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813.1 Multiplexed Immunofluorescence Phenocycler-Fusion® Imaging of FFPE Lung Sections V.3

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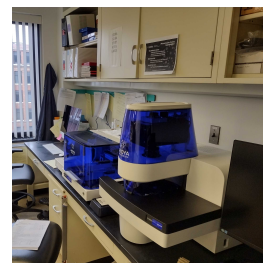
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We use this protocol and it's working

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Abstract

This protocol describes multiplexed immunofluorescent (MxF) staining and imaging of FFPE lung tissue sections utilizing the Phenocycler-Fusion platform (Akoya Biosciences). The approach is based on the CODEX multiplexed immunofluorescence staining technology developed by Garry Nolan and colleagues (1). The protocol is closely aligned with the attached Phenocycler-Fusion User Guide provided by Akoya (2).

The protocol includes A) FFPE lung tissue section preparation, B) Labeling of lung tissue sections with antibody-barcode conjugates, C) Barcode-Reporter plate and experiment design, D) Multiplexed imaging and analysis, E) Reference for H&E stain to follow MxF and F) Reference to custom antibody conjugation. Thus, the protocol is designed to provide information regarding specific reagents (i.e., antibodies), conditions (i.e., dilutions), and procedures used in multiplexed immunofluorescent staining of lung tissue.

Attachments



PhenoCycler-Fusion U...

10.9MB

Guidelines

An overview of Multiplexed Imaging using barcode conjugated antibodies can be found in the attached Phenocycler-Fusion User Guide. Onsite training in staining procedures, experimental setup and design, as well as image acquisition is provided by Field Application Scientists from Akoya Biosciences.

Materials

Key equipment, reagents, buffers, and supplies can be found in the attached Phenocycler-Fusion User Guide (Akoya Biosciences).

1. TruBOND™ 380 Adhesion Slides; P/N: 63700G10 Green (Electron Microscopy Sciences, Hatfield, PA)
2. AR9 buffer; PN#AR9001KT, (Akoya Biosciences, Malbororough, MA)
3. Paraformaldehyde (20% solution); P/N: 15713-S EM Grade (Electron Microscopy Sciences, Hatfield, PA)
4. dPBS; 17-512Q (Lonza, Walkerville, MD).
5. Sample Kit for PhenoCycler-Fusion; P/N:7000017 (Akoya Biosciences)
Contains Hydration, Staining, and Storage buffers, blocking buffers N, Gv2, J, and S, and final fixative reagent.
6. 10X Buffer Kit for PhenoCycler-Fusion; P/N; 7000019 (Akoya Biosciences)
Contains 10x Buffer for Phenocycler and Buffer Additive
7. Assay Reagent; P/N; 7000002 (Akoya Biosciences)
8. DMSO, ACS Reagent Grade (≥99.9%); 472301-1L Sigma-Aldrich.
9. Flow Cells (10 pack) Akoya Biosciences

Safety warnings

- ❗ Procedures involving xylenes and paraformaldehyde should be performed in a chemical fume hood. Nitrile gloves are permeable to xylenes therefore change gloves promptly upon contact with xylenes.

Use heat resistant gloves to handle Coplin jars after antigen retrieval.

Ethics statement

The protocol does not utilize laboratory animals.

Before start

The Phenocycler Fusion system must be setup and calibrated by an installation engineer. Akoya Biosciences will provide a list of materials required for training.



Lung section preparation

3h

1 Bake Sections to promote tissue adherence to the slide.

Since stained slides can be stored in storage buffer for a maximum 5 days without diminution of staining signal intensity, and Phenocycler-Fusion 2.0 imaging of two slides with a 45-50 marker panel requires ≥ 24 h, staining more than 10 slides at a time is not recommended.

1.1 FFPE Lung sections are prepared as described in dx.doi.org/10.17504/protocols.io.kxygxejwdv8j/v2 and mounted on TruBond™ 380 adhesion slides (Electron Microscopy Sciences, Hatfield, PA).

1.2 Heat Slide(s) in oven at 60 °C Overnight .

Labeling of lung tissue sections with antibody-barcode conjugates

7h 35m

2 Deparaffination

15m

Paraffin wax is removed from lung sections by incubation in xylene (3X)

- Xylene #1 00:05:00
- Xylene #2 00:05:00
- Xylene #3 00:05:00

3 Rehydration

40m

The tissue sections are rehydrated through descending ethanol series followed by molecular biology grade distilled water (2X).

