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.

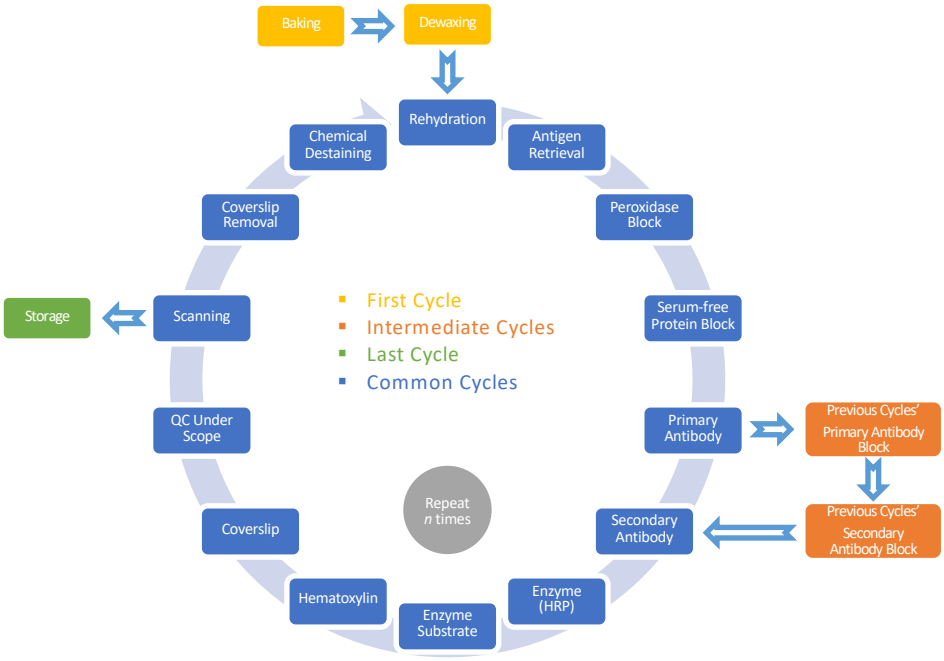

Figure 1: MICSSS Pipeline

2. MATERIALS AND EQUIPMENT

2.1. Critical Reagents

2.1.1. Antigen Retrieval

2.1.1.1. Target Retrieval Solution, pH9 (10x), 500ml (Agilent Cat# S236784-2)

2.1.1.2. Dako Target Retrieval Solution, Citrate pH6 (x10), 500ml (Agilent Cat# S236984-2)

2.1.2. Blocking Reagents

2.1.2.1. Peroxidase Suppressor (Thermo Scientific Cat# 35000)

2.1.2.2. Protein Block Serum-Free Ready-to-Use, 100ml (Agilent Cat# X090930-2)

2.1.2.3. Normal Rabbit Serum (Jackson Immuno Research Cat# 011-000-120)

2.1.2.4. Normal Mouse Serum (Jackson Immuno Research Cat# 015-000-120)

2.1.2.5. Biotin Blocking System (Agilent Cat# X059030-2)

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

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

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

2.1.3 Secondary Antibody and Horse Radish Peroxidase (HRP)

2.1.3.1. EnVision+System-HRP Labelled Polymer Anti-mouse, 110ml (Agilent Cat#K400111-2)

2.1.3.2. EnVision+System-HRP Labelled Polymer Anti-Rabbit, 15ml (Agilent Cat# K400211-2)

2.1.3.3. ImmPRESS REAGENT KIT anti-Rat (Vector MP-7444)

2.1.3.4. Goat anti-Rat whole serum (Jackson Immuno Research Cat#112-001-001)

2.1.3.5. Streptavidin/HRP (Agilent Cat# P039701-2)

2.1.3.6. Biotin-SP-conjugated AffiniPure Fragment Donkey anti-Rabbit IgG (H+L) (Jackson Immuno Research Cat# 711-065-152)

2.1.4. Chromogen

2.1.4.1. AEC Peroxidase Substrate Kit (Vector Laboratories SK4200)

2.1.5. Counterstain

2.1.5.1. Hematoxylin Solution, Harris Modified (Sigma-Aldrich HHS16-500ML)

2.1.6. Mounting Media

2.1.6.1. Glycergel Mounting Media (Agilent Cat# C056330-2)

2.2. Other Reagents

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

2.2.2. Dako Antibody Diluent (Agilent Cat# S080983-2)

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

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

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

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

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

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

2.3. Other Materials and Equipment

2.3.1. Beakers and Measuring Cylinders

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

2.3.3. H_2O milliQ

2.3.4. Vortex machine

2.3.5. Refrigerator (2°- 8°)

2.3.6. Slide Stain Tray

2.3.7. Water Bath (Fisherbrand-Isotemp)

2.3.8. Staining Racks

2.3.9. Slide Boxes

2.3.10. Slide scanner Nanozoomer S60 (Hamamatsu)

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

2.3.12. NUOVA II stir plate (Temrolyne)

Reagent Name	Vendor	Cat#	Dilution	Preparation / Notes
Target Retrieval solution, pH 9.0, conc (x10), 500 mL	Agilent	S236 784-2	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	S236 984-2	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.

