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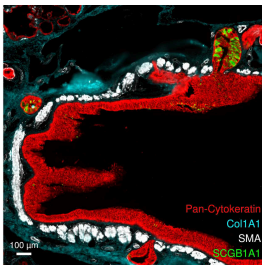
812.2 Lung FFPE Multiplexed Immunofluorescence Phenocycler-Fusion® Antibody Validation Protocol



Forked from 812.1 Lung FFPE OMAP Multiplexed Immunofluorescence Phenocycler-Fusion® Antibody Validation Protocol

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We use this protocol and it's working

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Abstract

This protocol describes validation of a 45-antibody panel used for multiplexed immunofluorescent (MxF) staining and imaging of FFPE lung tissue sections utilizing the Phenocycler-Fusion® 2.0 platform (Akoya Biosciences). The validation strategy involves staining two serial lung sections, one with a cocktail of 45 antibodies either custom (in-house) barcode-conjugated antibodies or antibody-barcoded conjugates sourced from Akoya Biosciences, and the second with the respective carrier-free, unconjugated (prior to conjugation with oligo barcode tag) control antibodies. The commercially conjugated Akoya-sourced antibodies were omitted from the second section.
In addition, a section of kidney cortex was exposed to the fully conjugated antibody cocktail to confirm specificity of antibodies directed against lung specific markers, and specificity of pan-epithelial, endothelial, megakaryocyte, and immune cell markers in 2 different tissues (e.g. lung, kidney) as cross-organ, high level cell type validation. The protocol includes sections describing 1) Tissue sections, 2) Labeling with barcode-conjugated or unconjugated Ab, 3) Reporter Plate and Experimental Design, 4) Multiplexed Imaging and Image Upload to OMERO for review, and 5) Review criteria. Detailed descriptions of staining, imaging and conjugation procedures can be found in a companion protocol **dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v3**.

Attachments

PDF

SOP_ Construction of...

285KB

Guidelines

In order to validate both commercial and custom conjugated antibody specificity, control tissue sections were selected for the expected presence of specific tissue cells and structures, expected to be selectively marked by the panel of antibodies to be tested. In multiplexed Ab panels, inclusion of antibodies directed against general or pan-markers of epithelial, endothelial, and immune cells is recommended, as they will serve as positive controls for tissue section quality and effectiveness of the staining protocol when assessing the presence or absence of staining by tissue specific markers. No primary antibody tests, in this case, Reporters-only without conjugated-antibodies, are also recommended to assess non-specific background.

Materials

A complete list of materials and supply can be found in the companion protocol referenced within.

Before start

Review HubMAP guidelines for Ab validation in the attached SOP for OMAP construction

Tissue Sections

1 Lung serial sections, and a kidney section were prepared in FFPE as described in dx.doi.org/10.17504/protocols.io.kxygxejwdv8j/v2

Labeling of Tissue Sections with barcode conjugated Ab or unconjugated Ab

2 One of two serial healthy Lung sections (D438-RLL-16B4-17), and a Kidney cortex section (K2300768-5-3) was labeled with a 45-marker panel (Tables 1 &2) of barcode antibodies as described in dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v3 The other lung serial section (D438-RLL-16B4-16) was labeled, for non-specific staining control, with a panel of carrier free, unconjugated antibodies used for custom conjugations from step 18.1 in the above protocol and as listed in Table 1.

Table 1. Antibodies used for custom conjugations.

