May 06, 2025

FIMM Digital Microscopy and Molecular Pathology Core Unit

Theranostics

DOI
https://dx.doi.org/10.17504/protocols.io.rm7vz6775gx1/v1

Teijo Pellinen[1], Nora Linnavirta[1], Annabrita Schoonenberg[1], Katja Välimäki[1]

[1]University of Helsinki

Teijo Pellinen
University of Helsinki, Institute for Molecular Medicine Fin...

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.rm7vz6775gx1/v1

External link: https://doi.org/10.7150/thno.118400

Protocol Citation: Teijo Pellinen, Nora Linnavirta, Annabrita Schoonenberg, Katja Välimäki 2025. FIMM Digital Microscopy and Molecular Pathology Core Unit. **protocols.io** https://dx.doi.org/10.17504/protocols.io.rm7vz6775gx1/v1

Manuscript citation:
Pellinen T, Luomala L, Mattila KE, Hemmes A, Välimäki K, Arjama M, Brück O, Paavolainen L, Kankkunen E, Nisén H, Järvinen P, Castillon L, Vanharanta S, Vainio P, Kallioniemi O, Jaakkola PM, Mirtti T (2026) Tumor cell FAP orchestrates EMT and immune suppression in aggressive localized ccRCC. Theranostics 16(7). doi: 10.7150/thno.118400

Protocol status: Working
We use this protocol and it's working

Created: April 01, 2025

Last Modified: May 06, 2025

Protocol Integer ID: 131026

Keywords: FIMM Digital Microscopy, Molecular Pathology Core Unit, AlexaFluor stainings, Antigen retrieval, Antibodies detection, multiplex immunofluorescence workflow, molecular pathology core unit this protocol, fimm digital microscopy, more protein marker, molecular pathology core unit, iterative rounds of antibody, antibody denaturation, mediated antibody denaturation, antibody, secondary staining cycles without tsa, antigen retrieval, seamless integration with scalable bioinformatics pipeline, scalable bioinformatics pipeline, secondary staining cycle, profiling formalin, induced antigen retrieval, tumor microenvironment, embedded tissue section, marker, immunofluorescence

Funders Acknowledgements:
Research Council of Finland
Grant ID: 363152

Abstract

This protocol describes a multiplex immunofluorescence workflow developed at the FIMM Digital Microscopy and Molecular Pathology Core Unit for profiling formalin-fixed, paraffin-embedded tissue sections. It combines dual-color tyramide signal amplification (TSA) with iterative rounds of antibody stripping and non-TSA HRP-based secondary detection to visualize six or more protein markers on a single slide. Key steps include slide mounting and drying; deparaffinization and rehydration; heat-induced antigen retrieval; endogenous peroxidase blocking; two sequential TSA reactions; heat-mediated antibody denaturation; and up to four additional HRP-secondary staining cycles without TSA. After each cycle, slides are scanned at high resolution for automated image analysis. This highly flexible system permits user-defined marker panels, fluorophores and cycle numbers. Although staining can be extended to include more rounds, repeated stripping may eventually weaken or detach tissue, depending on sample quality. Optimized for reproducibility and seamless integration with scalable bioinformatics pipelines, this protocol provides a versatile tool for detailed spatial mapping of immune and stromal populations in the tumor microenvironment.

Materials

Protocol design

Antibodies (Ab1, Ab2...) preferably in mouse-rabbit pairs

	A	B
	Detection	**Multiplex Panel**
	TSA488	Ab1
	TSA555	Ab2
		Boil
	AF647	Ab3
	AF750	Ab4
	scan 1	Bleach and boil
	AF647	Ab5
	AF750	Ab6
	scan 2	Bleach and boil
	AF647	Ab7
	AF750	Ab8
	scan3	Bleach and boil
	AF647	Ab9
	AF750	Ab10
	scan4	

Reagents and materials

	A	B	C	D
	Category	**Name**	**Company/ cat.**	**Number Dilution/ lot.nr.**
	rehydration Alcohol Absolute	VWR/ 20821.365		
	rehydration	Alcohol 96%	VWR/ 20824.365	
	rehydration	Xylene	VWR/534056	

