1. PURPOSE

Pipeline described here is a simple, highly sensitive, multiplexed immunohistochemistry chromogen-based staining on a single slide method, which is called MICSSS, to comprehensively characterize tissue cell phenotype, state, and spatial distribution in inflammatory lesions. The MICSSS method uses consecutive cycles of staining and destaining with primary and secondary antibodies to characterize up to 10 markers on a single formalin-fixed paraffin-embedded (FFPE) tissue slide (Figure 1). It can be applied to a variety of FFPE tissues, including whole sections of tumor and inflamed tissues, as well as tissue microarrays (TMA). The MICSSS method does not lead to antigenicity loss, steric hindrance, or increased cross-reactivity. MICSSS uses similar conditions to IHC protocols used in routine clinical pathology laboratories (antigen retrieval, primary antibody (Ab), secondary Ab, chromogen revelation), with added steps to allow reusing slides after chemical destaining and blocking steps. MICSSS has been developed as a new tool to describe the spatial immune microenvironment of tissues in depth at baseline, prior to treatment, and track immune change upon therapy, providing a unique sample-sparing analytical tool to characterize limited tissue samples obtained during clinical studies.

2. SPECIMEN SPECIFICATIONS

2.1. FFPE sections should be cut at a thickness of 4um.

2.2. Optimally, 5 unstained slides should be provided (3 slides at minimum).

2.4. A physical H&E slide or a .svs digital image of the H&E stain must be provided.

2.5. Sections should be mounted on Avantik Superfrost Plus slides (Avantik Cat# SL6021-2) as depicted in Figure 1 below. If Avantik slides are unavailable VWR Superfrost Plus slides (VWR Cat#

48311-703), Fisherbrand Superfrost Plus slides (Fisherbrand Cat# 12-550-15), or Superfrost Plus slides from other vendors are adequate.

