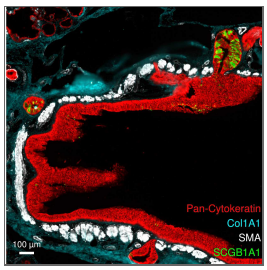

DEC 28, 2023

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

Jeffrey Purkerson[1,2], Gloria S Pryhuber[3,2], Heidie Huyck[3,2], gail.deutsch[4,5,2]

[1]University of Rochester; [2]URMC HuBMAP Lung TMC; [3]University of Rochester Medical Center; [4]Seattle Children's Hospital; [5]University of Washington Department of Pathology

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Gloria S Pryhuber
University of Rochester Medical Center

ABSTRACT

This protocol describes validation of a 34-antibody panel used for multiplexed immunofluorescent (MxF) staining and imaging of FFPE lung tissue sections utilizing the Phenocycler-Fusion® platform (Akoya Biosciences).
The validation strategy involves staining two serial lung sections, one with cocktail of custom barcode-conjugated antibodies plus antibody-barcoded conjugates sourced from Akoya Biosciences and the second with the respective unconjugated, carrier-free control antibodies and omitting the Akoya antibodies.
In addition, a tonsil section was exposed to the all-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, tonsil).
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 and imaging procedures can be found in a companion protocol dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v2.

ATTACHMENTS

SOP_ Construction of OMAPs.pdf

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 specifically 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, in this case, Reporters-only, tests 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 INSTRUCTIONS

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

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.36wgq3b9olk5/v1

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We use this protocol and it's working

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Tissue Sections

1 Lung serial sections, and a tonsil 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 (D016-RLL-11B2-6), a known diseased Lung section (D115-RLL-11A2-19) and a Tonsil Section (Tonsil-11-5) was labeled with a current panel (Table 1) of barcode antibodies as described in dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v2. The other lung serial section (D016-RLL-11B2-5) was labeled with a panel of carrier free, unconjugated antibodies used for custom conjugations from step 18.1 in the above protocol and are 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
SFTPC-BX020	SFTPC	1:500	Invitrogen	PA5-71842
ß-III-Tubulin-BX055	ß-III-Tubulin	1:400	R&D Systems	MAB1195
SCGB1A1-BX043	SCGB1A1	1:400	R&D Systems	MAB4218
SCGB3A2-BX002	SCGB3A2	1:400	Abcam	ab240255
CXCL4-BX004	CXCL4	1:200	Peprotech	500-P05
PROX1-BX050	PROX1	1:200	R&D Systems	AF2727
LYVE1-BX025	LYVE1	1:100	R&D Systems	AF2089
RAGE-BX028	RAGE	1:100	Abcam	ab228861
CD298-BX005	CD298	1:100	Abcam	ab167390
MUC5AC-BX040	MUC5AC	1:100	Abcam	ab212636
SCEL-BX052	SCEL	1:100	Abcepta	AP11564C
TP63-BX006	TP63	1:100	Abcam	ab214790
COL1A1-BX054	COL1A1	1:100	Abcam	ab88147
EDNRB-BX027	EDNRB	1:50	R&D Systems	MAB4496
CD1c-BX016	CD1c	1:50	Novus	ab156708

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, Thermoscience) equilibrated in PBS in accordance with the manufacturer's recommendations.

Table 2. Antibodies commercially conjugated (Akoya Biosciences).

Ab-Barcode	Dilution	Vendor	Cat #
PanCK-BX019	1:200	Akoya	4450020
COLIV-BX042	1:200	Akoya	4450122