	A	B	C	D
	HIER	100mM Tris-10mM EDTA, pH9	Home made	Dilute 10x in Milli-Q
	wash	10x TBS	Home made	Dilute 10x in Milli-Q
		Tris Base	Fisher BP152-5	
		NaCl Fisher	BP358-212	
	wash	Tween	Fisher/10113103	
	block	H2O2	Fisher/ 10736291	
	block	Normal goat serum (NGS)	Gibco/ 16210-064	
	secondary antibodies	G-a-M-AF 647	Life/ A21236	
	secondary antibodies	G-a-R-AF 647	Life/ A21245	
	secondary antibodies	G-a-M-AF 750	Life/ A21037	
	secondary antibodies	G-a-R-AF 750	Life/ A21039	
	secondary antibodies	G-a-M-poly HRP	Immunologic/ DPVM55HRP	
	secondary antibodies	G-a-R-poly HRP	Immunologic/ DPVR110HRP	
	Fluorescent substrate	Tyramide 488	Life/ B40953	
	Fluorescent substrate	Tyramide 555	Life/ B40955	
	Counter staining	Dapi	Roche/ 10236276001	
	Mounting medium (fluorescence)	Prolong Gold	Life/ P36934	
	Microscope slides	SuperFrost Plus/ Fisher J3800AMNZ or similar		
		PT Module	Thermo Scientific	
	Fluorescent scanner	3D Histech; Pannoramic Zeiss; Axioscan z.1		

Buffer/ reagent Preparation

Xylene/ alcohol series

Change the solutions every month!

Wash buffers

TBS: Prepare 10x TBS pH 7.6 from Tris-Base and NaCl. Store at Room temperature max 6 months. Dilute 10x with Milli-Q to 1x TBS buffer, store at Room temperature max 1 week.

TBST: Dilute 10x TBS in Milli-Q and add 1 mL 50% Tween (in Milli-Q)/ 1 L (end conc. tween= 0.05%), store at Room temperature max 1 week.

Endogenous peroxidase block

0.9% H_2O_2 in TBS

	A	B
	TBS	250 ml
	30% H_2O_2	7.5 ml

Protein block: 10% NGS in TBST

	A	B
	TBST	45 ml
	NGS (fresh aliquot from -20°C)	5 ml

Mix and filter (low protein binding 0.22 µm or 0.45 µm), store at 4 °C, max 1 week

Primary Antibodies:

Dilute in 10% NGS in TBST

Secondary Antibodies:

Dilute in TBST

TSA (tyramide) reagents:

Prepare 0.3% H_2O_2 just before use!

Dilute 30% H_2O_2 stock 1:100 in TBST!

For 100 µL TSA reagent: add 0.5 µL 1:100 diluted H_2O_2 (final dilution 1:20.000) and 1 µL Tyramide fluorochrome (final dilution 1:100), mix well.

Note

Use within 01:00:00, avoid exposure to light.

Bleaching solution

for 200 ml: 30 mL 30% H_2O_2+ 4.8 mL [M] 1 Mass Percent NaOH, add TBS to 200 mL.

- Ethanol absolute ≥99.8% **VWR International (Avantor) Catalog #**20821.365
- Ethanol GPR RECTAPUR® **VWR International (Avantor) Catalog #**20824.365
- Xylene (mixture of isomers) for histology **VWR International (Avantor) Catalog #**534056-4L
- Tris Base (White Crystals or Crystalline Powder/Molecular Biology) **Fisher Scientific Catalog #**BP152-5
- Sodium Chloride **Fisher Scientific Catalog #**BP358-212
- Polysorbate 20/Tween **Fisher Scientific Catalog #**10113103
- Hydrogen Peroxide 30-32% (w/w) (100 Volumes), Certified AR for Analysis, Fisher Chemical™ **Fisher Scientific Catalog #**10736291
- Goat Serum **Gibco - Thermo Fisher Scientific Catalog #**16210-064
- Goat anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 **Thermo Fisher Scientific Catalog #**A-21236
- Goat anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 **Thermo Fisher Scientific Catalog #**A-21245
- Goat anti-Mouse IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 750 **Thermo Fisher Scientific Catalog #**A-21037
- Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 750 **Thermo Fisher Scientific Catalog #**A-21039
- BrightVision, 1 step detection system Goat Anti- Mouse HRP / Goat Anti- Rabbit AP **Immunologic a WellMed Company Catalog #**DPVM55HRP
- BrightVision, one component detection system Goat Anti- Rabbit HRP / Mouse AP **Immunologic a WellMed Company Catalog #**DPVO110-rHRP-mAP
- Tyramide Conjugates **Thermo Fisher Scientific Catalog #**B40953
- Alexa Fluor™ 555 Tyramide Reagent **Thermo Fisher Catalog #**B40955
- DAPI **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**10236276001
- ProLong™ Gold Antifade Mountant **Thermo Fisher Scientific Catalog #**P36934