Figure 1: Tissue Placement on Slide

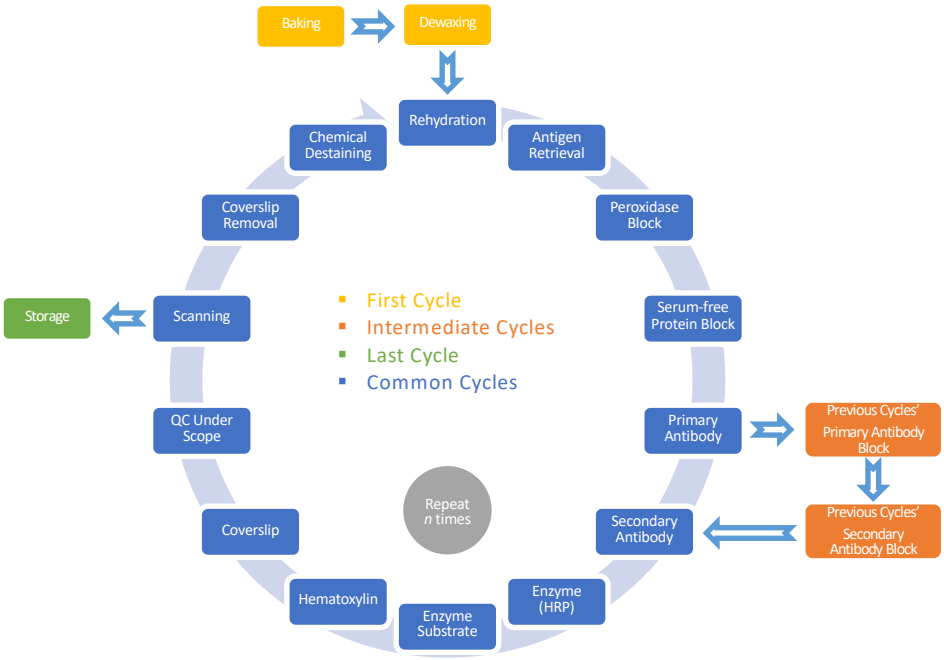

Figure 2: MICSSS Pipeline

3. MATERIALS AND EQUIPMENT

3.1. Critical Reagents

3.1.1. Antigen Retrieval

3.1.1.1. Bond TM Epitope Retrieval 1, pH6, 1L (Leica Biosystems Cat# AR9961)

3.1.1.2. Bond TM Epitope Retrieval 2, pH9, 1L (Leica Biosystems Cat# AR9640)

3.1.2. Blocking Reagents

3.1.2.1. Protein Block Serum-Free Ready-to-Use, 110ml (Agilent Cat# X0909)

3.1.2.2. AffiniPure Fab Fragment Donkey anti-mouse IgG (H+L) 1mg (Jackson Immuno Research Cat# 715-007-003)

3.1.2.3. AffiniPure Fab Fragment Donkey anti-rabbit IgG (H+L) 1mg (Jackson Immuno Research Cat# 711-007-003)

3.1.2.4. AffiniPure Fab Fragment Donkey anti-rat IgG (H+L) 1mg (Jackson Immuno Research Cat# 712-007-003)

3.1.3 Buffers

3.1.3.1. BOND TM Wash Solution, 10x Concentrate, 1L (Leica Biosystems Cat# AR9590)

3.1.3.2. BOND Dewax Solution, 1L (Leica Biosystems Cat# AR9222)

3.1.4 Primary Antibody, Secondary Antibody, and Horse Radish Peroxidase (HRP)

3.1.4.1. BOND Primary Antibody Diluent, 500mL (Leica Biosystems Cat# AR9352)

3.1.4.2. ImmPRESS Goat Anti-Rat IgG Polymer Kit, Peroxidase (Vector MP-7444)

3.1.4.3. VisUCyte HRP Polymer Goat IgG Antibody, 25mL (R&D Systems Cat# VC004-025)

3.1.5. Chromogen

3.1.5.1. ImmPACT AEC Substrate Kit, Peroxidase (Vector Laboratories SK-4205)

3.1.6. Mounting Media

3.1.6.1. Glycergel Mounting Medium, 15mL (Agilent Cat# C05633)

3.1.7 Staining Kit

3.1.7.1. BOND Polymer Refine Detection Kit (Leica Biosystems Cat# DS9800)

3.2. Other Reagents

3.2.1. Ethanol, Absolute (200 Proof), Molecular Biology Grade, Fisher BioReagents™ (Fisher Scientific BP-2818-4)

3.2.2. Tween™ 20, Fisher BioReagents™ (Fisher Scientific BP337-500)

3.2.3. Tris-Hydrochloride 500g (Fisher Scientific BP153-500)

3.2.4. Sodium Chloride, Fisher BioReagents (Fisher Scientific BP358-1)

3.2.5. Sodium Hydroxide (NF/EP/BP/FCC) 10N, Fisher Chemical (Fisher Scientific S399-500)

3.2.6. Hydrochloric Acid Solution 6N (Fisher Scientific SA56-500)

3.2.7. Alfa Aesar 3P Xylenes MIXED 97+%2.5L (Fisher Scientific 50-703-1590)

3.3. Other Materials and Equipment

3.3.1. BOND RX Fully Automated Research Stainer (Leica Biosystems)

3.3.2. Beakers and Measuring Cylinders

3.3.3. Microscope Cover Glass (Size 24x40-2) (10 pack in 1 box) (Fisher Scientific 12-543B)

3.3.4. H_2O milliQ

3.3.5. Vortex machine

3.3.6. Refrigerator (2°- 8°)

3.3.7. Slide Stain Tray

3.3.8. Water Bath (Fisherbrand-Isotemp)

3.3.9. Staining Racks

3.3.10. Slide Boxes

3.3.11. Slide scanner Nanozoomer S60 (Hamamatsu)

3.3.12. Scott C-fold paper towels (Fisher Scientific Cat# 06-666-32B)

3.3.13. NUOVA II stir plate (Temrolyne)

Reagent Name	Vendor	Cat#	Dilution	Preparation / Notes
Bond TM Epitope Retrieval 1, pH6, 1L	Leica Biosystems	AR9961	ready-to-use	pH9
Bond TM Epitope Retrieval 1, pH9, 1L	Leica Biosystems	AR9640	ready-to-use	pH6
BOND TM Wash Solution, 10x Concentrate, 1L	Leica Biosystems	AR9590	1:10 in water	Wash buffer solution needs to be diluted in distilled water following the manufacturer's procedures
Prot Block, Serum Free, 110 mL	Agilent	X090909	ready-to-use	0.25% casein in PBS, stabilizing protein 0.015 mol/L sodium azide. Ready-to-use following manufacturer's procedures