Ab-Barcode	Dilution	Vendor	Cat #
Keratin5-BX101	1:200	Akoya	4450090
ECAD-BX014	1:200	Akoya	4250021
CD45-BX021	1:200	Akoya	4550121
Ki67-BX047	1:200	Akoya	4250019
CD68-BX015	1:200	Akoya	4550113
CD14-BX037*	1:200	Akoya	4450047
CD163-BX069	1:200	Akoya	4250079
CD4-BX003	1:200	Akoya	4550112
CD8-BX026	1:200	Akoya	4250012
CD3e-BX045	1:200	Akoya	4550119
FOXP3-BX031	1:200	Akoya	4550071
CD20-BX007	1:200	Akoya	4450018
HLADR-BX033	1:200	Akoya	4550118
CD11c-BX024	1:200	Akoya	4550114
MPO-BX098	1:200	Akoya	4250083
CD31-BX001	1:200	Akoya	4450017
SMA-BX013	1:200	Akoya	4450049

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. *CD14-BX037 was omitted from the OMAP-34 validation as the Ab was temporarily unavailable. However, this Ab has been validated on FFPE tissue by others and in prior experiments.

Reporter Plate and Experiment Design

3 Reporter plate design and Phenocycler-Fusion run protocols were developed using the PhenoCycler Experiment Designer Software (Akoya Biosciences) as described in dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v2. The Reporter Plate design and exposures used for validation of the Lung FFPE OMAP-34 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 OMAP-34 Reporter Plate Design and exposures
Cycle #, reporters and exposure times

ATTO550	Exp (ms)	Cy5	Exp (ms)	AF750	Exp (ms)
Ki67-RX047	125	CD68-RX015	25	PanCK-RX019	150
CD8-RX026	150	CD4-RX003	75	CD20-RX007	150
Col1A1-RX054	400	CD3e-RX045	50	CD31-RX001	150
SFTPC-RX020	100	HLADR-RX033	50	SMA-RX013	150
CD298-RX005	100	CD45-RX021	75	CXCL4-RX004	150
TPSAB1-RX041	10	FOXP3-RX031	150	LYVE1-RX025	400
CD163-RX069	250	CD11c-RX024	125	RAGE-RX028	200
MPO-RX098	25	COIIV-RX042	150	Keratin5-RX101	75
ß-III-Tub-RX055	250	CD1c-RX016	300	SCEL-RX052	400
ECAD-RX014	200	TP63-RX006	150	SCGB1A1-BX043	125
SCGB3A2-RX002	75	ENDRB-RX027	250	MUC5AC-RX040	150
PROX1-RX050	150	None	150	None	150

DAPI exposure 3 milliseconds (ms), CD14-RX037 omitted due unavailability of Ab-barcode.

Multiplexed Imaging and Upload to OMERO for review

4 Tonsil and Lung Sections were imaged as described in dx.doi.org/10.17504/protocols.io.6qpvr38dpvmk/v2.

4.1 OMAP-34 Validation consisted of the following slide runs.

1. Tonsil 11-5 (OMAP-34 Antibody Panel)
2. D016-RLL-11B2-5 (Unconjugated Ab panel)
3. D016-RLL-11B2-6 (OMAP-34 Antibody Panel)
4. D115-RLL-11A2-19 (OMAP-34)

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. OMAP34validation) 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. Gloria Pryhuber (Principal investigator), and Dr. Gail Deutsch (Board Certified Pathologist, Pulmonary specialist).

5.1 Lung and tonsil 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, vascular endothelium, immune cell clusters), colocalization with appropriate pan-markers (e.g. CD4 with CD3/CD45, CD11c with HLADR/CD45; SCEL with RAGE and pan-cytokeratin (pan-CK), 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, with the exception of EDNRB-BX027, fulfilled this criterion. As a result, EDNRB was omitted from the Lung OMAP-34 panel.

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

Expected result

Unconjugated Ab, or barcode contribute little if any fluorescent signal associated with cellular and structural features in lung tissue sections. The D016-RLL-11B2-5 section was essentially devoid of feature specific staining (compare left and right panels in Figure 1) and thus fulfilled this criterion.