Protocol

1 Mount sections on microscope slides and attach by drying Overnight at Room temperature or 37 °C .

2 **Optional**: keep slides 15-30 min at 60 °C before starting paraffin removal.

Paraffin removal and rehydration 23m

3 Place the slides in a plastic slide holder!

4 Immerse the slides in xylene and alcohol solutions in the following order.

4.1 Xylene for 00:05:00 . (1/3) 5m

4.2 Xylene for 00:05:00 . (2/3) 5m

4.3 Xylene for 00:05:00 . (3/3) 5m

4.4 Alcohol absolute for 00:01:00 . (1/3) 1m

4.5 Alcohol absolute for 00:01:00 . (2/3) 1m

4.6 Alcohol absolute for 00:01:00 . (3/3) 1m

4.7 96% alcohol for 00:01:00 . (1/2) 1m

4.8 96% alcohol for 00:01:00 . (2/2) 1m

4.9 70% alcohol for 00:01:00 . 1m

4.10 Milli-Q water for 00:01:00 . (1/2) 1m

4.11 Milli-Q water for 00:01:00 . (2/2) 1m

Antigen retrieval 30m

5 HIER:

5.1 Place the slides in [M] 10 millimolar (mM) Tris and [M] 1 millimolar (mM) EDTA buffer pH 9 in the PT module.

5.2 Heat for 00:20:00 at 99 °C , total program lasts 01:10:00 . 20m

5.3 Wash 00:05:00 in Milli-Q and 00:05:00 in TBS. 10m

Note

NOTE: The staining can be performed using an autostainer, for which the following steps of the manual protocol must be programmed to the autostainer that is used.

Endogenous peroxidase block 15m

6 Immerse the slides in 0.9% H_2O_2 in TBS for 00:15:00 at Room temperature . 15m

7 Wash.

7.1 Wash 2× 3-5 minutes in TBS.

7.2 Wash 1x TBST.

Staining protocol: First 2 antibodies, detected with TSA reagents

3h 45m

8 Dry the slides around the specimen using lint-free tissue.

9 Apply 100-300 µL 10% NGS in TBST per slide, make sure the specimen is covered completely.

10 Incubate for 00:15:00 at Room temperature .

15m

11 Dilute the primary antibody or antibodies in 10% NGS in TBST.

12 Tap off the solution and apply 100-300 µL diluted primary antibody.

13 Incubate for 01:00:00 at Room temperature or Overnight at 4 °C (depending on the antibody).

1h

14 Wash 3× 3-5 min TBST.

15 Apply 100-300 µL 1:5 diluted (in TBST) Bright Vision goat anti mouse or rabbit HRP per slide.

16 Incubate 00:30:00 Room temperature .

30m

17 Wash 3× 3-5 min TBST.

18 Prepare the TSA-AF488 reagent just before use.

19 Pipet 100-300 µL reagent per slide and incubate 00:15:00 Room temperature . 15m

20 Wash 3× 3-5 min TBST.

21 **Note**: In case the second primary antibody is made in the same species as the first: 20m
- Denature the previous antibody by heating the slides in **freshly prepared** [M] 10 millimolar (mM) Tris and [M] 1 millimolar (mM) EDTA pH 9 in the PT module at 99 °C for 00:20:00 .
- Cool in MilliQ-water 5min. Perform endogenous peroxidase block and then follow steps 8-14 of the staining protocol.
- Continue for TSA-AF555 detection with step 24.