BOND Dewax Solution, 1L	Leica Biosystems	AR9222	ready-to-use	Ready to use per manufacturer's procedures to remove paraffin wax from tissue sections
BOND Primary Antibody Diluent, 500 mL	Leica Biosystems	AR9352	ready-to-use	Tris-buffered saline, surfactant, protein stabilizer and 0.35% ProClinTM 950. Ready-to-use following manufacturer's procedures
VisUCyte HRP Polymer Goat IgG Antibody, 25mL	R&D Systems	VC004-025	ready-to-use	Biotin-free HRP polymer Anti-Goat IgG reagent made in donkey. Ready-to-use following manufacturer's procedures
ImmPRESS REAGENT KIT anti-RAT (mouse adsorbed)	Vector Laboratories	MP-7444	ready-to-use	Peroxidase) Polymer Anti-Rat IgG (mouse adsorbed) Reagent (made in goat, ready-to-use)
ImmPACT AEC Substrate Kit, Peroxidase	Vector Laboratories	SK-4205	Prepare per manufacturer's recipe	3-amino-9-ethylcarbazole HRP substrate that produces a red reaction product. To prepare: 5mL of Diluent, 2 drops of Reagent 1, 3 drops of Reagent 2, 2 drops of Reagent 3
BOND Polymer Refine Detection Kit	Leica Biosystems	DS9800	ready-to-use	Contains a peroxide block, post primary anti-mouse IgG, anti-rabbit polymer reagent with conjugated HRP, and hematoxylin counterstain. Ready-to-use following manufacturer's procedures
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.

Table 1: Critical Regaents

4. SAFETY

4.1 Wear protective gloves and lab coat while performing this procedure.

4.2 Hematoxylin is toxic and corrosive reagent. Wear protective gloves/protective clothing/eye protection face. Do not get in eyes, on skin or on clothing. Do not breathe dust/fume/gas/mist/vapors/spray.

4.3 Xylene is a flammable, corrosive and toxic reagent. Perform all slide incubations within a fume hood. Wear protective gloves/protective clothing/eye protection face. Do not breathe dust/fume/gas/mist/vapors/spray. Keep container tightly closed. Keep away from heat/sparks/open flames/ hot surfaces.

4.4 Sodium hydroxide is a base. It should be stored away from oxidizing agents, reducing agents, metals, acids, and alkalis. Never add water to sodium hydroxide.

4.5 AEC Peroxidase Substrate Kit is a flammable, corrosive and toxic reagent. Keep away from heat/sparks/open flames/hot surfaces. Wear protective gloves, protective clothing, eye and face protection. Wash skin thoroughly after handling. IF AEC gets ON SKIN (or hair), immediately, take off all contaminated clothing, rinse skin with water or shower if necessary. IF INHALATED, remove person to fresh air. IF AEC gets into EYES, rinse cautiously with water for 10 minutes. Remove contact lenses, if present and continue rinsing for 10 minutes. If exposed or concerned: seek medical attention. If eye irritation persists: seek medical attention immediately.

4.6 Hydrochloric acid is a corrosive and irritant reagent. Wear protective gloves/protective clothing/eye protection/face protection.

5. REAGENT PREPARATION

5.1. TBS 10x, pH7.4 (3 Liters):

Tris-Hydrochrloride (Fw 157.6), 47.28g + Sodium Chloride (Fw 58.44), 262.98g in 3 L H_2O milliQ

Add Sodium Hydroxide 10N, 3.3 ml

Measure the pH

5.2. Wash Buffer TBS 1X:

18 L H_2O milliQ + 2 L TBA 10X.

5.3.Wash Buffer (TBS 1x + 0.04% Tween20)