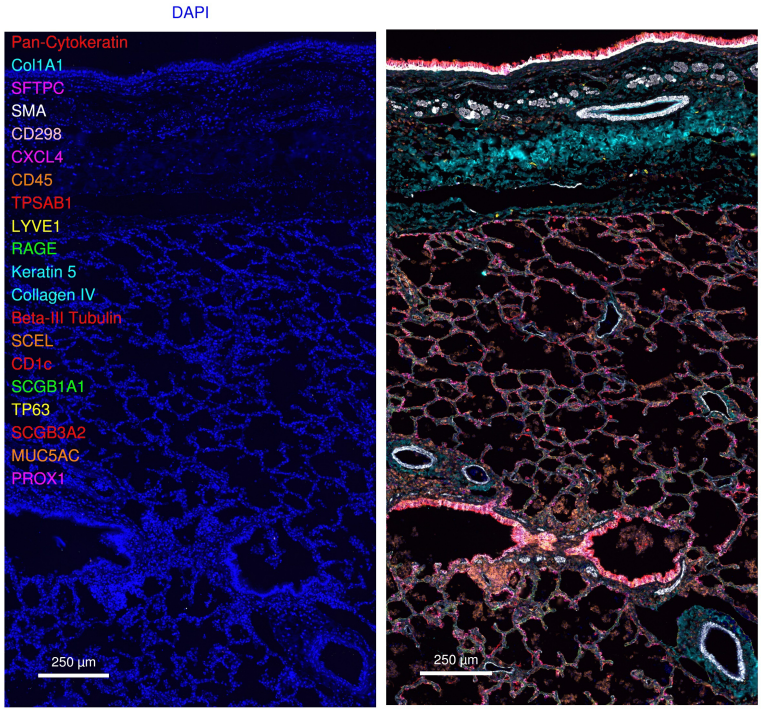

Figure 1. D016-RLL-11B2-5 (section 5, Left Panel) labeled with unconjugated antibodies (Table 1) and imaged with the full Reporter panel (Table 3). D016-RLL-11B2-6 (section 6, Right panel) was labeled with the Barcode-Ab conjugated panel (Tables 1 and 2) and imaged with the full Reporter panel (Table 3), as for section 5 (left panel). The indicated channels are active in both left and right panel images.

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

Expected result

Lung specific markers do not stain cellular or structural markers in tonsil (See Figure 2). Ab-barcode conjugates identify the same cellular and anatomical features when present in both lung and tonsil (e.g., CD45, panCK). All Ab-barcode conjugates, with the exception of EDNRB-BX027 fulfilled this criterion.

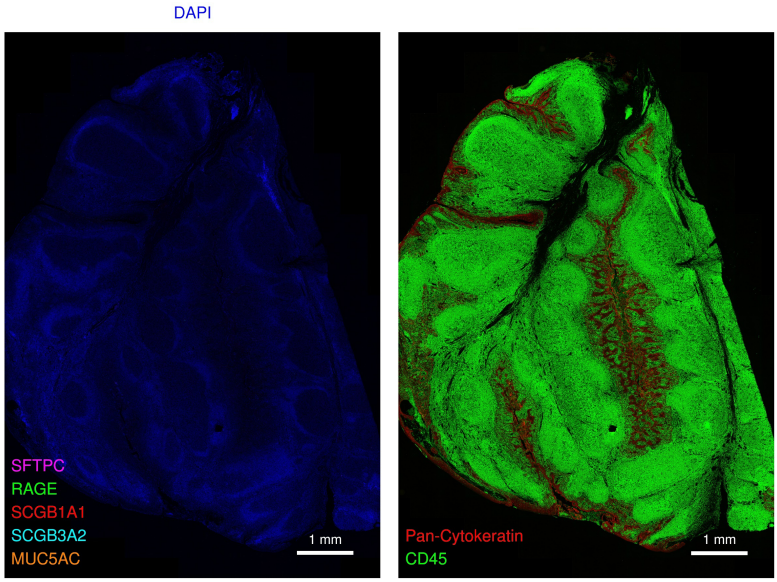

Figure 2. Ab-barcode conjugates targeting lung specific markers did not stain cellular features in tonsil (Left Panel). Pan markers for epithelial (pan-cytokeratin) and immune cells (CD45) identify tonsil cell populations as expected (Right panel). The latter was true also for immune cell markers including CD20, CD3e, CD4, CD8, CD68 (data not shown).