- 100% Ethanol #1 00:05:00
- 100% Ethanol #2 00:05:00
- 90% Ethanol 00:05:00
- 70% Ethanol 00:05:00
- 50% Ethanol 00:05:00
- 30% Ethanol 00:05:00
- ddH2O #1 00:05:00
- ddH2O #1 00:05:00

4 High pH Antigen Retrieval 9

Heat-Induced Epitope Retrieval (HIER) reverses protein crosslinking in FFPE tissue

4.1 Dilute AR9 buffer (Akoya Biosciences) 1/10 in ddH₂O and fill plastic Coplin Jar to 90-95% volume. Add slides containing tissue sections to be stained. Cover entire Coplin Jar with aluminum foil. **(Do not Cap)**

4.2 Place Coplin Jar in an InstantPot® pressure cooker containing ddH₂O to 1/3-1/2 depth of Coplin Jar.

4.3 Heat on high pressure setting  00:20:00 (turn off keep warm button)

20m

4.4 After releasing pressure per pressure cooker directions, remove Coplin Jar, partially unwrap foil without uncovering slides and allow slides to cool for a minimum of  01:00:00 . Attempting to rinse/wash without allowing slides to cool may reduce tissue adherence to glass slide.

1h



5 Prepare Blocking Buffer

Prepare Blocking Buffer no earlier than 1 h before staining (i.e. while slide are cooling in AR9 buffer and/or incubating in Staining buffer, see below) and keep on ice.

5.1 Table 1. Blocking Buffer Component Table

Component	2 Slides	5 slides	N Slides
Staining Buffer	 362 µL	 905 µL	 181 µL x N
N Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
G Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
J Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
S Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
Total Volume	 400 µL	 1000 µL	 200 µL x N

5.2 ■ **Optional Preblock Step:** If non-specific binding results in low signal-noise for marker signals a preblock incubation step may be included prior to adding antibody cocktail to slide. Prepare preblock buffer ( 5 µL each of N, J, S Blocker to  185 µL staining buffer) X N slides). Add  190 µL of Preblock buffer to slides while avoiding pipetting directly onto tissue and incubate for  00:45:00  Room temperature in humidified chamber. Pour off excess blocking buffer and soak up remaining excess with a Kimwipe™, prior to adding antibody cocktail.

45m

5.3 Prepare Antibody Cocktail

In a 1.5 ml microfuge tube, mix volume of blocking buffer =

 200 µL x N slides to be stained **minus total volume of antibodies (µl/slide x N)** to be used in experiment (See Table below). The Blocking buffer volume must be **≥ 60% of total antibody cocktail volume** for effective blocking. If needed, reduce staining buffer volume to achieve 60% blocking buffer volume in Ab solution.

Example antibody panel (final column = microliter of antibody per single slide):

	Antibody	Vendor	Cat#	Barcode	Dilution	µl/200µl/1slide
	CD31	Akoya	4450017	BX001	1:200	1
	KRT14	Akoya	4450031	BX002	1:200	1
	CD4	Akoya	4550112	BX003	1:200	1
	CD20	Akoya	4450018	BX007	1:200	1
	SMA	Akoya	4450049	BX013	1:200	1
	E-Cadherin	Akoya	4250021	BX014	1:200	1
	CD68	Akoya	4550113	BX015	1:200	1
	PanCK	Akoya	4450020	BX019	1:200	1
	CD45	Akoya	4550121	BX021	1:200	1
	CD11c	Akoya	4550114	BX024	1:200	1
	CD8	Akoya	4250012	BX026	1:200	1
	FOXP3	Akoya	4550071	BX031	1:200	1
	HLADR	Akoya	4550118	BX033	1:200	1
	CD14	Akoya	4450047	BX037	1:200	1
	ColIV	Akoya	4550122	BX042	1:200	1
	CD3e	Akoya	4550119	BX045	1:200	1
	Ki67	Akoya	4250019	BX047	1:200	1
	CD163	Akoya	4250079**	BX069	1:200	1
	CAV1	Akoya	4450084	BX086	1:200	1
	MPO	Akoya	4250083	BX098	1:200	1