	Ab-Barcode	Unconjugated Ab	Dilution	Vendor	Cat #
	TPSAB1-BX041	TPSAB1	1:1000	Abcam	ab2378
	SOX9-BX046	SOX9	1:1000	Cell Signaling	74021SF
	SFTPC-BX020	SFTPC	1:500	Invitrogen	PA5-71842
	MUC5AC-BX040	MUC5AC	1:500	Abcam	ab212636
	FOXJ1-BX036	FOXJ1	1:500	Thermofischer	14-9965-82
	GRP-BX030	GRP	1:500	LSBio	LS-C755064
	TUBB3-BX055	TUBB3	1:400	R&D Systems	MAB1195
	SCGB3A2-BX034	SCGB3A2	1:400	Abcam	ab240255
	KRT8-BX022	KRT8	1:400	Biolegend	904804
	CD55-BX027	CD55	1:200	R&D Systems	AF2009
	PF4-BX004	PF4	1:200	Peprotech	500-P05
	LYVE1-BX025	LYVE1	1:200	R&D Systems	AF2089
	ATP1A1-BX005	ATP1A1	1:200	Abcam	ab167390
	SFTPB-BX010	SFTPB	1:200	ThermoFischer	PA5-42000
	SCGB1A1-BX043	SCGB1A1	1:100	R&D Systems	MAB4218

	Ab-Barcode	Unconjugated Ab	Dilution	Vendor	Cat #
	AGER-BX028	AGER	1:100	Abcam	ab228861
	COL1A1-BX054	COL1A1	1:100	Abcam	ab88147
	TP63-BX006	TP63	1:75	Abcam	ab214790
	CD1c-BX016	CD1c	1:75	Novus	ab156708
	SCEL-BX052	SCEL	1:75	Abcepta	AP11564C
	MUC5B-BX049	MUC5B	1:75	Abcam	AB87376
	PROX1-BX050	PROX1	1:50	Abcam	ab236026

For antibodies containing sodium azide (0.05-0.1%) or trehalose (5%), buffer exchange was performed utilizing Zeba™ Spin Desalting columns 7K MWCO (89890, 2ml, Thermo-Fisher Scientific) equilibrated in PBS in accordance with the manufacturer's recommendations prior to conjugation with nucleic acid tag (**ref)**.

Table 2. Antibodies commercially conjugated (Akoya Biosciences).

	Ab-Barcode	Dilution	Vendor	Cat #
	CD31-BX001	1:200	Akoya	4450017
	KRT14-BX002	1:200	Akoya	4450031
	CD4-BX003	1:200	Akoya	4550112
	CD20-BX007	1:200	Akoya	4450018
	SMA-BX013	1:200	Akoya	4450049
	ECAD-BX014	1:200	Akoya	4250021
	CD68-BX015	1:200	Akoya	4550113
	PanCK-BX019	1:200	Akoya	4450020
	CD45-BX021	1:200	Akoya	4550121
	CD11c-BX024	1:200	Akoya	4550114
	CD8-BX026	1:200	Akoya	4250012
	FOXP3-BX031	1:200	Akoya	4550071
	HLADR-BX033	1:200	Akoya	4550118
	CD14-BX037	1:200	Akoya	4450047

	Ab-Barcode	Dilution	Vendor	Cat #
	COLIV-BX042	1:200	Akoya	4450122
	CD3e-BX045	1:200	Akoya	4550119
	Ki67-BX047	1:200	Akoya	4250019
	CD163-BX069	1:200	Akoya	4250079
	CAV1-BX086	1:200	Akoya	4550084
	MPO-BX098	1:200	Akoya	4250083
	Keratin5-BX101	1:200	Akoya	4450090
	SOX2-BX102	1:200	Akoya	4250075
	PDPN-BX121	1:200	Akoya	4250094

Note that unconjugated forms of barcode-conjugated antibodies purchased from Akoya Biosciences are not included for validation. The majority of Akoya-sourced antibody clones have previously been validated for Akoya multiplexed immunofluorescence assessment of FFPE tissue.
The antibody dilutions correspond to working dilutions for the respective barcode-conjugated Ab.

Reporter Plate and Experiment Design

3 Reporter plate design and Phenocycler-Fusion run protocols were developed using the PhenoCycler Experiment Designer Software 2.1.0 (Akoya Biosciences) as described in **dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v3**. The Reporter Plate design and exposures used for validation of the Lung FFPE OMAP-45 Panel is shown in Table 3. Note that all 3 slides included in the validation were exposed to the same reporter panel.