Table 1: Critical Regaents

3. SAFETY

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

3.2 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.

3.3 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.

3.4 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.

3.5 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.

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

4. REAGENT PREPARATION

4.1. 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

4.2. Wash Buffer TBS 1X:

18 L H_2O milliQ + 2 L TBA 10X.

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

1L TBS 1X + 0.4 ml Tween20

4.4. Deparaffinization solution 90% Ethanol Absolute

180ml Ethanol Absolute + 20 ml H_2O milliQ

4.5. Deparaffinization solution 70% Ethanol Absolute

140ml Ethanol Absolute + 60ml H_2O milliQ

4.6. Deparaffinization solution 50% Ethanol Absolute

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

4.7. Destaining solution 50% Ethanol Absolute

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

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

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

4.9. Destaining solution 70% Ethanol Absolute

140ml Ethanol Absolute + 60ml H2O milliQ

4.10. Target Retrieval Solution, pH9 (1x)

20ml Target Retrieval Solution, pH9 (10x) + 180ml H2O milliQ

4.11. Dako Target Retrieval Solution, Citrate pH6 (x1)

20ml Dako Target Retrieval Solution, Citrate pH6 (x10) + 180ml H2O milliQ

4.12. Mouse, Rabbit or Rat Serum block solution

Dilute 1:20 Mouse, Rabbit or Rat serum in TBS 1X

4.13. 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

4.14. Primary Antibody Dilution

Dilute antibody at the suggest dilution in Dako Antibody Diluent

4.15. 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

5. PROCEDURE

Day-1 – Bake slides

5.1. Bake slides overnight at 37°C in a slide box, leaving it a bit open. In this way, slides can get dry.

Day-2 – Antibody 1 Staining

Deparaffinization and Rehydration Steps

5.2. 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.

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

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

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

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

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

5.8. Immerse slides in dH_2O for 5 mins (2^{nd} wash) using a staining rack

Heat-induced Epitope Retrieval

5.9. Dilute 10X Target Retrieval solution (RS) (DAKO) to 1X in dH_2O

- Pay close attention to the correct pH for each antigen
- Use pH6, pH8, or pH9 depending on the antigen
- Prepare 40ml for up to 3 slides or 240 ml for the staining rack

5.10. Pre-heat the RS to 95°C in a water bath

5.11. Immerse slides in the 50ml conical tube of 95°C RS

5.12. Incubate the conical tube closed immersed in 95°C water bath for 30 mins

5.13. Remove conical tubes from water bath and place at RT

5.14. Open caps of conical tubes and incubate at RT for 30 mins

5.15. Rinse slides with Tris Buffered Saline (TBS) 1x (prepared in dH_2O) using a staining rack

5.16. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- **CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE**

Blocking

5.17. Cover tissue with 3% peroxide (H_2O_2) and incubate for 15 mins in the humid slide stain tray

- This quenches endogenous peroxidase activity
- Generally 1-3 drops covers tissue

5.18. Rinse slides with TBS 1x using a staining rack

5.19. Dry the back of the slides and around the tissue section Scott C-fold paper towel

- **CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE**

5.20. Cover tissue with Serum-Free- Protein Block (SFPB, Dako), incubate for 30 mins in the humid slide stain tray

5.21. Rinse slides with TBS 1x using a staining rack

5.22. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- **CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE**

Primary Staining

5.23. Dilute primary antibody pre-determined strength in Dako Antibody Diluent to working concentration

5.24. Cover tissue with primary antibody solution and incubate for 1h in the humid slide stain tray

5.25. Rinse slide briefly with TBS 1x using a staining rack

5.26. Immerse slides in TBS + 0.04% Tween 20 for 5 min

5.27. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- **CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE**

Secondary Staining

5.28. Dilute secondary antibody pre-determined strength in TBS 1x to working concentration

5.29. Cover the tissue with secondary antibody solution (Biotinylated Rb or Mouse 1:200 instead Rat 1:500 in TBS 1x) and incubate for 30 mins in the humid slide stain tray or you can use a EnVision Polymer anti-mouse or anti-rabbit ready to use.

