected and results were therefore discarded.

Analyte	CD68										
Clone	KP1										
FDA Class	FDA Class I IVD										
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining	
6/7/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Histiocytes	positive	3+	cytoplasmic	Histiocyte distribution	n/a	minimal in epithelium	n/a
4/21/17	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Histiocytes	positive	2 to 3+	cytoplasmic	Histiocyte distribution	n/a	1+ in epithelium	blur areas exist in certain parts of the digital section
6/7/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Histiocytes	positive	3+	cytoplasmic	Histiocyte distribution	n/a	minimal in epithelium	n/a
4/26/18	3DHistech Pannoramic Scanner	Qupath	Tonsil #1	Histiocytes	positive	1+ to 2+	cytoplasmic	Histiocyte distribution	n/a	none	n/a
7/3/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Histiocytes	positive	3+	cytoplasmic	Histiocyte distribution	n/a	minimal in epithelium	n/a
7/3/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Histiocytes	positive	3+	cytoplasmic	Histiocyte distribution	n/a	none	n/a
15/2/18	Hamamatsu Nanozoomer	Qupath	Tonsil #2	Histiocytes	positive	3+	cytoplasmic	Histiocyte distribution	n/a	minimal in epithelium	blur areas exist in certain parts of the digital section

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

Analyte	CD138										
Clone	MI15										
FDA Class	FDA Class I IVD										
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining	
10/30/17	3DHistech Pannoramic Scanner	Qupath	Tonsil #1	plasma cells	positive	3+	membranous	plasma cell distribution	n/a	none	n/a
7/25/17	3DHistech Pannoramic Scanner	Qupath	Tonsil #1	plasma cells	positive	3+	membranous	plasma cell distribution	n/a	3+ in epithelium, 1+ in lymphoid zones	n/a

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

Analyte	CD66b											
Clone	G10F5											
FDA Class	FDA Class I IVD											
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment	
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining		
7/12/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	no proper control
7/12/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	no proper control
3/01/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	no proper control

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

Analyte	FOXP3											
Clone	236A/E7											
FDA Class	FDA Class I IVD											
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment	
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining		
3/22/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	subpopulation of T lymphocytes (T-reg's)	positive	minimal to 1+	nuclear	T lymphocyte distribution	n/a	1+	n/a	n/a
1/18/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	subpopulation of T lymphocytes (T-reg's)	positive	minimal	nuclear	T lymphocyte distribution	n/a	minimal	rare nuclear stained lymphocytes are detectable	

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

Analyte	IgA											
Clone	Polyclonal											
FDA Class	FDA Class I IVD											
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment	
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining		
12/11/17	Hamamatsu Nanozoomer	Qupath	Tonsil #1	plasma cells	positive	3+	membranous	plasma cell distribution	n/a	minimal to 1+	n/a	n/a

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

<b>Analyte</b>	<b>DC-LAMP</b>										
<b>Clone</b>	1010E1.01										
<b>FDA Class</b>	FDA Class I IVD										
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining	
3/19/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Dendritic cells	positive	3+	cytoplasmic	dendritic cell distribution	n/a	none	n/a
3/13/18	Hamamatsu Nanozoomer	Qupath	Tonsil #1	Dendritic cells	positive	3+	cytoplasmic	dendritic cell distribution	n/a	none	n/a

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

<b>Analyte</b>	<b>PD-L1</b>										
<b>Clone</b>	E1L3N										
<b>FDA Class</b>	FDA Class II IVD										
Date	Scanner	Image Analysis Software	Tested Specimen Type	Results							Comment
				Stained Cell Type	Positivity	Intensity	Cellular Staining Pattern	Zonal Staining Pattern	Percentage	Background Staining	
1/26/18	Hamamatsu Nanozoomer	Qupath	Placenta	trophoblastic cells	positive	2+	cytoplasmic and membranous	n/a	n/a	none	n/a
15/2/18	Hamamatsu Nanozoomer	Qupath	Placenta	trophoblastic cells	positive	2+	cytoplasmic and membranous	n/a	n/a	none	n/a
11/12/17	Hamamatsu Nanozoomer	Qupath	Placenta	trophoblastic cells	positive	2+	cytoplasmic and membranous	n/a	n/a	none	n/a

\*Interpreted in 5 randomly selected areas which are selected from better-stained parts of the section.

