IHC Multiplex

Introduction

IHC multiplex protocol for immune and cancer cells markers plus protein of interest. paraffin-embedded cuts on Tomo slides were used.

Materials

- Xylene
- Ethanol
- ddH2O
- Hematoxylin (T865.2)
- Eosin (HT110216-500ML)
- Aqueous mounting medium
  - 70% Glycerol
  - 30% ddH_2O
- PBS
- PBS-T
  - 0.1% Tween in PBS
- 10X Antigen Retrieval Buffer
  - 100mM citric acid
  - 20mM EDTA
  - in 800ml ddH2O
  - pH to 6.2 with NaOH
  - 0.5% Tween20
  - fill up to total 1000ml (can be stored at 4°C)
- 3% H_2O_2 (1A8Y.1)
- Aurion BSA 10% (900.022)
- Antibody stripping buffer
  - 1% SDS
  - 25mM Glycine
  - set pH to 2
  - in ddH2O
- Picro-sirius red stain solution
  - 0.1% direct red 80 (365548-5G)
  - 0.1% fast green FCF (F7252-5G)
  - Dissolved in saturated picric acid solution 1.3% in H2O (P6744-1GA)
- Secondary Antibodies ready to use

- DAKO EnVision+/HRP, Rabbit (K4003)
- DAKO EnVision+/HRP, Mouse (K4001)

- Primary Antibodies and the sequence of staining: please look at the end of protocol
- ImmPACT AMEC (SK-4285)
- Fast Red Substrate Kit (ab64254)
- VectaMount Permanent Mounting Medium (H-5000)

Procedure

Day 1

Day 1

	A	B	C	D	E	F
1	**Deparaffinize**	1x	10min	r.t.	Xylene	
2		1x	10min	r.t.	Xylene	
3		1x	10min	r.t.	Xylene	
4	**Rehydrate**	2x	5min	r.t.	100% Ethanol	
5		1x	5min	r.t.	95% Ethanol	
6		1x	5min	r.t.	70% Ethanol	
7		1x	5min	r.t.	50% Ethanol	
8		1x	5min	r.t.	ddH2O	
9	**H&E**	1x	1min	r.t	Hematoxylin	
10			2min	r.t	Running tab water	
11		1x	4min	r.t.	Eosin	
12		2x	10sec	r.t.	ddH2O	
13	**Mounting**				Aqueous mounting medium	
14	**Scanning**					
15	**Remove coverslip**	1x	>20min	r.t.	PBS	Do not pull the coverslip off!
16	**washing**	1x	2min	r.t.	ddH2O	
17	**Antigen Retrieval**	1x	35min	125°C	1X Antigen Retrieval Buffer (ARB)	in a pressure cooker
18	**Cool down**		30min		Remaining in the ARB	
19	**Washing**		5min		ddH2O	
20	**All of the following steps are carried out in a humidified chamber in the dark**					
21	**H2O2 Blocking**	1x	20min	r.t.	3% H2O2	in the dark
22	**Washing**	1x	5min	r.t.	ddH2O	
23		1x	2min	r.t.	PBS	
24	**Blocking**	1x	60min	r.t.	Blocking buffer	see primary ABs table
25	**Primary Ab**		o.n.	4°C	1:x	see primary ABs table

Day2

Day 2

	A	B	C	D	E	F
1	Washing	3x	7min	r.t.	PBS-T	
2	Secondary Ab	1x	60min	r.t.	Ready-to-use	Based on host of the primary Ab
3	Washing	3x	7min	r.t.	PBS-T	
4	Staining development	1x		r.t.	AMEC staining solution	Under the microscope
5	Stopping HPO reaction	1x	>1min	r.t.	ddH2O	
6	Hematoxylin	1x	1min	r.t.	hematoxylin	
7	Washing		2min	r.t.	Running tab water	
8	Mounting				Aqueous mounting medium	
9	Scanning					
10	Remove coverslip	1x	>20min	r.t.	PBS	Do not pull the coverslip off!
11	washing	1x	2min	r.t.	ddH2O	
12	AMEC distaining	1x	1min	r.t.	70% Ethanol	
13		1x	3min	r.t.	95% Ethanol	
14		1x	1min	r.t.	70% Ethanol	
15		1x	2min	r.t	ddH2O	
16	Antibody stripping	1-2x	20min	55°C	Antibody stripping buffern pH 2	in shaking waterbath (90 rpm), also see primary ABs table
17	PH balancing	1x	7min	r.t.	Antibody stripping buffer before pH adjusting (pH 6-7)	
18	Washing	1x	5min	r.t.	ddH2O	
19		1x	2min	r.t.	PBS	
20	Blocking	1x	60min	r.t.	Blocking buffer	see primary ABs table
21	Primary Ab		o.n.	4°C	1:x	see primary ABs table
22	Go back to step 1 in day 2 (washing) and continue the cycle of multiplex					

Primary Antibodies and the sequence of staining

	A	B	C	D	E	F	G	H	I
1	**Antibody**	**Reactivity**	**Host**	**Application**	**Company**	**#Cat**	**Blocking in Aurion BSA**	**Dilution in Aurion BSA**	**Stripping**
2	**SMPD1**	H	R	Ihc-miltiplex	LSBio	LS-C334919	5% 1h R.T.	1:500 in 3% o.n.	2x 20min
3	**CD8**	H	M	Ihc-miltiplex	Cell Marque	108M-94	1% 1h R.T.	1:100 in 1% o.n.	1x 20min
4	**CD206**	H	M	Ihc-miltiplex	R&D	MAB25341	3% 1h R.T.	1:50 in 3% o.n.	2x 20min
5	**PHH3**	H	R	Ihc-miltiplex	cell signaling	cs: 53348	1% 1h R.T.	1:100 in 1% o.n.	1x 20min
6	**CD68**	H	M	Ihc-miltiplex	Dako	M0876	2% 1h R.T.	1:100 in 2% o.n.	1x 20min
7	**CK19**	H	M	Ihc-miltiplex	Dako	M0888	2% 1h R.T.	1:85 in 0.5% o.n.	stained with fast red no stripping
8	**CD3**	H	M	Ihc-miltiplex	Dako	M7254	1% 1h R.T.	1:70 in 1% o.n.	1x 20min
9	**CD45**	H	M	Ihc-miltiplex	Dako	M0701	1% 1h R.T.	1:70 in 1% o.n.	no stripping, only destaining of Amec
10	**Picro-sirius red staining**								

Picro-sirius red staining

	A	B	C	D	E	F
1	Picro-sirius red staining	1x	60min	r.t.	Picro-sirius red solution	in the dark
2	Washing	2x	30sec	r.t.	ddH2O	
3	Dehydration	1x	30sec	r.t.	50% Ethanol	
4		1x	30sec	r.t.	70% Ethanol	
5		1x	30sec	r.t.	95% Ethanol	
6		2x	30sec	r.t.	100% Ethanol	
7		3x	5min	r.t.	Xylene	
8	Mounting				VectaMount Permanent Mounting Medium	
9	Scanning					

Notes

1. CK19 AB is hard to strip, so we stained it with the Fast red (permanent stain), which has a different color from ImmPACT AMEC (washable stain). the following ABs were in different cell compartment.

2. After the last AB cycle, we only destain AMEC and procende with Picro-sirius red staining.