5.30. Immerse slides in TBS + 0.04% Tween20 for 5 mins in a staining rack

5.31. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

5.32. Dilute streptavidin – HRP (only if you used Biotinilated antibody) at 1:300 in TBS 1x solution

5.33. Cover the tissue with HRP solution and incubate for 30 mins in the humid slide stain tray

5.34. Immerse slides in TBS + 0.04% Tween20 for 5 mins (1st wash) in a staining rack

5.35. Immerse slides in TBS + 0.04% Tween20 for 5 mins (2nd wash) in a staining rack

5.36. Immerse slides in TBS 1x for 5 mins (1st wash) in a staining rack

5.37. Immerse slides in TBS 1x for 5 mins (2nd wash) in a staining rack

5.38. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

Antigen Detection

5.39. Prepare (fresh) AEC solution (Vector):

- in 5ml of dH_2O:

2 drops of Buffer Stock Solution

3 drops of AEC Stock Solution

2 drops Hydrogen Peroxide Solution

5.40. Cover tissue with AEC solution and incubate for 4-5 mins in the humid slide stain tray

- This incubation step can be up to 30 mins as the color starts to develop in specific manner in the tissue.

- Background non-specific staining will appear homogenously red across the tissue

5.41. Check the intensity of the staining under the microscope

- Look under light microscope with solution still on slide to determine when to stop the color development with AEC solution.

- Short duration of chromogen revelation can cause faint staining (Figure 2).

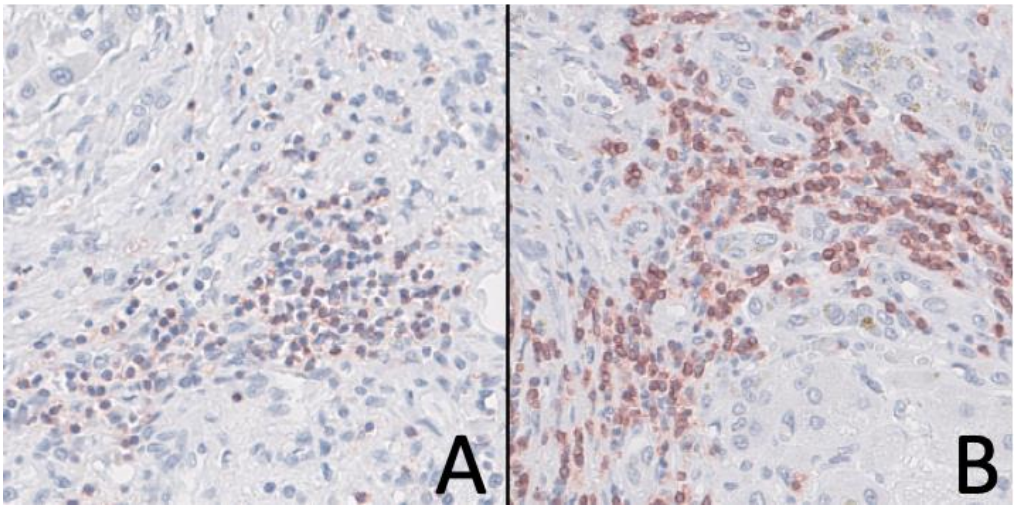

Figure 2: CD3 – hepatocellular carcinoma: A, Faint staining due to short duration of chromogen color development; B, optimal chromogen color development.

5.42. Rinse slides with dH_2O using a staining rack

5.43. Counterstain by immerse slides to 100% hematoxylin for 5 sec

- Hematoxylin is used in multiple times within 4 weeks

5.44. Rinse slides with dH_2O in two big beakers, one beaker of 5L and the other of 2L, if you are working with 24 slide.

- Rinse slides with a lot of hH_2O (around 2L-5L)

5.45. Mount the slides with a coverslip in Aqueous Mounting Medium (Dako)

- Prepare warm mounting medium before use. Warm up the mounting media using a beaker with dH_2O warmed using a NUOVA II stir plate (Temrolyne).

- 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.

5.46. Incubate slides at RT until mounting medium solidifies (or keep the slides at +4°C)

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

Day-3 – REPEAT the staining for the 2nd marker: Bleaching tissue and 2nd marker staining

Bleaching

5.48. 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

5.49. 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

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

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

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

-AEC chromogen and the mouting medium are removed

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

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

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

Heat-induced Epitope Retrieval

5.56. Dilute 10X Target Retrieval solution (RS) (DAKO) to 1X in dH_2O

- Use the correct pH each antigen

- Use pH6, pH8, or pH9 depending on the antigen

- Prepare 40ml for 3 slides or 240 ml for the staining rack

5.57. Pre-heat the Retrieval Solution to 95°C in a water bath

5.58. Immerse slides in 50ml conical tube with pre-heated retrieval solution

5.59. Immerse the conical tube capped in 95°C water bath for **10 mins**

- All hematoxylin is removed

- HRP enzyme is inactivated

5.60. Open caps of conical tubes and incubate at RT for 30 mins

5.61. Rinse slides with Tris Buffered Saline (TBS) 1x (prepared in dH_2O) using a staining rack

5.62. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

Blocking

5.63. Cover tissue with 3% peroxide (H_2O_2) and incubate for 15 mins in the humid slide stain tray

- This quenches endogenous peroxidase activity
- Generally 1-3 drops covers tissue