## 2G. Sample storage conditions to ensure data consistency and tissue integrity

Similarly to any IHC protocol, FFPE specimen preparation and storage should follow best practices outlined in the CIMAC biospecimen protocols. It is recommended that tissue blocks should be cut recently, with the oversight of a pathologist. Nevertheless, the following tumor tissues have been successfully tested in over 1000 individual slides and 20 different tissue microarrays so far with MICSSS: colorectal, non-small cell lung, breast, head and neck, adenomatous pancreatic, neuroendocrine pancreatic and small bowel cancers, and medulloblastoma, glioblastoma, hepatocarcinoma, multiple myeloma and mesothelioma. These tissues represent a variety of sources and storage conditions, and we are continuously logging information about potential issues encountered in staining when compared to reference tissues used as controls. We have occasionally observed that fixation conditions or age of slide could negatively detect sensitivity of staining, thereby underlining the importance of proper SOPs for biospecimen handling.

Importantly, tissue integrity can be a major issue with multiplex IHC approaches, in particular with ours because of the iterative cycles of staining, destaining, cover slip removal, and antigen retrieval. Remarkably however, in the majority of cases, was also preserved even for delicate tissues such as non-small cell lung cancer (**Figure 7**).

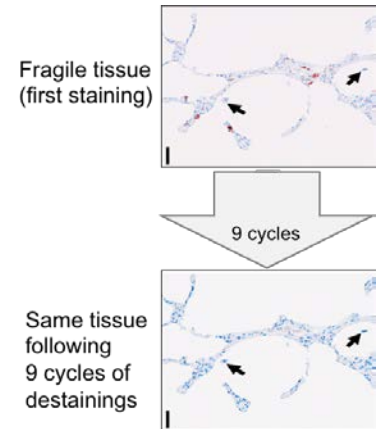


Figure 7. NSCLC tissue showing fragile areas still detectable even after 9 staining cycles.

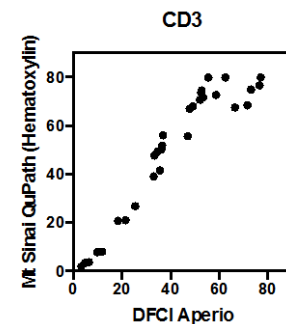
## 2H. Validation of inter-instrument reproducibility

The MICSSS method can be performed manually, except for the last step of image acquisition that is essential before moving to the next step. We have tested three separate slide scanners in our MICSSS workflow using instruments available at Mount Sinai: An Olympus slide scanner with OlyVIA software, a 3DHistech Panoramic Scanner with Panoramic viewer software, and a Hamamatsu NanoZoomer S60 with dedicated software. The preferred instrument is the Hamamatsu NanoZoomer, because it has multifocal focusing while still allowing automation of 30 slides at a time, with SOPs and use of calibrating slides to ensure reproducibility of scanning. Compared to Panoramic in particular, the NanoZoomer leads to fewer areas out of focus.

Nevertheless, if images are correctly acquired from any of the instruments above, or even from outside institutions that use Aperio files for example, the analytical workflow of image quantification is possible without significant difference.

Indeed, as part of a harmonization effort for quantification of chromogen staining, the DFCI, MDA, and Mt Sinai CIMAC have all quantified pre-stained slides independently on images captured from Aperio at DFCI, as well as from images captured by rescanning slides at each local institution. The results shown in **Figure 8** indicated excellent concordance for the CD3 marker between DFCI and Mt Sinai, and efforts are ongoing to harmonize results for other non-membrane markers.

These studies are currently being expanded to evaluate inter-instrument variability and reproducibility of data across the four CIMACs in an ongoing proficiency test for multiplex immunohistochemistry with the other CIMACS using samples distributed from DFCI. The data from these studies will be reported as soon as completed (expected in 10/2018)



	DFCI Aperio vs. Mt Sinai QuPath (Hematoxylin)
Spearman r	
r	0.9534
95% confidence interval	0.9014 to 0.9783
P value	
P (two-tailed)	< 0.0001
P value summary	****
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	30

Figure 8. Good correlation (Spearman  $r = 0.95$ ,  $p < 0.0001$ ) in CD3 counts from images acquired at DFCI and quantified independently at DFCI and Mt Sinai.

## 2I. Criteria and metrics for defining significant changes (e.g., between timepoints, between responders and non-responders).

Once images from each MICSSS staining step are acquired, they need to be analyzed by counting the number of cells positive for each marker, as well as potentially quantifying markers with co-expression. The analytical part can be performed manually by several independent investigators, as well as by image analysis algorithms that can segment cells based on nuclei staining or optical density and quantify chromogen staining on a per-cell basis. The eventual read-out is a number of stained cells out of number of total cells or out of a specific area of interest. **Figure 9** shows excellent concordance between manual counts and quantification by machine. The advantage of MICSSS is that it can be performed on whole tissues, without the need to preselect areas (which other computationally intense methods such as MIBI or VECTRA currently require). We therefore now rely on software to rapidly quantify staining and have used QuPath to do so.