	Antibody	Vendor	Cat#	Barcode	Dilution	μl/200μl/1slide
	Keratin5	Akoya	4450090	BX101	1:200	1
	SOX2	Akoya	4250075	BX102	1:200	1
	PDPN	Akoya	4250094	BX121	1:200	1
	TPSAB1*	Abcam	ab2378	BX041	1:1000	0.2
	SOX9*	Cell Signaling	74021SF	BX046	1:1000	0.2
	SFTPC*	Invitrogen	PA5-71842	BX020	1:500	0.4
	MUC5AC*	Abcam	ab212636	BX040	1:500	0.4
	FOXJ1*	ThermoFischer	14-9965-82	BX036	1:500	0.4
	GRP*	LSBio	LS-C755064	BX030	1:500	0.4
	TUBB3*	R&D Systems	MAB1195	BX055	1:400	0.5
	SCGB3A2*	Abcam	ab240255	BX034	1:400	0.5
	SFTPB*	ThermoFischer	PA5-42000	BX010	1:400	0.5
	KRT8*	Biolegend	904804	BX022	1:400	0.5
	CD55*	R&D Systems	AF2009	BX027	1:200	1
	PF4*	Peprotech	500-P05	BX004	1:200	1
	LYVE1*	R&D Systems	AF2089	BX025	1:200	1
	ATP1A1*	Abcam	ab167390	BX005	1:200	1
	SCGB1A1*	R&D System	MAB4218	BX043	1:100	2
	AGER*	Abcam	ab228861	BX028	1:100	2
	COL1A1*	Abcam	ab88147	BX054	1:100	2
	TP63*	Abcam	ab214790	BX006	1:80	2.5
	SCEL*	Abcepta	Abcepta	BX052	1:80	2.5
	CD1c*	Novus	ab156708	BX016	1:80	2.5
	MUC5B	Abcam	AB87376	BX049	1:80	2.5
	PROX1*	R&D Systems	AF2727	BX050	1:50	4

Note: Whenever possible barcodes and reporters were assigned to specific antibodies based on predicted antigen abundance and relative channel sensitivity in accordance with the



PhenoCycler-Fusion User Guide (Akoya Biosciences).

*Denotes custom-conjugated antibody. Refer to custom-conjugation section at the end of the protocol.

** CD163-BX069 (Cat# 4250079, Akoya Biosciences) has been discontinued. EPR19518 can be sourced from Abcam Cat# AB213612 for custom conjugation.

6 Incubate with Ab + Blocking Buffer Cocktail Solution

6.1 Wash slides in Coplin Jars containing dd H₂O

2m

- dd H₂O #1 - 3 dips
- dd H₂O #2 - immersion  00:02:00

6.2 Immerse Slides in sequential Coplin jars containing the following buffers from Akoya Biosciences:

54m

- **Hydration buffer #1**  00:02:00
- **Hydration buffer #2**  00:02:00
- **Staining buffer**  00:20:00 -  00:30:00 **max.**

6.3 Carefully dry slide around tissue with a Kimwipe™. Then pipette 190 µL Ab cocktail solution onto slide to cover tissue section. Avoiding pipetting directly onto tissue.

6.4 Incubate slides, covered in a lightly humidified chamber, for 03:00:00

3h

 Room temperature .

7 Post Stain Wash and Multiple Fixations

Tissue slides are briefly washed in staining buffer followed by sequential fixation with paraformaldehyde, ice-cold methanol, and final fixation solution.

7.1 Incubate in sequential Coplin Jars containing:

19m

- **Staining Buffer #1.**  00:02:00
- **Staining Buffer #2.**  00:02:00
- 1.6% **paraformaldehyde** (Diluted in Storage buffer, Akoya Biosciences from 20% stock).  00:10:00
- Rinse slides sequentially in 3 Coplin Jars containing 1 x **PBS** (3 dips each)
- pre-chilled,  -20 °C , **methanol on ice**  00:05:00
- Rinse slides sequentially in 3 Coplin Jars containing 1 x **PBS** (3 dips each)



- 7.2 Prepare Final Fix dilution (2%)
20 µl aliquot of **Final Fix** (Akoya Biosciences) diluted in 1 ml PBS

Carefully dry slide around tissue with a Kimwipe™

Then pipette 190 µL of **Final Fix solution** onto slide to cover tissue section while avoiding pipetting directly onto tissue.