1L TBS 1X + 0.4 ml Tween20

5.4. Bond TM Wash Solution 1X:

900mL Bind TM Wash Solution 10X + 100ml milliQ water

5.4. Deparaffinization solution 90% Ethanol Absolute

180ml Ethanol Absolute + 20 ml H_2O milliQ

5.5. Deparaffinization solution 70% Ethanol Absolute

140ml Ethanol Absolute + 60ml H_2O milliQ

5.6. Deparaffinization solution 50% Ethanol Absolute

100ml of 100% Ethanol absolute+100ml H_2O milliQ

5.7. Destaining solution 50% Ethanol Absolute

100ml of 100% Ethanol absolute+100ml H2O milliQ

5.8. Destaining solution 70% Ethanol Absolute+ 1% HCl 12N

140ml Ethanol Absolute + 56ml H2O milliQ + 4ml Hydrochloric Acid Solution 6N

5.9. Destaining solution 70% Ethanol Absolute

140ml Ethanol Absolute + 60ml H2O milliQ

5.10. AffiniPure Fab Fragment Donkey anti-mouse, or anti-rabbit or anti-rat block solution

Dilute 1:50 AffiniPure Fab Fragment Donkey anti-mouse, or anti-rabbit or anti-rat in TBS 1X

5.11. Primary Antibody Dilution

Dilute antibody at the suggested dilution in BOND Primary Antibody Diluent

5.12. AEC chromogen solution, make 5 ml total, add

- 2 drops of Buffer Stock solution
- 3 drops of AEC Stock solution
- 2 drops of Hydrogen Peroxide Solution

in 5ml of H2O milliQ

6. PROCEDURE

Day-1 – Bake slides

6.1. Bake slides for 1 hour at 60°C in a slide box, leaving it a bit open. In this way, slides can get dry.

Day-2 – Antibody 1 Staining

Autostainer Set up

6.2. Turn on the autostainer prior to use so that the machine has plenty of time to boot up, perform any updates, and back up data.

6.3. Insert the Epitope Retrieval 1 and Epitope Retrieval 2 Containers into the autostainer.

6.4. Refill any Bulk Reagent Containers that are more than half empty. Bulk Reagent Containers are labelled as follows and contain the specified reagent:

- Dewax Solution: Dewax Solution bought from Leica Biosystems, RTU
- DI Water: Deionized water
- Wash Buffer: Bond TM Wash Buffer 1x
- Alcohol: 100% Ethanol
- Epitope Retrieval 1: Epitope Retrieval 1 bought from Leica Biosystems, RTU
- Epitope Retrieval 2: Epitope Retrieval 2 bought from Leica Biosystems, RTU

6.5. Empty any Waste Containers that are more than half full.

- Waste from the Bulk Waste containers can be disposed of in the sink
- Waste from the Hazardous Waste container must be disposed off in the Mt. Sinai Hazardous Waste Bin

6.6. Open the lid to each of the containers in the Bond Polymer Refine Detection kit and insert it into the autostainer

- If the reagent levels of the Bond Polymer Refine Detection Kit are low remove this detection kit and load a new one instead
- Scan the 2 barcodes on the new Bond Polymer Refine Detection Kit to program it into the computer

6.7. Scan the barcode on the front of each of the Bond Titration Containers that will be used for this run. Make sure there is a current value of 6 mL. If there isn't, click on 'Refill'.

- If there isn't enough volume left in the Bond Titration Container to refill the 'Current Volume' to 6mL then the Bond Titration Container can be thrown away, and a new Bond

Titration Container needs to be programmed. Scan the front barcode of a new Bond Titration Container. In the 'Reagent Name' drop down box select the reagent that will be assigned to this Bond Titration Container. Set the expiration date to 1 year from today. Click 'OK'. Label with front of the New Bond Titration Container with the name of the reagent that has been assigned to it.

6.8. Add a study under the 'Slide Setup' tab, name it the name of the experiment, select the user's name under the 'Researcher' drop down menu, and select the preparation protocol that is appropriate for the experiment.

6.9. Add a slide in the study that was created and type their name in the textbox. Select the Bond Titration Container that will contain the primary antibody from the 'Marker' dropdown menu. Select the appropriate staining protocol from the 'Staining' drop down menu. Select the appropriate antigen retrieval protocol from the 'HIER' drop down menu. Repeat these steps for all the slides.

