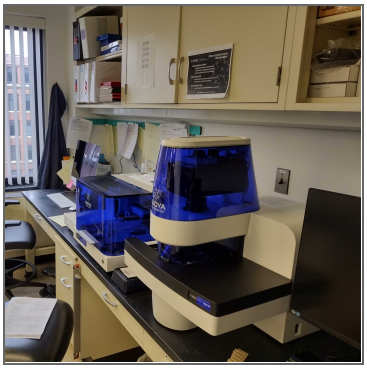

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Protocol status: Working
We use this protocol and it's working

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Multiplexed Immunofluorescence Staining and Imaging of Lung Sections

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ABSTRACT

This protocol describes multiplexed immunofluorescent 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 Phenocycler-Fusion User Guide provided by Akoya (2), and includes sections that describe A) lung tissue preparation, B) Integration of multiomic analysis (e.g., MALDI-Mass Spectroscopy) of lung tissue section into the Multiplex immunofluorescent staining protocol, C) Labeling of lung tissue sections with antibody-barcode conjugates, D) Reporter plate and experiment design, E) Multiplexed imaging and analysis, F) 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 User Guide.pdf

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.

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PROTOCOL integer ID: 87058

Keywords: lung, multiplexed immunofluorescence, Phenocycler-Fusion, FFPE, tissue sections, CODEX

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MATERIALS

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

1. Surgipath Apex Superior Adhesive Slides; P/N: 3800086 Orange (Leica® Biosystems, Richmond, IL)
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 buffer N,Gv2,J, and S blockers, and final fixative reagent).
6. 10X Buffer Kit for PhenoCycler-Fusion; P/N; 7000019 (Akoya Biosciences)
7. DMSO, ACS Reagent Grade (≥99.9%); 472301-1L Sigma-Aldrich.
8. Flow Cells (10 pack) Akoya Biosciences

PROTOCOL MATERIALS

Sodium azide **P212121** Step 14.1

Trehalose **Contributed by users** Step 14.1

SAFETY WARNINGS

Procedures involving Xylenes and paraformaldehyde should be performed in a chemical fume hood. Nitrile gloves are not impermeant 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 INSTRUCTIONS

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.**

1.1 FFPE Lung sections are prepared as described in dx.doi.org/10.17504/protocols.io.kxygxejwdv8j/v2; and mounted on Leica® Surgipath Apex Superior Adhesive Slides.

1.2 Heat Slide in oven at 60 °C Overnight .

N-glycan analysis prior to labeling with antibody-barcode con...

2 **Slide Preparation for MALDI Mass Sprectrometry includes the following steps**

2.1 Deparaffinization in Xylene (See Steps 3.1-3.3)

2.2 Delipidation in Ethanol and Rehydration (See Step 3.4-3.11)

2.3 Antigen Retrieval via immersion of slides in a Coplin jar containing 50mM citraconic buffer pH 3 and heating in a vegetable steamer 100 °C 00:30:00 30m

2.4 Washing in Water (See Step 6)

2.5 Spraying Enzyme for N-Glycan Removal

2.6 Spraying Maldi-Matrix in 50% Acetonitrile

2.7 MALDI Analysis (PNNL-protocol)

2.8 Removal of MALDI matrix with 50% Acetonitrile 2X for 00:02:00 2m

2.9 Slides are air-dried and shipped for multiplexed immunofluorescence staining.

2.10 Preparation of slides for staining post MALDI Analysis begin at step 3.4 below.

Labeling of lung tissue sections with antibody-barcode conjug... 2h 15m

3 **Deparaffination and Rehydration**
Incubate slides (5 min) in Coplin Jars containing Xylene (3X) followed by a descending ethanol series followed by molecular biology grade distilled water (2X).

3.1 Xylene #1- 00:05:00 5m

3.2 Xylene #2 00:05:00 5m

3.3 Xylene #3 00:05:00 5m

3.4 100% Ethanol #1 00:05:00 5m

3.5 100% Ethanol #2 00:05:00 5m

3.6 90% Ethanol 00:05:00 5m

3.7 70% Ethanol 00:05:00 5m

3.8 50% Ethanol 00:05:00 5m

3.9 30% Ethanol 00:05:00 5m

3.10 ddH20 00:05:00 5m

3.11 ddH20 00:05:00 5m

4 High pH Antigen Retrieval pH 9

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

4.1 Dilute AR9 buffer (Akoya Biosciences) 1/10 in ddH20 and fill plastic Coplin Jar to 90-95% volume and cover entire Coplin with aluminum foil. **(Do not Cap)**

4.2 Place Coplin Jar in an InstantPot® pressure cooker with ddH20 to 1/3-1/2 depth of Coplin Jar.

4.3 Heat on high pressure setting 00:20:00 20m

4.4 After releasing pressure 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. 1h

5 Prepare Antibody 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 (Vol. in µL)

Component	2 Slides	5 slides

Staining Buffer	362 µL	905 µL
N Blocker	9.5 µL	23.75 µL
G Blocker	9.5 µL	23.75 µL
J Blocker	9.5 µL	23.75 µL
S Blocker	9.5 µL	23.75 µL
Total Volume	400 µL	1000 µL

Note

Since stained slides can be stored in storage buffer for a maximum 5 days without diminution of staining signal intensity, and Phenocycler fusion imaging typically range from 16-20h, staining more than 5 slides at a time is not recommended.