Table 3. Lung FFPE 45 Marker Panel Plate Design and exposures
Cycle #, reporters and exposure times

	ATTO550	Exp (ms)	Cy5	Exp (ms)	AF750	Exp (ms)
	Col1A1-RX054	150	HLADR-RX033	50	CD31-RX001	100
	SFTPC-RX020	125	CD45-RX021	50	CXCL4-RX004	150
	ATP1A1-RX005	125	FOXP3-RX031	150	LYVE1-RX025	200
	TPSAB1-RX041	15	CD11c-RX024	125	AGER-RX028	200

	ATTO550	Exp (ms)	Cy5	Exp (ms)	AF750	Exp (ms)
	CD163-RX069	150	COLIV-RX042	150	KRT5-RX101	50
	MPO-RX098	35	CD1c-RX016	150	SCEL-RX052	250
	TUBB3-RX055	125	TP63-RX006	75	SCGB1A1-BX043	75
	CDH1-BX014	150	FOXJ1-RX036	150	MUC5AC-RX040	75
	KRT14-RX002	100	CD55-RX027	100	SCGB3A2-RX002	100
	PROX1-RX050	300	GRP-BX030	150	CAV1-BX086	25
	PDPN-BX121	150	SFTPB-BX010	50	MUC5B-BX049	250
	SOX2-BX102	50	SOX9-BX046	50	KRT8-BX022	50
	Ki67-RX047	75	CD68-RX015	10	CD20-RX007	150
	CD14-BX037	75	CD4-RX003	75	ACTA2-RX013	100
	CD8-RX026	150	CD3e-RX045	75	PanCK-RX019	50

DAPI exposure 3 milliseconds (ms)

Multiplexed Imaging and Upload to OMERO for review

4 Kidney and Lung Sections were stained and imaged as described in **dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v3**.

4.1 45 Marker Panel Validation consisted of the following slide runs.

	Source ID	Sample ID	Lab ID	Tissue	Panel
	TBD	TBD	K2300768-5-3	Kidney Cortex	45-marker
	NA	NA	D438-RLL-16B4-16	Normal Lung	Unconjugated Ab

	Source ID	Sample ID	Lab ID	Tissue	Panel
	HBM365.VQTL.758	HBM958.DRDX.792	D438-RLL-16B4-17	Normal Lung	45-marker
	TBD	TBD	D260-RLL-15C3-2	Normal Lung	45-marker
	TBD	TBD	D264-RLL-12B3-2	Normal Lung	45-marker
	TBD	TBD	D271-RLL-15B7-3	Normal Lung	45-marker
	TBD	TBD	D273-RLL-15B3-2	Normal Lung	45-marker
	TBD	TBD	D292-RLL-19B4-2	Normal Lung	45-marker
	TBD	TBD	D341-RLL-17C4-2	Normal Lung	45-marker
	TBD	TBD	D342-RLL-18A6-11	Normal Lung	45-marker
	TBD	TBD	D346-RLL-21C5-2	Normal Lung	45-marker
	NA	NA	D288-RLL-10B2-2	Asthmatic Lung	45-marker
	NA	NA	D294-RLL-7A2-3	Asthmatic Lung	45-marker
	NA	NA	D376-RLL-7A2-2	Asthmatic Lung	45-marker

TBD= To be determined; NA= Not Applicable

4.2 The resulting .qptiff images are converted to ome.tiffs utilizing command line: C:\>bfconvert -no-sas -series 0 -compression LZW -pyramid-resolutions 5 -pyramid-scale 2 INPUT.qptiff OUTPUT_ome.tiff.

4.3 Utilizing Omero importer, ome.tiff files are uploaded to the URMC Omero-CODEX folder and the respective validation subfolder (i.e. OMAP45 validation) for review.

Review Criteria

5 Validation of marker signals is reviewed by a 3-person panel that includes the protocol author (MxIF Research Scientist, Dr. Jeff Purkerson), Dr. Gloria Pryhuber (Principal investigator), and Dr. Gail Deutsch (Board Certified Pathologist, Pulmonary specialist).