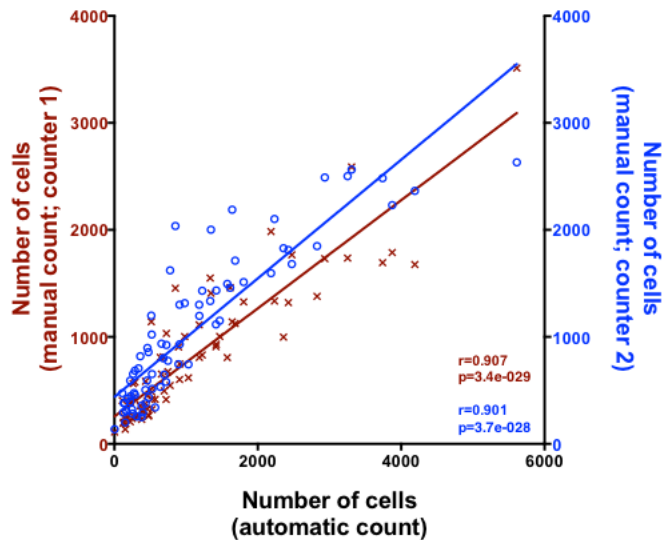


Figure 9. High correlation between manual counts of positive cells between two operators (y axes) and automated counts by cell segmentation software (x axis).  $r=0.907$ ;  $p=3.4 \times 10^{-29}$  and  $r=0.901$ ;  $p=3.7 \times 10^{-28}$  between automated and manual counting performed by counter #1 and counter #2, respectively (Spearman test).

The criteria for measuring immune infiltration in relation to clinical outcomes are not absolute and depend on markers of interest as well as on the cohorts analyzed. There is no pre-set threshold to define a highly vs. poorly infiltrated tumor, and even best practice multicenter studies. While most studies report infiltration as high vs. low based on median values across samples analyzed, others use the lowest p-value method where various cutoffs are tested to find the value that will give best results.

A minimum p value-based cutoff was used for the original immunoscore study<sup>4</sup> and its latest multicenter validation in colorectal cancer<sup>17</sup>. The minimum p value is an attractive method provided the results show a wide area of possible cutoffs with similar associated p values, to avoid random overfitting of data. From our own studies, we have found that both methods can be used. In the example shown in **Figure 10**, a cohort of 398 head and neck cancer patients were analyzed for infiltration of CD3 and CD20 cells, using tissue microarrays with triplicate cores, and results were correlated with overall survival using the Kaplan-Meier Rank Log method. Cutoffs for high vs. low infiltration were tested either using the median value of CD3 density (cells/mm<sup>2</sup>) of the entire cohort in primary tumors, or using the minimal p value method. For CD3, we found that the median and the cutoff based on minimal p value were the same, at 253 cells/mm<sup>2</sup>. For CD20 however, the results differed and the median value (56 cells/mm<sup>2</sup>) did not achieve statistical difference in survival while a higher cutoff determined by minimal p value (330 cells/mm<sup>2</sup>) did.

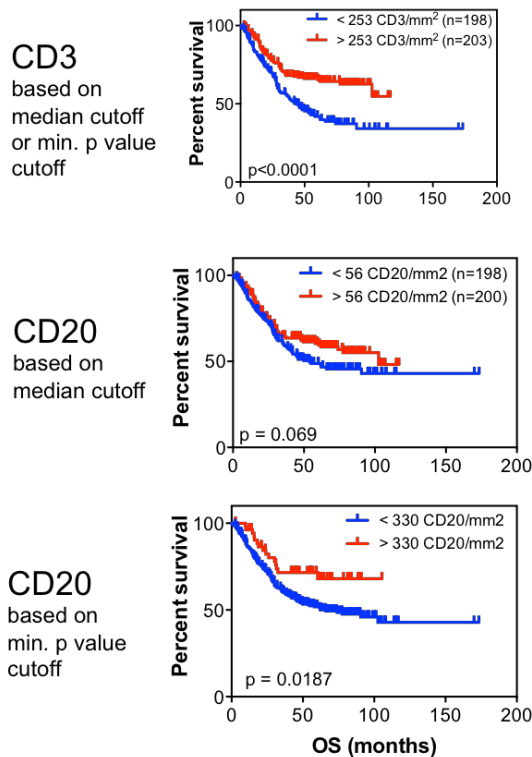


Figure 10. Selecting a cutoff to show low vs. high density of immune infiltration in relation to overall survival of patients with head and neck tumors, based on either median values or cutoffs determined by minimal p value method. Log Rank p value indicated in each plot.