Incubate  00:20:00 .

- 7.3 Rinse slides sequentially in 3 Coplin Jars (3 dips each) PBS

8 **Autofluorescence Imaging prior to Photobleaching**

The slide is stained with DAPI to facilitate tissue detection by the Fusion algorithm prior to scanning the slide in the Opal480 channel with the **AF480** protocol. Since the Fusion algorithm is designed to subtract autofluorescence (AF) from Opal fluorescent channels, the AF subtraction exposure setting in the algorithm is reduced such that the Opal480 channel signal reflects biomolecule autofluorescence in this channel without algorithm-based subtraction.

- 8.1 Stain slide with 14.3 micromolar (uM) DAPI in PBS for  00:05:00 .

- 8.2 Rinse slides sequentially in 3 Coplin Jars containing 1 x **PBS** (3 dips each)

- 8.3 Carefully pipette ~  100 µL of PBS (not directly over tissue) onto slide and gently overlay a 24 X 50 (1.5 mm thick) glass coverslip while avoiding capturing air bubble over the tissue.

- 8.4 Carefully place the slide in the holder without displacing the coverslip.

- 8.5 Open the scan slide tab Fusion software and select the "**Scan Slide**" task "**Autofluorescence**" study and the "**AF480**" protocol. The AF480 protocol and the following exposure settings: DAPI (10 ms), Opal480 (75 ms), Algorithm autofluorescence subtraction (10 ms).

- 8.6 After imaging carefully remove coverslip by submerging the slide in a Coplin Jar containing PBS and proceed with photobleaching.

9 **Photobleaching and Storage**



- 9.1 Slides may be stored for up to 5 days in a Coplin Jar containing **Storage buffer** 🌡️ 4 °C .
- 9.2 In order to reduce autofluorescence from the tissue, on the day prior to imaging, lay the slide to be imaged flat in a 100 cm² dish containing **Storage Buffer** (Akoya Biosciences). Photobleach by illumination with a 200 mA, 15 watts, 1600 lumens bulb 🌡️ 4 °C
- 🕒 Overnight .

Reporter Plate and Experiment Design

- 10 Reporter plate design and Phenocycler-Fusion run protocols are developed using the PhenoCycler Experiment Designer Software (Akoya Biosciences).
- 11 **Prepare 1x Buffer Phenocycler Buffer and Reporter Stock Solution**
Reporter Stock Solution is prepared according to guidelines in the attached Phenocycler-Fusion User Guide (Akoya Biosciences®). In PCF 2.0, low and high DMSO buffers are prepared prior to the imaging run. **Note that the phenocycler buffers are stable for up to 14 days, after which they must be disposed as hazardous waste. Do not use phenocycler or DMSO buffers stored in opaque or brown bottles longer than two weeks.**
- 11.1 Open the calculate buffer tab in the Phenocycler Experiment Designer 2.1.0 software and import reporter plate design for one or two flow cells into the buffer calculator which in turn will output total volumes and component volumes required for 1X Phenocycler buffer, low DMSO buffer, and high DMSO buffer for the imaging run (see attached example below). Multiply volumes by the anticipated number of imaging runs. 1 x PCF Buffer with Buffer additive is used in the side-car PCF Buffer bottle, in the reporter stock solution and in the mounting of the flow cell. Do not filter. Store at room temperature for up to **14 days**. Combine in clean glass container, mix well with magnetic stirrer while avoid bubbles:

PhenoCycler Experiment Designer

Home

Experiments to Run:

Position 1 Clear Load

Study: HuMAP
Experiment: OMAP-458_P1
Total Number of Cycles: 17

Position 2 Clear Load

Study: HuMAP
Experiment: OMAP-458_P2
Total Number of Cycles: 17

Run Buffers Required:

1x PCF Buffer		Recipe	
Total volume:	850 mL	10x Buffer for PhenoCycler:	85 mL
For making High and Low DMSO solutions:	460 mL	Buffer Additive:	85 mL
Remainder volume for bottle:	390 mL	D ₂ O water:	680 mL

High DMSO Buffer		Recipe	
Total volume for bottle:	200 mL	DMSO:	180 mL
		1x PCF Buffer:	20 mL

Low DMSO Buffer		Recipe	
Total volume for bottle:	550 mL	DMSO:	110 mL
		1x PCF Buffer:	440 mL

The Buffer Calculator tab in the PhenoCycler Experiment Designer facilitates calculation of required volumes for preparation of PhenoCycler buffers.