5.1 Lung and kidney sections are reviewed for marker fluorescence signal localization to appropriate anatomical structures (e.g. airway, vasculature) and cell types within anatomical structures (e.g. alveolar epithelia, submucosal glands, vascular and lymphatic endothelium, immune cell clusters), colocalization with appropriate pan-markers (e.g. MUC5AC or/and MUC5B with PanCK; SCEL with RAGE, SFTPB with SFTPC etc.).

Expected result

All Barcode-Ab conjugates identify specific cellular and anatomical features in lung tissue sections. Note that all Ab barcode conjugates in Table 1 fulfilled this criterion.

5.2 Slides labeled with unconjugated Ab are assessed for the contribution, if any, of nonspecific reporter binding of fluorescent signals.

Expected result

Unconjugated Ab plus reporter barcodes contribute little if any fluorescent signal associated with cellular and structural features in lung tissue sections. The section was essentially devoid of feature specific staining (compare Upper and Lower panels in Figure 1) and thus fulfilled this criterion.

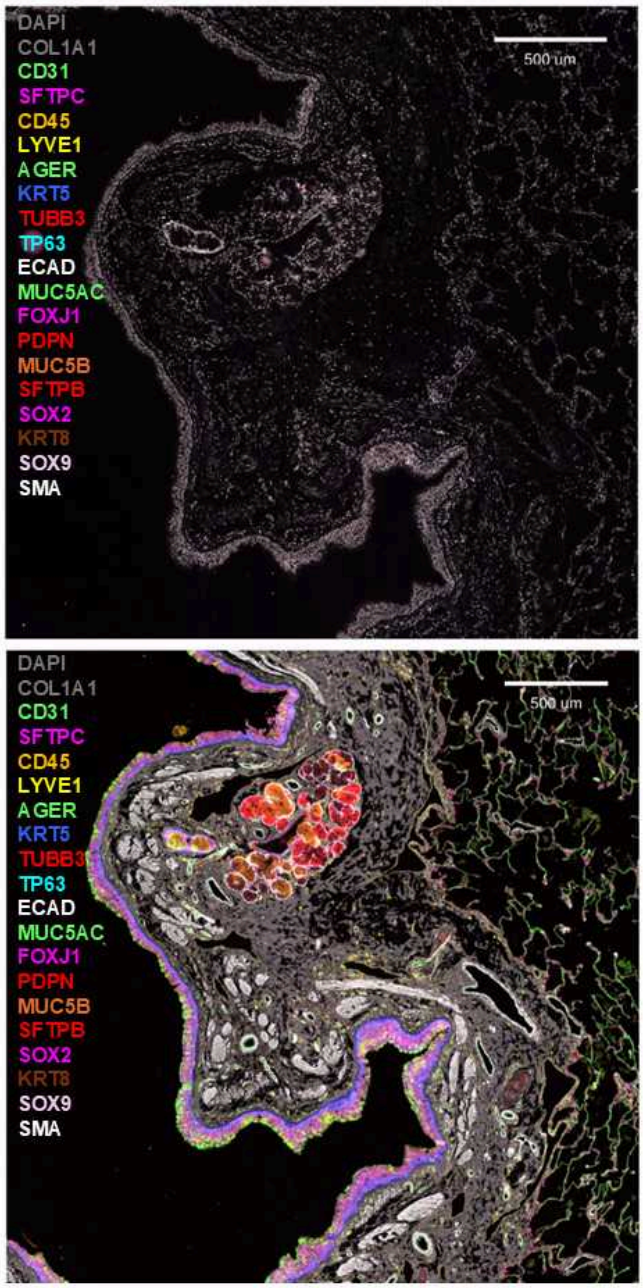

Figure 1. D438-RLL-16B4-16 (section 16, Top Panel) was labeled with unconjugated antibodies (Table 1) and imaged with the full Reporter panel (Table 3). D439-RLL-16B4-17 (section 17, Bottom panel) was labeled with the Ab-Barcode-conjugated panel (Tables 1 and 2) and imaged with the full Reporter panel (Table 3), as for section 16 (Top panel). The indicated channels are active in both panel images.