5.2 Pipette volume of blocking buffer corresponding to total buffer volume minus volume of antibodies to be added to blocking buffer in a 1.5 ml microfuge tube (See Table below). The Blocking buffer volume must be **60% of total antibody buffer volume** for effective blocking. If needed reduce staining buffer volume (µL) to achieve 60% blocking buffer volume in Ab solution.

Antibody	Vendor	Cat#	Barcode	Dilution
SMA	Akoya	4450049	BX013	1:200
PanCK	Akoya	4450020	BX019	1:200
MPO	Akoya	4250083	BX098	1:200
Ki67	Akoya	4250019	BX047	1:200
Keratin5	Akoya	4450090	BX101	1:200
HLADR	Akoya	4550118	BX033	1:200
FOXP3	Akoya	4550071	BX031	1:200
ColIV	Akoya	4550122	BX042	1:200
CD8	Akoya	4250012	BX026	1:200
CD68	Akoya	4550113	BX015	1:200
CD45	Akoya	4550121	BX021	1:200
CD4	Akoya	4550112	BX003	1:200

Antibody	Vendor	Cat#	Barcode	Dilution
CD3e	Akoya	4550119	BX045	1:200
CD31	Akoya	4450017	BX001	1:200
CD20	Akoya	4450018	BX007	1:200
CD163	Akoya	4250079	BX069	1:200
CD14	Akoya	4450047	BX037	1:200
CD11c	Akoya	4550114	BX024	1:200
E-Cadherin	Akoya	4250021	BX014	1:200
TPSAB1*	Abcam	ab2378	BX041	1:1000
SFTPC*	Invitrogen	PA5-71842	BX020	1:500
SCGB1A1*	R&D System	MAB4218	BX043	1:400
β-III-Tubulin*	R&D Systems	MAB1195	BX055	1:400
ENDRB*	R&D Systems	MAB4496	BX027	1:50
SCEL*	Abcepta	Abcepta	BX052	1:100
RAGE*	Abcam	ab228861	BX028	1:100
LYVE1*	R&D Systems	AF2089	BX025	1:100
COL1A1*	Abcam	ab88147	BX054	1:100
CD298*	Abcam	ab167390	BX005	1:100
CD1c*	Novus	ab156708	BX016	1:50
SCGB3A2*	Abcam	ab240255	BX002	1:400
TP63*	Abcam	ab214790	BX006	1:100
MUC5AC*	Abcam	ab212636	BX040	1:100
PROX1*	R&D Systems	AF2727	BX050	1:200
CXCL4*	Peprotech	500-P05	BX004	1:200

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-conjugated section at the end of the protocol.

6 **Wash Slides and Incubate with Ab Solution** 2m

Remove slides from cooled AR9 buffer and rinse briefly by dipping slides(3X) in Coplin Jar ddH20 followed by immersion in a second Coplin Jar ddH20 00:02:00 .

6.1 Immerse Slides in sequential Coplin jars containing the following buffers from Akoya Biosciences: 54m

Hydration buffer 00:02:00

Hydration buffer 00:02:00

Staining buffer 00:20:00 - 00:30:00 **max**.

6.2 Carefully dry slide around tissue with a Kimwipe™ and then pipette 190 µL Ab solution onto slide to cover tissue section while avoiding pipetting directly onto tissue.

6.3 Incubate slides covered in a humidified chamber for 03:00:00 Room temperature . 3h

7 **Post Stain Wash-Fixation**

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 Coplin Jar #1 containing Staining buffer 00:02:00 2m

7.2 Incubate in Coplin Jar #2 containing Staining Buffer 00:02:00 2m

7.3 Incubate slides in Coplin Jar containing 1.6% paraformaldehyde (Diluted from 20% stock) 10m

00:10:00

7.4 Rinse slides sequentially in 3 Coplin Jars (3 dips each) containing PBS.

7.5 Incubate slides in Coplin jar on ice containing pre-chilled (-20 °C methanol 00:05:00 5m

7.6 Rinse slides sequentially in 3 Coplin Jars (3 dips each) PBS.

7.7 Carefully dry slide around tissue with a Kimwipe® and then pipette 190 µL Final Fix solution (20 ul of aliquot of final fix (Akoya Biosciences) diluted in 1 ml PBS onto slide to cover tissue section while avoiding pipetting directly onto tissue. Incubate 00:20:00 20m

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

8 Photobleaching and Storage

8.1 Prior to imaging the next day immerse a slide in a 100 cm2 dish containing **Storage Buffer** (Akoya Biosciences), and photobleached by illumination with a 200 mA, 15 watts, 1600 lumens bulb 4 °C Overnight .