11.2 Prepare Reporter Stock Solution

Combine, mix gently by inversion:

- 1 x PhenoCycler Buffer with Buffer additive 265 μL X N
- Assay Reagent 25 μL X N
- Nuclear Stain 10 μL X N

Total volume should equal $300\ \mu\text{L} \times \mathbf{N}$ = **number of cycles plus two blank cycles** (# reporter plate wells) to be done in the imaging run.

12 Prepare Reporter Solutions for each cycle in an opaque or foil wrapped tube.

Keep Reporters on ice.

Centrifuge Reporter stock tubes briefly prior to removing aliquot.

Protect the fluorescent dye-containing Reporters from photobleaching.

Avoid introducing bubbles into the solutions.

Important to keep the reporter plate dust-free.

An imaging cycle = 4 channel detection including nuclear stain and 3 reporters, depending on filter set chosen, for example one each of ATTO550, Cy5/AF647, AF750. A multi-cycle experiment composes an imaging run.

- ### 12.1
- Label an opaque microtainer for each well, each well = one imaging cycle of the imaging run. To each tube add **235 μL** Reporter Stock Solution X N imaging runs and **5 μL** X N



imaging runs of each predetermined reporters complementing the antibody barcodes to be detected in that cycle to make total volume **250 µl** X N imaging runs per microtainer

- 12.2 Pipette 245 µl of each reporter mix into a well of a light-opaque 96 well microtiter plate (Akoya Biosciences) according to the Experimental Design developed in the experimental design app.

Also, pipette 245 ul reporter stock solution (blanks) into two wells of row H to create 2 blank wells. Seal the solution-containing wells with Akoya foil seals. The plate may be stored @ 4⁰C for up to 14 days in accordance with the attached PhenoCycler-Fusion User Guide (Akoya Biosciences).

Multiplexed Imaging and Analysis

13 Image Acquisition via Phenocycler-Fusion® 2.0 (i.e., CODEX V2)



- 13.1 Use the Phenocycler Experiment Designer 2.1.0, located on the PhenoCycler-Fusion Acquisition PC desktop, to define the design of the Reporter plate and imaging parameters to be used in the experiment. Follow details provided in the attached PhenoCycler-Fusion User Guide.

- 13.2 If necessary, warm reporter plate to Room temperature

- 13.3 After photobleaching in storage buffer, wash slides in PBS (250 ml; Coplin Jar)

10m

Room temperature 00:10:00

- 13.4 After the wash, dry the bottom of the slide and around the edges of the tissue with a kimwipe.

30s

Attach a flow cell using the flow cell assembly device (Akoya Biosciences). Center and press the flow cell onto the slide for 00:00:30

- 13.5 Cure the flow cell adhesive by incubating the slide in 1X phenocycler buffer (Akoya biosciences) 00:10:00 Room temperature

10m

- 13.6 Fill respective Reagent reservoirs on the Phenocycler side car with low DMSO, high DMSO, and 1X Phenocycler buffers (prepared in step 11.1), and ddH₂O. Place a one or two blank flow cells in the flow cell slide carrier attached to the fluidics tubing. If imaging a single flow cell slide load the blank and subsequently the sample flow cell in



the blue (1) imaging window and replace the magnetic slide retainer for the Red (2) imaging window with a **blue blocking tray**.

- 13.7 Start an imaging run by turning on the Phenocycler fluidics system and the Phenoimager, followed by launching the Fusion 2.2.0 software. Select Start experiment and follow the prompts.
- 13.8 Images are acquired utilizing the 20X (0.5 $\mu\text{M}/\text{pixel}$) objective and the Fusion 2.2.0 software.
- 13.9 Image processing is automated via the Fusion 2.2.0 software and completed at the end of the experiment run.