6.9. Print the slide labels

Deparaffinization and Rehydration Steps

6.10. Immerse slides (using a staining rack) in 100% xylene for 5 minutes, 3X each for 5 mins

- Gently drain excess liquid between each step

- Do not let the tissue get dry once staining has started

- Do steps 5.2 – 5.6 in chemical fume hood

- Can reuse solutions from steps 5.2 – 5.6 up to 20 times

- After xylene step, remove the excess of xylene shaking very well the staining rack on a Scott C-fold paper.

6.11. Immerse slides in 100% ethanol for 5 mins using a staining rack

6.12. Immerse slides in 90% ethanol for 5 mins using a staining rack

6.13. Immerse slides in 70% ethanol for 5 mins using a staining rack

6.14. Immerse slides in 50% ethanol for 5 mins using a staining rack

6.15. Immerse slides in dH_2O for 5 mins (1^{st} wash) using a staining rack

6.16. Immerse slides in TBS 1X until ready to be loaded into the autostainer

Reagent Set Up

6.17. Insert all the Bond Titration Containers that will be used in this experiment into the Bond Reagent Tray. Insert a Bond Titration Insert into each one of the Bond Titration Containers

6.18. Prepare the primary antibody solution in the Bond Titration Insert that is in the corresponding Bond Titration Container. Prepare this solution at the dilution recommended by the manufacturer's procedures using the Bond Primary Antibody Diluent.

- Prepare enough volume for every slide to receive 200ul. If staining less than 8 slides make a total volume of at least 1600ul

6.19. Add enough Serum Free Protein Block in the Bond Titration Insert that is in the corresponding Bond Titration container for each slide to receive 200ul

- If staining less than 8 slides add a total volume of at least 1600ul

6.20. Prepare 5ml of FAB block in the Bond Titration Insert that is in the corresponding Bond Titration Container

- This reagent is not used for the first stain but is added for every stain thereafter to block out the previous stain.

6.21. Prepare the AEC solution in the Bond Titration Insert that is in the corresponding Bond Titration Container

- Do not excessively expose this reagent to light as it is photosensitive

6.22. Insert the Bond Reagent Tray into the autostainer

Slide Set Up

6.23. Remove the first slide from the TBS, wipe the back of the slide with a Kimtech wipe until dry, and place the slide face up on the first slot of the BOND Slide Tray making sure that the slide fits inside of the notches. Repeat this for all the slides.

6.24. With a Kimtech wipe dab the label of each slide on the BOND Slide Tray to dry and then stick the proper printed label on the corresponding slide.

6.25. Place a BOND Universal Covertile on top of the first slide such that the “foot” of the covertile fits properly into the notch on the BOND Slide Tray. Repeat this for all the slides on the tray.

6.26. Insert the BOND Slide Tray with the slides into the first staining assembly on the autostainer until a click is felt. Then push the button underneath the staining assembly.

6.27. Wait for the BOND Slide Tray to lock in place and for the scanner to scan all the slides. Then click the play button that appears on the computer to initiate the stain.

6.28. Repeat steps 5.23-5.27 for any additional BOND Slide Trays that you will be loading for this stain.

Mounting Coverslips on Slides

6.29. 30 minutes prior to the end of the stain, submerge a vial of Glycergel Mounting Media in a small beaker with hot water and place the beaker on a hot plate set to just under 100°C. This will melt the mounting media in preparation for slide mounting.

6.30. Once the stain has completed push the button underneath the first staining assembly on the autostainer. Wait for the tray to unlock and then pull it out.

6.31. Gently remove the first covertile off of the first slide making sure not to disturb the tissue underneath and submerge the covertile in 100% Ethanol to clean. Repeat for all the remaining covertiles on that Bond Slide Tray.

6.32. Submerge the slides in TBS

6.33. Repeat steps 35-37 for the remaining BOND Slide Trays / slides

6.34. Mount coverslips on the slides using the warmed Glycergel Mounting Media.

- Pipette 100ul of aqueous mounting medium on the coverslip carefully without making bubbles
- Carefully place the slide supporting the tissue on the top of the coverslip, making the contact between the tissue and the mounting media.