5.64. Rinse slides with TBS 1x using a stain rack

5.65. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- **CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE**

5.66. Cover tissue with Serum-Free- Protein Block (SFPB, Dako), incubate for 30 mins at RT

5.67. Rinse slides with TBS 1x using a staining rack

5.68. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

5.69. Cover the tissue with Mouse, Rabbit or Rat **SERUM** (depending on your **Marker #1 primary Ab** antibody) **dilution 1:20 in TBS 1x** in the humid slide stain tray **(this step is in order to block FAB fragment free from the secondary antibody)-**

Ex: If **Marker #1 primary Ab it is a mouse antibody you should use mouse serum, instead if Marker #1 primary Ab it is a rabbit you should use a rabbit serum**

5.70. Incubate 30 mins at RT in the humid slide stain tray

5.71. Immerse slides in TBS + 0.04% Tween20 for 5 mins in the staining rack (1st wash)

5.72. Immerse slides in TBS + 0.04% Tween20 for 5 mins in the staining rack (2nd wash)

5.73. Cover the tissue with **FAB-Fragment Donkey** anti-mouse (Life Technology) or anti-Rabbit or anti-Rat (depending of your previous antibody (Marker #1 primary antibody) dilution **1:50 in TBS 1x in the humid slide stain tray (to block Fc fragment from primary antibody)**

5.74. Incubate 30 mins at RT in the humid slide stain tray

5.75. Immerse slides in TBS + 0.04% Tween 20 for 5 mins in the staining rack (1st wash)

5.76. Immerse slides in TBS + 0.04% Tween20 for 5 mins in the staining rack (2nd wash)

Primary Staining

5.77. Dilute primary antibody in Dako Antibody Diluent to working concentration

5.78. Cover tissue with primary antibody solution and incubate for 1h or 2h at RT (depending on the setting please see the table below)

5.79. Rinse slide with TBS 1x – dip the slides gently in 1x TBS in the staining rack

5.80. Immerse slides in TBS +0.04% Tween 20 for 5 min in the staining rack

5.81. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

Secondary Staining

5.82. Dilute secondary antibody in TBS 1x to working concentration (Biotinylated Rb or Mouse 1:200 instead Rat 1:500)

5.83. Cover the tissue with secondary antibody solution (1:200 in TBS 1X) or you can use the EnVision polymer anti-mouse or EnVision polymer anti-rabbit ready to use. and incubate for 30 mins at RT in the humid slide stain tray

5.84. Immerse slides in TBS + 0.04% Tween 20 for 5 mins at RT in the staining rack

5.85. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

5.86. Dilute streptavidin – HRP (only if you used Biotinilated antibody) 1:300 in TBS 1x

5.87. Cover the tissue with HRP solution and incubate for 30 mins at RT in the humid slide stain tray

5.88. Immerse slides in TBS + 0.04% Tween 20 for 5 mins (1^{st} wash) in the staining rack

5.89. Immerse slides in TBS + 0.04% Tween 20 for 5 mins (2^{nd} wash) in the staining rack

5.90. Immerse slides in TBS 1x for 5 mins (1^{st} wash) in the staining rack

5.91. Immerse slides in TBS 1x for 5 mins (2^{nd} wash) in the staining rack

5.92. Dry the back of the slides and around the tissue section using Scott C-fold paper towel

- CAREFUL: DO NOT TOUCH/DISTURB THE TISSUE

Antigen Detection

5.93. Prepare (fresh) AEC solution (Vector):

- in 5ml of dH_2O: add
    - 2 drops of Buffer Stock Solution
    - 3 drops of AEC Stock Solution
    - 2 drops hydrogen Peroxide Solution

5.94. Cover tissue with AEC solution and incubate for 4-5 mins

- This incubation can be up to 30 mins depending on color development
- Background staining will appear homogenously red

5.95. Check the intensity of the staining under the microscope

- Look under light microscope with solution still on slide to determine when to stop the color development

5.96. Rinse slides with dH_2O using the staining rack

5.97. Counterstain by adding slides to 100% hematoxylin for 5 sec using the staining rack

- Hematoxylin can be used multiple times within 2 weeks.

5.98. Rinse slides with dH_2O with wash bottle

- Rinse slides with a lot of hH_2O (around 2L - 5L) using a big beaker

5.99. Mount the slides using a coverslip in Aqueous Mounting Medium (Dako)

- Need to warm mounting medium before use
- Use 100ul per cover slip, without making bubbles, and changing tip
- Then add slide on top

5.100. Incubate slides at RT until mounting medium solidifies (leaves the slides +4°C)

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

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

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.