Expected result

- A. Folder with slide/sample name containing:
- The respective Ab-Reporter (.xpd) file generated by the Phenocycler Experiment designer.
 - Akoya whole slide scan .qptiff (~10-15 GB for a 45-marker marker panel; ~1 cm^2 lung section)
- B. The following .temp file contents:
- qptiff.raw files: 10-15 GB for each cycle (45 marker panel; 1 cm^2 section)
 - qptiff.intermediate: 10-15 GB for each cycle (45 marker panel; 1 cm^2 section)
 - CombineInputs
 - FlowCellOverview
 - FocusMap
 - FocusTable
 - Label
 - LastLabel0
 - LastLabel1
 - MarkerList
 - Overview BF
 - Position1CarrierBarcode
 - Position1TrayBarcode
 - Position2CarrierBarcode
 - Position2TrayBarcode
 - SampleMask
 - SampleValMask

- 13.10 **Do not attempt to open raw or intermediate cycle.qptiff during the imaging run.**
- 13.11 Rapid review of the resultant image.qptiffs is performed utilizing PhenoChart 2.2.0 software. If necessary, exposure time (ms) is adjusted in the Phenocycler Experiment



Designer to obtain readily detectable, specific marker signals that are below saturation. Note that after viewing qptiffs in Phenochart an_annotations.xml.lock file will appear in the image folder.

- 13.12 After the run return the tissue slide with attached flow cell to storage buffer  4 °C . If necessary, slides can be reimaged with a new set of reporters up to 5 days post staining without loss of signal.

14 Record Imaging Run MetaData

- 14.1 Record Slide ID, staining and imaging date in the Metadata Excel file located in the Experiments_Protocols_Metadata subfolder in the CODEX Akoya folder located on BOX.
- 14.2 Likewise record the dates for biweekly Brightfield reference calibration and maintenance washes as well as monthly fluorescent and phenocycler reference calibration.
- 14.3 The "source storage time" = length of time (Days) between slide staining date and imaging date.
- 14.4 The image acquisition time = length of time between time stamp on the MarkerList or CombineInputs files (**START**) and the time stamp on the Akoya whole slide scan file .qptiff (**FINISH**).
- 14.5 The time_since_acquisition_instrument_calibration_value is the time interval (days) between the phenocycler reference calibration date and the imaging run date.

Data backup and storage

15 Upload files from Phenocycler-Fusion runs to the URM local Bluehive-Archive/archive/lungmap_lab codex folder.

- 15.1 Maintain directory structure for each run by creating a new folder labeled with the slide ID containing a scan# subfolder, and a temp subfolder within the scan folder.
- 15.2 Include the following files in the upload:
Slide folder:
Scan Folder:
A. (.xpd) file (Phenocycler Experiment designer)
B. **Akoya whole slide scan .qptiff**