6.35. Capture the image using IHC scanner Nanozoomer S60 (Hamamatsu)

Bleaching

6.36. Remove the coverslip from the slides

- put the slide in the rack and immerse in the hot Tap water (56°C) until the mounting media is dissolved and you are able to remove carefully the coverslip

- Very gently tug the coverslip to see whether it moves off away from the tissue without stickiness.

-push out the coverslip kindly using your hands

- Do not touch and damage the tissue

6.37. Take a COLD water (dH_2O) with the empty staining rack inside and transfer the slide one by one without the coverslip in the cold water

6.38. Immerse the slide in 50% EtOH for 2 mins using a staining rack

6.39. Immerse the slide in 70% EtOH + 1% HCl for 2 mins using a staining rack

6.40. Immerse the slide in 100% EtOH for 5 mins using a staining rack

-AEC chromogen and the mouting medium are removed

6.41. Immerse the slide in 70% EtOH for 2 mins in a staining rack

6.42. Immerse the slide in 50% EtOH for 2 mins in a staining rack

6.43. Immerse the slides in dH_2O for 5 mins in a staining rack

6.44. Repeat steps 5.3-4.43 for all subsequent stains.

Table 1											
Tier	**Marker**	**Cat#**	**Company**	**Clone**	**Species**	**Isotype**	**Antigen retrieval**	**Time for AR**	**Dilution**	**Incubation**	**Concentrations (for current batch)**
1	CD3	790-4341	Ventana	2GV6	Rabbit	IgG	pH9	30 min	RTU	2h @RT	0.4 µg/mL
1	CD8	M7103	Dako	C8/144b	Mouse	IgG1	pH9	30 min	1/100	1h @RT	200 mg/L
1	CD20cy	M0755	Dako	L26	Mouse	IgG2a	pH6	30 min	1/250	1h @RT	126 mg/L
1	CD68	M0814	Dako	KP1	Mouse	IgG1	pH6	30 min	1/100	1h @RT	n/a
1	CD66b	555723	BD Pharmingen	G10F5	Mouse	IgM	pH9	30 min	1/600	1h @RT	0.5 mg/mL
1	Ki-67	790-4286	Ventana	30-9	Rabbit	IgG	pH9	30 min	RTU	1h @RT	2 µg/mL
1	HLA-DR	ab20181	Abcam	TAL1B5	Mouse	IgG1	pH6	30 min	1/500	1h @RT	0.9 mg/mL
1	DC-LAMP	DDX0191P	Novus biologicals	1010E1.01	Rat	IgG2a	pH9	30 min	1/80	1h @RT	0.5 mg/mL
1	CD138	M7228	Dako	MI15	Mouse	IgG1	pH6	30 min	1/100	1h @RT	n/a
1	HLA-ABC (class I)	ab70328	Abcam	EMR8-5	Mouse	IgG1	pH6	30 min	1/200	1h @RT	1 mg/mL
1	PanCK	M3515	Dako	AE1/AE3	Mouse	IgG1	pH6	30 min	1/50	1h @RT	101.6 mg/mL
1 Pre	FoxP3	ab20034	Abcam	236A/E7	Mouse	IgG1	pH6	30 min	1/80	2h @RT	1 mg/mL
1 Pre	PD-1	ab52587	Abcam	EPR4877 (2)	Rabbit	IgG	pH6	20 min	1/250	30 mins @RT	1 mg/mL
1 Pre	PD-L1	13684S	CST (Cell Signaling)	E1L3N	Rabbit	IgG	pH9	30 min	1/100	1h @RT	0.74 µg/mL
2	CD1a	M3571	Dako	O10	Mouse	IgG1	pH6	30 min	1/50	1h @RT	816 mg/L
2	CD2	M7309	Dako	AB75	Mouse	IgG1	pH9	30 min	1/40	1h @RT	72 mg/L
2	T-bet	760-4598	Cell Marque	MRQ46	Rabbit	IgG1	pH9	30 min	RTU	1h @RT	n/a
2	CD103	ab129202	Abcam	EPR4166 (2)	Rabbit	IgG	pH9	30 min	1/500	1h @RT	0.894 mg/mL