22 Immerse the slides in 0.9% H_2O_2 in TBS for 00:15:00 Room temperature . 15m

23 Wash 1× 3-5 minutes in TBS and 1x TBST.

24 Apply 100-300 µL 1:5 diluted (in TBST) Bright Vision goat anti mouse or rabbit HRP per slide.

25 Incubate 00:30:00 Room temperature . 30m

26 Wash 3× 3-5 min TBST.

27 Prepare the TSA-AF555 reagent just before use.

28 Pipet 100-300 µL reagent per slide and incubate 00:15:00 Room temperature . 15m

29 Wash 3× 3-5 min TBST and store at 4 °C Overnight if not proceeding immediately.

30
- After the second TSA reaction denature the previous antibody or antibodies by heating the slides in **freshly prepared** [M] 10 millimolar (mM) Tris and [M] 1 millimolar (mM) EDTA pH 9 in the PT module at 99 °C for 00:20:00 .
- Cool in MilliQ water 00:05:00 .

25m

31 Continue to AlexaFluor staining.

Staining protocol: AlexaFluor staining

45m

32 Dry the slides around the tissue.

33 Apply 100-300 µL 10% NGS in TBST per slide, make sure the tissue is covered completely.

34 Incubate 00:15:00 Room temperature .

15m

35 Dilute the primary antibodies in 10% NGS in TBST.

36 Tap off the blocking solution and apply 100-300 µL diluted primary antibodies.

37 Incubate 1-2h Room temperature or Overnight 4 °C .

38 Wash 3× 3-5 min TBST.

39 Dilute the secondary, AlexaFluor conjugated, antibodies in TBST, 1:300, add Dapi, final concentration 1.7 µL .

40 Apply 100-300 µL of the secondary reagent solution per slide and incubate 00:30:00 at Room temperature . 30m

41 Wash 3× 3-5min TBST and 1x 00:05:00 Milli-Q.

42 Dry slides at Room temperature .

43 Mount sections in ProlongGold anti-fade mounting medium, use coverslips with thickness: no. 1.5.

44 Dry the slides before scanning, avoid exposure to light.

45 Scan/image the fluorescence using the appropriate filters.

46 After scanning, soak off the coverslips in TBST at Room temperature or 4 °C .

Staining protocol: Second to fourth round AlexaFluor stainings 45m

47 Wash the slides after the coverslips are soaked off in TBS.

48 Bleach the attached AF647 and AF750 by soaking the slides in TBS/NaOH/H_2O_2, 30-60 min Room temperature .

49 Rinse in 1x TBS and MilliQ.

50
- Denature the previous antibodies by heating the slides in **freshly prepared** [M] 10 millimolar (mM) Tris and [M] 1 millimolar (mM) EDTA pH 9 in the PT module at 99 °C for 00:20:00 . 30m
- Cool in MilliQ-water 00:05:00 and wash in TBS 00:05:00 .

51 Apply 100-300 µL 10% NGS in TBST per slide, make sure the tissue is covered completely.

52 Incubate 00:15:00 Room temperature . 15m

53 Dilute the primary antibodies in 10% NGS in TBST.

54 Tap off the blocking solution and apply 100-300 µL diluted primary antibodies.

55 Incubate 1-2h Room temperature or Overnight 4 °C .

56 Wash 3× 3-5min TBST.

57 Dilute the secondary, AlexaFluor conjugated, antibodies in TBST, 1:300, add Dapi, final concentration 3.4 µL (1:150).

58 Apply 100-300 µL of the secondary reagent solution per slide and incubate 30-45 minutes at Room temperature .

59 Wash 3× 3-5min TBST and 1x 00:05:00 Milli-Q.

60 Dry slides at Room temperature .

61 Mount sections in ProlongGold anti-fade mounting medium, use coverslips with thickness: no. 1.5.

62 Dry the slides before scanning, avoid exposure to light.

63 Scan/image the fluorescence using the appropriate filters.

64 After scanning, soak off the coverslips in TBST if more rounds are stained.