C. **Optional:** The ome.tiff generated via bfconvert (See step 17)

D. Temp folder contents including **qptiff raw files**, **Optional:** qptiff.intermediate files.

Multiplexed Imaging and Analysis

16 Image Analysis and Segmentation

- 16.1 Analysis of processed image.qptiff files can be performed utilizing QuPath 4.3-5.0 (See Reference 3), and subsequent analysis steps describe key facets and output using this approach. Additional image analysis pipelines are in development and include utilization of: DeepCell (Mesmer) **DeepCell** for cell segmentation; scanpy <https://scanpy.readthedocs.io/en/stable/> for clustering and cell type annotation utilizing marker expression signals within segmentation masks and squidpy <https://squidpy.readthedocs.io/en/stable/> for additional downstream analysis; Napari <https://napari.org/stable/> for visualization of marker expression and cell type annotation; Banksy <https://www.nature.com/articles/s41576-024-00743-9> or equivalent for cell neighborhood analysis.
- 16.2 Cell segmentation based on (DAPI) stained nuclei is performed utilizing the respective StarDist extension (See Reference 4) in QuPath.
- 16.3 Cell detection measurements (e.g. X,Y coordinates, fluorescent intensity data for all channels) are saved as a text file, opened in Excel as a tab delimited file, and resaved as a CSV (comma delimited) file.
- 16.4 Cell segmentation masks can be exported from QuPath running the following ExpCell script within Script Editor:

```
import qupath.lib.images.servers.LabeledImageServer
import qupath.lib.roi.RectangleROI
import qupath.lib.scripting.QP

def imageData = getCurrentImageData()
def project = getProject()
def server = imageData.getServer()

// Define output path (relative to project)
def name =
GeneralTools.getNameWithoutExtension(imageData.getServer().getMeta
data().getName())
def pathOutput = buildFilePath(PROJECT_BASE_DIR, 'export')
mkdirs(pathOutput)
// Define output resolution
double requestedPixelSize =
server.getPixelCalibration().getAveragedPixelSize()
// Convert to downsample
double downsample = requestedPixelSize /
imageData.getServer().getPixelCalibration().getAveragedPixelSize()
println('Downsample factor : '+downsample)
// Create an ImageServer where the pixels are derived from
annotations
def labelServer = new LabeledImageServer.Builder(imageData)
    .backgroundLabel(0, ColorTools.BLACK) // Specify background
label (usually 0 or 255)
    .downsample(downsample) // Choose server resolution; this
should match the resolution at which tiles are exported
    .useCells()
    .useInstanceLabels() //Export as unique label
    .multichannelOutput(false) // If true, each label refers to
the channel of a multichannel binary image (required for
multiclass probability)
    .build()

annotation = getSelectedObject()

//Export the mask
def region = RegionRequest.createInstance(labelServer.getPath(),
downsample, annotation.getROI())
def outputPath = buildFilePath(pathOutput, 'labels_mask.ome.tiff')
writeImageRegion(labelServer, region, outputPath)

resetSelection()
```

- 16.5 Vascular smooth muscle and airway epithelial features are annotated and classified by thresholding the ACTA2 and PanCK signals utilizing the Pixel classifier in QuPath.
- 16.6 Annotation masks can also be exported from QuPath running the following ExpCell script within Script Editor:

```
def imageData = getCurrentImageData()

// Define output path (relative to project)
def outputDir = buildFilePath(PROJECT_BASE_DIR, 'export')
mkdirs(outputDir)
def name =
GeneralTools.getNameWithoutExtension(imageData.getServer().getMeta
data().getName())
def path = buildFilePath(outputDir, name + "-labels.png")

// Define how much to downsample during export (may be required
for large images)
//double downsample = 8

// Create an ImageServer where the pixels are derived from
annotations
def labelServer = new LabeledImageServer.Builder(imageData)
    .backgroundLabel(0, ColorTools.WHITE) // Specify background
label (usually 0 or 255)
    //.downsample(downsample)    // Choose server resolution; this
should match the resolution at which tiles are exported
    // Choose output labels (the order matters!)
    .addLabel('airway', 1)
    .addLabel('Vasculature', 2)
    .addLabel('Other', 3)
    .multichannelOutput(false) // If true, each label refers to the
channel of a multichannel binary image (required for multiclass
probability)
    .build()

// Write the image
writeImage(labelServer, path)
```



16.7 Object Data can also be exported from QuPath as a GeoJSON file

Conversion of .qptiff files to ome.tiff for upload to Omero utilizing bfconvert

17 **Download Command Line Tools from <https://www.openmicroscopy.org/bio-formats/downloads/>**

18 **Execute the following in command prompt:**

18.1 C:\>Set BF_MAX_MEM=10240M

18.2 C:\>bfconvert -no-sas -series 0 -compression LZW -pyramid-resolutions 5 -pyramid-scale 2 INPUT.qptiff OUTPUT_ome.tiff

18.3 **Upload the resulting ome.tiff file to the CODEX folder in Omero and the respective tissue donor subfolder (e.g. D265) utilizing the Omero importer.**