For clinical studies looking at infiltration changes before and after treatment, descriptive statistics should be used. Changes between time points or other paired features are measured by paired tests and are facilitated by assessing slides whenever possible in the same assay, in a batched manner, to minimize technical and experimental variation. Pre-determining significant changes even in paired specimens before and after treatment may not be straightforward, because of potential heterogeneity in areas of immune infiltration. MICSSS can be performed on entire tissue slides, but there is still a risk that the area chosen by the pathologist

may not be representative of the entire tumor. Nevertheless, changes observed in paired specimens from no infiltration to presence of immune infiltration, or at least 2x higher than baseline, may be noted and tested by Wilcoxon and Spearman tests, as was done in the pembrolizumab study of melanoma patients<sup>11</sup>. Comparing populations across cohorts, there is no predetermined criteria or metrics for significant differences, which would need to be addressed in either unpaired t tests or mixed models and placed in the context of normal intra-individual and inter-individual baseline variation of a given parameter.

The criteria for positivity for the PD-L1 marker is much more strictly defined, but is highly dependent on the primary antibody clones used in analysis. Because PD-L1 is used as a complementary as well as companion test in immunotherapy, we do not propose to use PD-L1 in MICSSS as a clinical test because it should be done in a CLIA environment. The best we can do is include our PD-L1 reagent for information only, to compare with existing assays. We chose clone E1L3N from Cell Signaling Technologies because it has been reported<sup>11</sup> as being very similar in magnitude and intensity of staining to the commercially developed clinical tests. Harmonization of PD-L1 staining has been the subject of extensive studies from the Blueprint project<sup>18</sup>, though extending this to research labs is hampered by the availability of proprietary reagents.

## 2J. Transfer of MICSSS Protocol to External Labs

MICSSS protocol is performed by several labs successfully. Hands on training by using the MICSSS SOP is provided by our lab. Since MICSSS protocol is very simple and depends on the rules of singleplex immunohistochemistry with some additional steps, the adoption process usually works seamlessly (Figure 11).

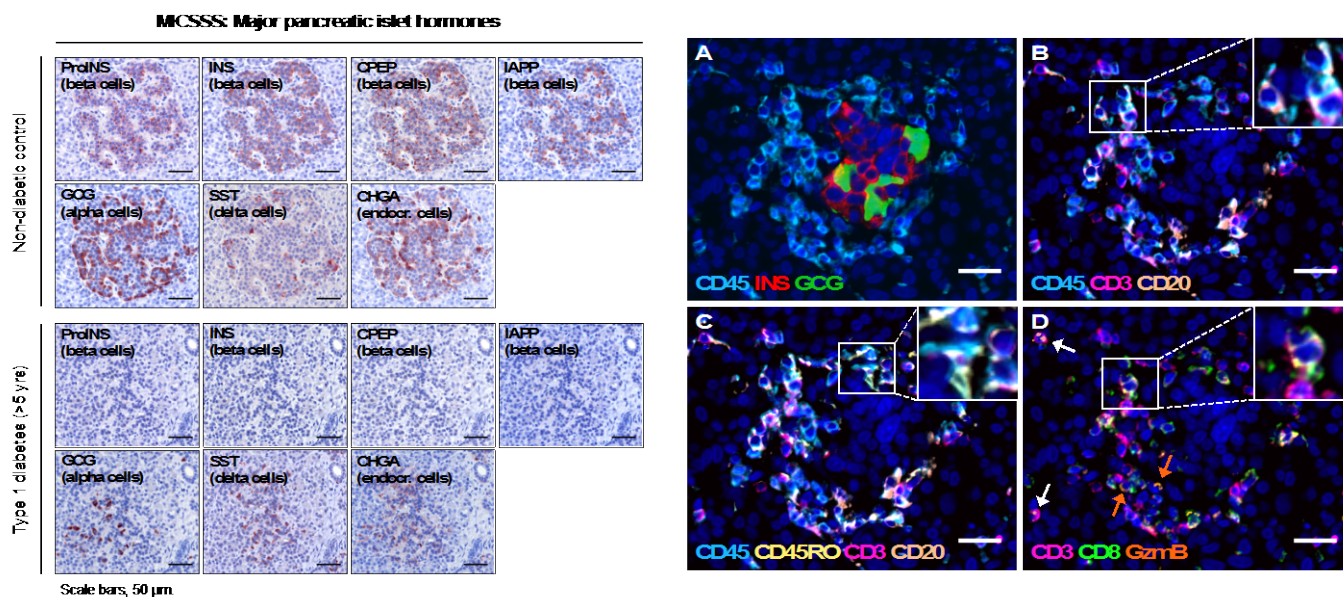


Figure 11: Example of MICSSS protocol transfer. The pancreatic islet cell hormone profile MICSSS panel demonstrated in this figure is performed by an external laboratory successfully. Hands-on training and SOP are provided for the transfer of MICSSS protocol.