8.2 Slides may be stored for up to 5 days in a Coplin Jar containing **Storage buffer** 4 °C .

Reporter Plate and Experiment Design

9 Reporter plate design and Phenocycler-Fusion run protocols are developed using the PhenoCycler Experiment Designer Software (Akoya Biosciences).

10 **Prepare Reporter Stock Solution**

Report Stock Solution is prepared according to guidelines in the Phenocycler-Fusion User Guide (Akoya Biosciences®)

11 **Prepare Reporter Solutions for each cycle**

Cycle (N=# of imaging runs)

	Stock Vol. (uL)	ATTO550 Reporter	AF647 Reporter	AF750 Reporter
	235 X N	5 ul X N	5 ul X N	5 ul X N
	235 X N	5 ul X N	5 ul X N	5 ul X N
	235 X N	5 ul X N	5 ul X N	5 ul X N

245 uL of reporter stock solution (blanks) or 245 uL reporter mix are aliquoted into light opaque microtiter plates, sealed, and stored @ 4 C in for up to 14 days in accordance with the PhenoCycler-Fusion User Guide (Akoya Biosciences)

Multiplexed Imaging and Analysis

12 **Image Acquistion via Phenocycler-Fusion (i.e., CODEX V2)**

12.1 If necessary, warm reporter plate to Room temperature

12.2 After photobleaching in storage buffer wash slides in PBS (250 ml; Coplin Jar) Room temperature

12.3 After the wash dry the bottom of the slide and around the edges of the tissue with a kimwipe and attach a flow cell using the flow cell assembly device (Akoya Biosciences). 00:00:30 30s

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

12.5 Fill respective Reagent reservoirs on the Phenocycler side car with DMSO, 1X phenocycler buffer, and ddH20, and place a blank flow cell in the attached flow cell carrier.

12.6 Start an imaging run by turning on the Phenocycler fluidics system and the Phenoimager, followed by launching the fusion software. Select Start experiment and follow the prompts.

12.7 Images are acquired utilizing the 20X (0.5 µM/pixel) objective and Fusion 1.0.8 software.

12.8 Image processing is automated via the Fusion 1.0.8 software and completed at the end of the experiment run.

Expected result

A. Folder with slide/sample Name Containing:
i. the respective (.xpd) file (Phenocycler Experiment designer)
ii. Akoya whole slide scan .qptiff (~8-12 GB for a 30-36 marker panel; 1 cm2 lung section

B. The following temp file contents:
i. CombineInputs
ii. Coverslip Mask
iii. qptiff raw files: 8-12 GB for each cycle (30-36 marker panel; 1 cm section)
iv. qptiff.intermediate: 8-12 GB for each cycle (30-36 marker panel; 1 cm section)
v. FocusMap
vi. Label
vii. MarkerList
viii. Overview BF
ix. Overview FL
x. SampleMask

12.9 Checking the Sample mask, BF overview, and FL overview, by dragging and dropping files into ImageJ after the first cycle is recommended. If major issues are observed, the run may be aborted to preserve reporters.
Do not attempt to open raw or intermediate cycle.qptiff during the imaging run.

12.10 Rapid review of the resultant image.qptiffs was performed utilizing PhenoChart 1.2.0 software. If necessary, exposure time (ms) was adjusted in the Phenocycler Experiment Designer to obtain readily detectable, specific marker signals that are below saturation.

12.11 After the run return the slide 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.

13 Image Analysis and Segmentation

13.1 Analysis of processed image.qptiff files is performed utilizing QuPath.

13.2 Cell segmentation based on DAPI stained nuclei is performed utilizing the respective StarDist extension (i.e., 0.3 or 0.4) in QuPath.

Custom antibody conjugation

14 **Custom Antibody Conjugation is performed as described** **dx.doi.org/10.17504/protocols.io.3fugjnw.**

14.1 For antibodies containing Sodium azide P212121 (0.05-0.1%) or Trehalose Contributed by users (5%) buffer exchange is performed utilizing Zeba Spin Desalting columns 7K MWCO (89890, 2ml, Thermoscience) equilibrated in PBS in accordance with the manufacturer's recommendations.

14.2 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).

H&E staining Post Phenocycler-Fusion

10m

15 **Slides with lung tissue sections covered with a flow cell (See Phenocycler-User Guide) are stained with H&E as described** dx.doi.org/10.17504/protocols.io.kqdg397yeg25/v1

10m

Eosin staining tends to be less intense, therefore recommended duration of eosin staining is 00:10:00 or longer.