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Protocol for Multiplexed Immunofluorescence Imaging and Analysis of the Tumor Microenvironment

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

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Abstract

High throughput multiplex immunofluorescent (mIF) image preparation and analysis provides a rapid, dependable, and informative method for clinical diagnosis and prognosis of diseases and therapeutic responses. This protocol describes the preparation of a melanoma mIF panel designed to detect six protein biomarkers along with DAPI in formalin-fixed paraffin-embedded (FFPE) human melanoma tumor sections. The method involves sequential staining of tissue sections using the Leica BOND™ RX autostainer through repeated cycles of heat-induced epitope retrieval (HIER), blocking, primary antibody incubation, secondary antibody binding, Opal fluorophore application, and antibody elution. The Opal fluorescent signal remains stable during the antibody stripping process, enabling additional rounds of staining for new targets without the risk of cross-reactivity, even for co-localized markers. The protocol also incorporates nuclear counterstaining with DAPI. While the protocol accommodates up to seven markers (six antigens plus DAPI), individual antigen targets can be substituted and re-optimized as needed. The outlined workflow includes the following steps: (1) Staining using the Leica Bond™ RX, (2) Scanning with the Akoya PhenoImager HT™ 2.0, and (3) image annotation and analysis using PhenoChart 3.0, inFORM® 3.0, and phenoptrReports 0.3.2.

This protocol leverages multispectral imaging and built-in spectral unmixing to facilitate efficient large-scale spatial signature analysis. The chosen antibodies—FoxP3, PD-L1, Sox10, PD-1, CD8, and CD68—enable a thorough assessment of PD-L1- and PD-1-mediated immune responses in the context of melanoma tumor cells.

Attachments

PDF

Protocol Multiplex P...

271KB

Guidelines

Guidelines from the CDC and CAP outline safe work practices for human medical and histopathological diagnostic laboratories. Any specimen suspected of containing tubercle bacilli must be processed in a BSL-3 laboratory using Class IIA2 biological safety cabinets that undergo annual inspections. Once the sample has been inactivated or its genetic material extracted, subsequent testing—including multiplex immunofluorescence (mIF) processing and data analysis—can be safely conducted in a BSL-2 laboratory or office environment.

Materials

Materials and Reagents

Antibodies

Item	Company	Catalog Number
Anti-FoxP3 D608R	Cell Signaling Technology	#12653
Anti-PD-L1 (SP263)	Roche Diagnostics	790-4905
Anti-Sox10 EP268-1	Cell Marque	383R-14
Anti-PD-1 EPR4877(2)	Abcam	ab137132
Anti-CD8 4B11	Leica Biosystems	CD8-4B11-L-U
Anti-CD68 PG-M1	Thermo Fisher Scientific	MA5-12407

mIF

Processing and Labeling Reagents

Item	Company	Product #
1X Plus Auto Amplification Diluent	Akoya Biosciences	FP1609
Opal Polymer HRP Ms + Rb	Akoya Biosciences	ARH1001EA
Antibody Diluent/Block buffer	Akoya Biosciences	ARD1001EA
Opal 480 Reagent	Akoya Biosciences	FP1500001KT
Opal 520 Reagent	Akoya Biosciences	FP1487001KT
Opal 540 Reagent	Akoya Biosciences	FP1494001KT
Opal 570 Reagent	Akoya Biosciences	FP1488001KT
Opal 620 Reagent	Akoya Biosciences	FP1495001KT
Opal 650 Reagent	Akoya	

Biosciences	FP1496001KT	
Opal 690 Reagent	Akoya Biosciences	FP1497001KT
Opal 780 Reagent Pack (Opal TSA-DIG, 1 vial, Opal Polaris 780, 1 vial)	Akoya Biosciences	FP1501001KT
Spectra DAPI	Akoya Biosciences	FP1501001KT

Instruments

Item	Company	Catalog Number
BOND RX Automated Stainer	Lecia Biosystems	21.2821
PhenoImager HT™ 2.0 Automated Quantitative Pathology Imaging System	Akoya Biosystems	CLS143455

Bond

RX Reagents and Materials

Item	Company	Catalog Number
Titration Kit	Leica Biosystems	OPT9049
Open container - 30 mL	Leica Biosystems	OP309700
Titration kit container - 6 mL	Leica Biosystems	OPT9049
Research Detection System 2	Leica Biosystems	DS9777
Universal Covertiles	Leica Biosystems	S21.4611
Slide Tray	Leica Biosystems	S21.0304
Reagent Tray	Leica Biosystems	S21.1003
Slide Labels and Printer Ribbon	Leica Biosystems	S21.4564

Apex Adhesive Slide	Leica Biosystems	3800040
Dewax Solution	Leica Biosystems	AR9222
Epitope Retrieval Solution 1 (pH6)	Leica Biosystems	AR9961
Epitope Retrieval Solution 2 (pH9)	Leica Biosystems	AR9640
BOND Wash Solution 10X Concentrate	Leica Biosystems	AR9590
BOND Aspirating Probe Cleaning System	Leica Biosystems	CS9100
Coverslip 24 × 32 mm #1 (0.13-0.16 mm)	Fisher Scientific	15717592
ProLong™ Diamond Antifade Mountant	Thermo Fisher Scientific	P36965

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Data Analysis

Item	Company
inFORM® software 3.0	Akoya Biosciences
PhenoChart™ 2.0	Akoya Biosciences
phenoptrReports 0.3.2	Akoya Biosciences
PhenoImager HT™ 2.0	Akoya Biosciences
R 4.4.2	open source
RStudio 2024.09.1+394	open source

FFPE

Melanoma Tissue Slides

Item	Company	Catalogue Number
Human Skin Tumor: Malignant Melanoma	BioChain	T2235218-3
Human Adult Normal: Tonsil	BioChain	T2234268