2	CD1c	ab156708	Abcam	2F4	Mouse	IgG1	pH9	30 min	1/150	1h @RT	1 mg/mL
2	Langerin	392M	Cell Marque	12D6	Mouse	IgG2b	pH6	30 min	1/50	1h @RT	n/a
2	CD206	ab64693	Abcam	polyclonal	Rabbit	IgG	pH6	30 min	1/500	1h @RT	1 mg/mL
2	Podoplanin	760-4395	Cell Marque	D2-40	Mouse	IgG1	pH9	30 min	RTU	1h @RT	n/a
2	Granzyme B	M7235	Dako	GrB-7	Mouse	IgG2a	pH9	45 min	1/50	1h @RT	40 mg/L
2	CD163	NB110-59935	Novus biologicals	10D6	Mouse	IgG1	pH6	30 min	1/50	1h @RT	unpurified
2	CK19	ab52625	Abcam	EP1580Y	Rabbit	IgG	pH9	30 min	1/400	1h @RT	0.838 mg/mL
2	CD21	M0784	Dako	1F8	Mouse	IgG1	pH6	30 min	1/25	1h @RT	199 mg/L
2	NY-ESO1	N2038	Sigma	E978	Mouse	IgG1	pH9	30 min	1/300	2h @RT	~1 mg/mL
2	Mage-A1	sc-20033	Santa Cruz	MA454	Mouse	IgG1	pH9	30 min	1/50	3h @RT	200 µg/mL
2	P53	M7001	Dako	DO-7	Mouse	IgG2b	pH9	30 min	1/50	1h @RT	237 mg/L
2	Melan-A	M7196	Dako	A103	Mouse	IgG1	pH9	30 min	1/50	1h @RT	96 mg/L
2	SURVIVIN	M3624	Dako	12C4	Mouse	IgG2a	pH6	45 min	1/100	1h @RT	73.6 mg/L
2	PNAd	553863	BD Pharmingen	MECA-79	Rat	IgM	pH6	30 min	1/50	1h @RT	0.5 mg/ml
2	IgA	A0262	Dako	polyclonal	Rabbit	IgG	pH9	30 min	1/100	1h @RT	4.2 g/L
2	ERG	M7314	Dako	EP111	Rabbit	IgG	pH9	30 min	1/50	1h @RT	258 mg/L
2	CTLA-4	sc-376016	Santa Cruz	F-8	Mouse	IgG1		30 min	1/50	1h @RT	200 µg/mL
2	Mage-A3/6	collaboration	LICR	M3H67	Mouse	IgG1	pH6	30 min	1/1000	3h @RT	2.7 mg/mL
2	VISTA	64953	Cell Signaling Technology	(D1L2G) XP	Rabbit	IgG	pH6	30 min	1/50	O.N	18.8 µg/mL

2	TLR7	ADI-CSA-824-E	Enzo lifesciences	polyclonal	Rabbit	IgG	pH6	30 min	1/50	1h @RT	n/a
2	TLR8	ALX-804-376-C100	Enzo lifesciences	44C143	Mouse	IgG1	pH6	30 min	1/100	1h @RT	n/a

Table 2: List of Antibodies

Note 1: Depending on the species of antibody clone, Fab Fragment Donkey anti-mouse/rabbit/rat is used to block the primary antibody on the next cycle of immunostaining. First cycle does not require this blocking step since there is no previous cycle that requires Fab Fragment Donkey anti-mouse/rabbit/rat blocking.

Example: CD8 is a mouse species primary antibody and it is blocked by Fab Fragment Donkey **anti-mouse**. Secondary antibody for CD8 must be antimouse and **mouse serum** is used to block secondary in this case.

Note 2: CD68, CD138, T-bet, langerin, podoplanin, TLR7 and TLR8 concentrations will be revised once we receive the new batches of antibodies. They are currently not available in our antibody bank.

Note 3: Concentrations in Table 1 represents our current batches.

Note 4: PD-L1 is always placed as first immunostain in MICSSS panels because interpretation of PD-L1 staining is quantitative and can affect treatment decisions. PD-1 and FOXP3 is sensitive to destaining and should be included as first immunostains if it's possible. If all these mentioned markers are included in the same panel for a given project, PD-L1 should be placed as the first immunostain and, PD-1/FOXP3 should follow PD-L1.