5.3 The Kidney section(s) was reviewed for any nonspecific interactions of respective Ab-barcode conjugates expected to uniquely target lung specific markers (e.g. AGER, SFTPC, SCGB1A1, SCGB3A2, MUC5AC, MUC5B, SFTPB, FOXJ1, TP63).

Expected result

Lung specific markers do not stain cellular or structural markers in Kidney Cortex (See Figure 2, Top Panel). Ab-barcode conjugates in the 45-marker panel identify appropriate cellular and anatomical features in kidney cortex. (Figure 2, bottom panel). All Ab-barcode conjugates fulfilled these criteria.

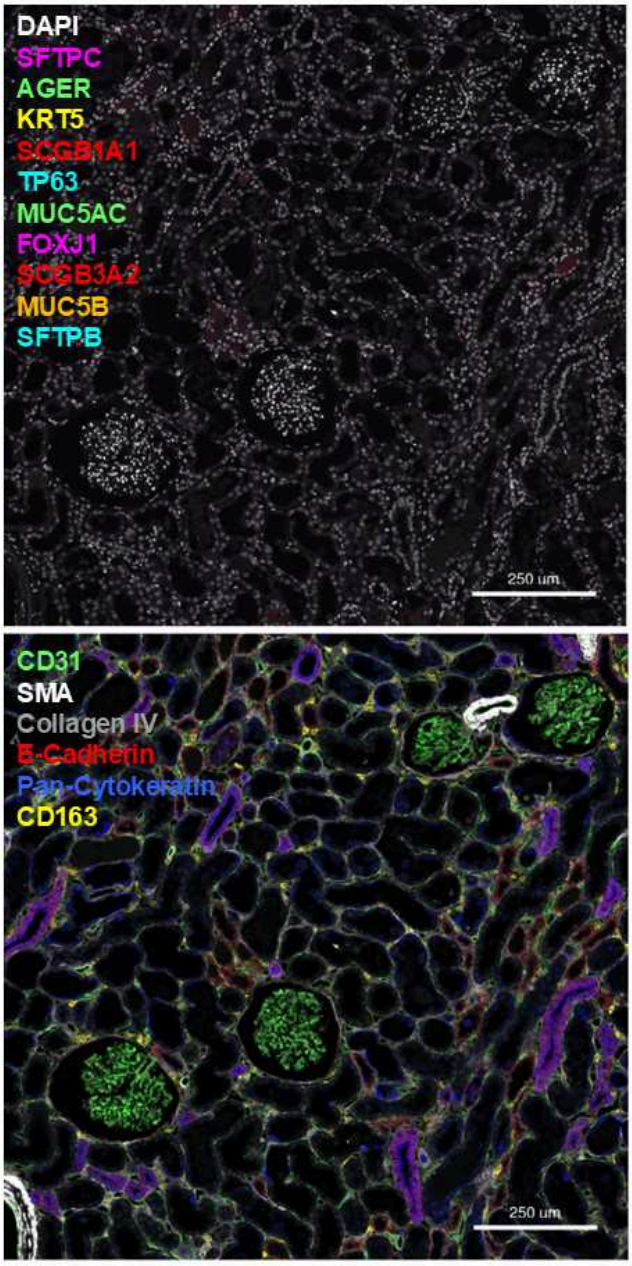

Figure 2. K2300768-5-3, Section 3) was labeled with barcode conjugated antibodies listed in Tables 1 and 2 and imaged with the full Reporter panel (Table 3). **Top Panel:** Ab-barcode conjugates targeting lung specific markers did not stain cellular features in Kidney Cortex. **Bottom Panel:** Several Ab-barcode conjugates labeled specific cell types, such macrophages (CD163), or structure features in kidney cortex such as vasculature (CD31, SMA), and renal tubule epithelium (PanCK, ECAD).

Protocol references

1. Andrea J. Radtke, Ellen M. Quardokus, Diane C. Saunders. SOP: Construction of Organ Mapping Antibody Panels for Multiplexed Antibody-Based Imaging of Human Tissues. V2.0.0 Approved by: Katy Börner, Neil Kelleher, Ronald N. Germain (11/30/2022) Supported by the HuBMAP Consortium. (See attached).

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