Custom antibody conjugation

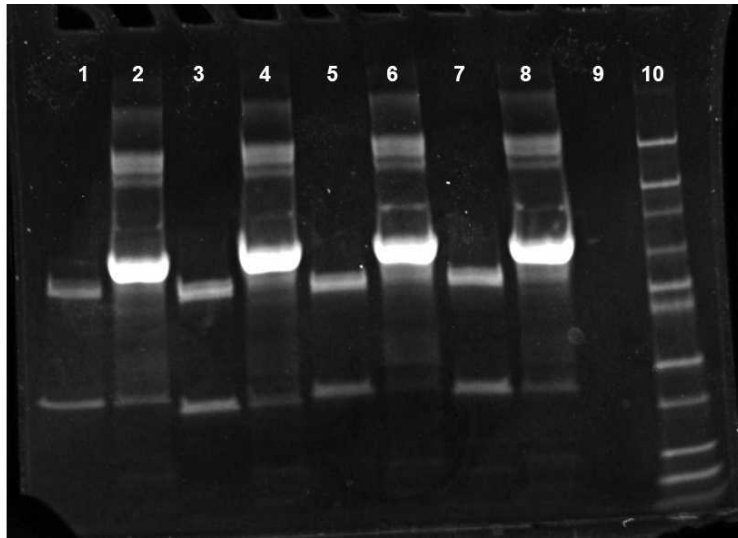
19 **Custom Antibody Conjugation is performed as described [dx.doi.org/10.17504/protocols.io.3fugjnw](https://doi.org/10.17504/protocols.io.3fugjnw).**

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

19.2 Success of Antibody-Barcode chemical conjugation is determined by resolving unconjugated and conjugated Ab's on BioRAD™s MiniProtean TGX Gel 4-15% Bis-Tris Protein Gels in accordance with Guidelines in the Phenocycler-Fusion User Guide (Akoya Biosciences®, Malborough, MA).

Expected result

Barcode conjugated Ig heavy and light chains exhibit an Electrophoretic Mobility Shift (EMS) relative to the corresponding unconjugated Ab.



1. Unconj. MUC5B
2. MUC5B-BX010
3. Unconj. SCGB3A2
4. SCGB3A2-BX002
5. Unconj. TP63
6. TP63-BX006
7. Unconj. RAGE
8. RAGE-BX028
9. Empty Well
10. Protein STD.

Figure 1. A representative SDS-PAGE Gel providing quality assurance of Ab barcode conjugation.

H&E staining Post Phenocycler-Fusion

10m

20 It is useful to stain the tissue sections with hematoxylin & eosin following the multiplexed fluorescence imaging in order to match histology with antibody staining. We have stained lung sections with an aqueous H&E stain as described dx.doi.org/10.17504/protocols.io.kqdg397yeg25/v1 but this resulted in a rather grey stain and so we have opted instead to remove the flow cell by soaking in xylene prior to H&E staining of lung sections as described in Step 19.1.

20.1 After MxIF imaging is completed for the slides, remove the flow cell covered slides from storage buffer (Akoya Biosciences), wipe dry with Kimwipe and, utilizing a vacuum-suction apparatus similar to what is described in dx.doi.org/10.17504/protocols.io.kqdg397yeg25/v1, remove buffer from the flow cell.

4h

- Incubate slides in a Coplin Jar containing Xylene for 04:00:00 to

Overnight in order to weaken or dissolve the flow cell adhesive.



- Carefully pry flow cell off the slide with razor blade or preferably a thin spatula.
- Proceed with H&E staining according to the standard protocol described here [dx.doi.org/10.17504/protocols.io.36wggjnj3vk5/v4](https://doi.org/10.17504/protocols.io.36wggjnj3vk5/v4) beginning at Step 5.

Protocol references

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2. **Phenocycler-Fusion User Guide.**
3. Bankhead, P., Loughrey, M.B., Fernández, J.A. *et al.* QuPath: Open-source software for digital pathology image analysis. *Sci Rep* **7**, 16878 (2017). <https://doi.org/10.1038/s41598-017-17204-5>.
4. Schmidt, U., Weigert, M., Broaddus, C., and Myers, G. (2018). "Cell detection with star-convex polygons," in *Medical Image Computing and Computer Assisted Intervention—MICCAI 2018*. Editors A. F. Frangi, J. A. Schnabel, C. Davatzikos, C. Alberola-López, and G. Fichtinger (Springer International Publishing) 11071, 265–273. doi:10.1007/978-3-030-00934-2_30; M. Weigert, U. Schmidt, R. Haase, K. Sugawara and G. Myers, "Star-convex polyhedra for 3D object detection and segmentation in microscopy", *Proc. IEEE Winter Conf. Appl. Comput. Vis. (WACV)*, pp. 3655–3662, Mar. 2020.