## 3. References

- 1 Wolchok, J. D. & Chan, T. A. Cancer: Antitumour immunity gets a boost. *Nature* **515**, 496-498, doi:10.1038/515496a (2014).
- 2 Remark, R. *et al.* The Non-Small Cell Lung Cancer Immune Contexture: a Major Determinant of Tumor Characteristics and Patient Outcome. *Am J Respir Crit Care Med*, doi:10.1164/rccm.201409-1671PP (2014).
- 3 Fridman, W. H., Pages, F., Sautes-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* **12**, 298-306, doi:10.1038/nrc3245 (2012).
- 4 Galon, J. *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964, doi:10.1126/science.1129139 (2006).
- 5 Fridman, W. H. *et al.* The Immune Microenvironment: A Major Player in Human Cancers. *Int Arch Allergy Immunol* **164**, 13-26, doi:10.1159/000362332 (2014).
- 6 Galon, J., Angell, H. K., Bedognetti, D. & Marincola, F. M. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* **39**, 11-26, doi:10.1016/j.immuni.2013.07.008 (2013).
- 7 Goc, J. *et al.* Dendritic Cells in Tumor-Associated Tertiary Lymphoid Structures Signal a Th1 Cytotoxic Immune Contexture and License the Good Positive Prognostic Value of Infiltrating CD8+ T Cells. *Cancer Res*, doi:10.1158/0008-5472.CAN-13-1342 (2014).
- 8 Remark, R. *et al.* Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. *Clin Cancer Res* **19**, 4079-4091, doi:10.1158/1078-0432.CCR-12-3847 (2013).
- 9 Galon, J. *et al.* Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med* **10**, 205, doi:10.1186/1479-5876-10-205 (2012).
- 10 Remark, R. *et al.* In-depth tissue profiling using multiplexed immunohistochemical consecutive staining on single slide. *Science Immunol* **1**, aaf6925, doi:10.1126/sciimmunol.aaf6925 (2016).
- 11 Tumeh, P. C. *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515**, 568-571, doi:10.1038/nature13954 (2014).
- 12 Cogswell, J. *et al.* An Analytical Comparison of Dako 28-8 PharmDx Assay and an E1L3N Laboratory-Developed Test in the Immunohistochemical Detection of Programmed Death-Ligand 1. *Mol Diagn Ther* **21**, 85-93, doi:10.1007/s40291-016-0237-9 (2017).
- 13 Salmon, H. *et al.* Expansion and activation of CD103(+) dendritic cell progenitors at the tumor site enhances tumor responses to therapeutic PD-L1 and BRAF inhibition. *Immunity* **44**, 924-938, doi:10.1016/j.immuni.2016.03.012 (2016).
- 14 Remark, R. *et al.* The non-small cell lung cancer immune contexture. A major determinant of tumor characteristics and patient outcome. *Am J Respir Crit Care Med* **191**, 377-390, doi:10.1164/rccm.201409-1671PP (2015).
- 15 de Moll, E. H. *et al.* Immune biomarkers are more accurate in prediction of survival in ulcerated than in non-ulcerated primary melanomas. *Cancer Immunol Immunother* **64**, 1193-1203, doi:10.1007/s00262-015-1726-0 (2015).
- 16 Trojanowski, J. Q., Obrocka, M. A. & Lee, V. M. A comparison of eight different chromogen protocols for the demonstration of immunoreactive neurofilaments or glial filaments in rat cerebellum using the peroxidase-antiperoxidase method and monoclonal antibodies. *J. Histochem. Cytochem.* **31**, 1217-1223, doi:10.1177/31.10.6350434 (1983).
- 17 Pages, F. *et al.* International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **391**, 2128-2139, doi:10.1016/S0140-6736(18)30789-X (2018).
- 18 Hirsch, F. R. *et al.* PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *J. Thorac. Oncol.* **12**, 208-222, doi:10.1016/j.jtho.2016.11.2228 (2017).