Flow Cytometry and High-Content Imaging to Identify Markers of Monocyte-Macrophage Differentiation

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BD Biosciences

Application Note

Contents

Abstract

The identification and characterization of cell surface markers present on different subtypes of cells of the immune system can broaden the understanding of immune cell function. The BD Lyoplate™ human cell surface marker screening panel is a collection of cell surface marker antibodies designed for screening cells for the expression of 242 human cell surface proteins by flow cytometry or fluorescence imaging. Using a human monocytic cell line, THP-1, undifferentiated (suspension) THP-1 cells were screened for surface marker expression using a BD FACSCanto™ II flow cytometer. Differentiated (adherent) THP-1 macrophages were screened using a BD Pathway™ high-content imaging system. The analysis of results from both proof-of-principle screening experiments identified 21 markers expressed on both THP-1 monocytes and macrophages, 23 markers uniquely expressed on THP-1 monocytes, and 20 markers uniquely expressed on THP-1 macrophages.

The expression of a subset of the identified markers was further investigated during a time course of differentiation of THP-1 monocytes into macrophages. In addition, expression of the cell surface marker CD54 was multiplexed with NFκB translocation and lysosomal tracking assays. This multiplexed assay was performed after lipopolysaccharide (LPS) activation of THP-1 macrophages using high-content imaging. The simplified workflow presented in this application note demonstrates how researchers can use BD Lyoplate screening panels with BD FACS™ flow cytometry systems with BD™ High Throughput Samplers (HTS) and BD Pathway high-content imaging systems. With these panels, researchers could quickly develop novel strategies to characterize and isolate not only specific cells of the immune system, but many types of suspension or adherent cells, based upon their unique protein expression profiles at various states of differentiation and culture conditions.

Introduction

Studies of complex eukaryotic cells have been made possible by flow cytometry and imaging technologies that enable single-cell analysis. These two powerful tools have led to the characterization and function of a multitude of cells for various basic, applied, and clinical research applications. Both technologies described here use fluorochrome-labeled reagents that can be used to label cells, and have their own advantages and disadvantages. Table 1 compares these technologies.

Table 1. Comparison of flow cytometry and high-content imaging technologies

Features	Flow Cytometry	High-Content Imaging
Parameters measured	Fluorescence intensity (up to 18 colors), forward scatter, and side scatter	Fluorescence intensity (up to 4 colors), xyz position, morphology, time lapse (kinetics, motility)
Cells analyzed	1,000s–100,000s of cells in single-cell suspension	1–1,000s of adherent (or settled, suspended) cells
Sample utilization	Analyzers: cells are discarded after acquisition. Sorters: cells can be collected following sorting.	Live cells in culture can be re-imaged over time. Fixed cells can be stored and re-imaged as needed.
Strengths	• Detection of rare events • Analysis of cells within heterogeneous populations • Statistical population analysis • Higher order multicolor analysis • Cell isolation (sorting)	• Subcellular resolution • Morphological and spatial analysis • Live single-cell kinetics • Visual confirmation of data

During the differentiation of various cell types, such as stem cells into specialized lineages, the phenotypic morphology of the cells might change substantially in both in vitro and in vivo models. For example, in in vitro differentiation models, suspension monocytic cells acquire adherent properties when differentiated into macrophages. Similarly, upon treatment with growth factors, neuronal cells such as PC12 cells differentiate into neurons displaying long neurites. To perform flow cytometric analysis on these adherent differentiated cells, the cells have to be detached from the growth surface. Although various enzymatic and physical treatments are commonly used to detach cells to perform flow cytometry, this treatment might alter the native state of the cell. Moreover, information such as colony size and cell shape is compromised. In vitro differentiation of cells using growth factors or agonists is also a lengthy process, and the differentiated cells are often available in limited numbers not sufficient for flow cytometry analysis. High-content imaging uses the same fluorescence technology as flow cytometry but is particularly useful for acquiring data on live or fixed adherent cell types, and when combined with image analysis, can provide quantitative output. The subcellular events, such as translocation of proteins, can also be analyzed using imaging. In addition, this technique also provides the ability to use combinations of markers (multiplexing) to simultaneously acquire data from multiple assays in a single experiment. Overall, the use of both flow and imaging techniques allows researchers to acquire more complete data from cells in their native state.

Monoclonal antibodies are powerful tools for studying cell surface protein expression signatures. The BD Lyoplate human cell surface marker screening panel is a collection of 242 lyophilized monoclonal antibodies for the direct profiling of cell surface proteins. This screening panel kit is available in the form of three 96-well plates that are ideally suited for high-content screening applications using both flow cytometry and imaging platforms. Researchers can perform high-throughput screening using flow cytometry with the BD High Throughput Sampler (HTS), which can be attached to many BD flow cytometers such as the BD FACSCanto II, BD™ LSR II, BD LSRFortessa™, and

BD FACSCalibur™ systems. The HTS provides fully automated and rapid sample acquisition from either 96- or 384-well microtiter plates. BD flow cytometers can analyze several thousand events (in suspension) every second and offer high-throughput automated quantification of various parameters. BD Pathway cell analyzers provide an alternative solution for high-content imaging whereby adherent cells, in multiwell-plate format, can be imaged and analyzed in a high-throughput manner.

Recent studies undertaken in collaboration with BD have shown that the screening of cell surface markers can provide powerful tools in stem cell research, for isolation and purification of stem cell populations, based on their surface marker signature.[1] In these experiments, we used the THP-1 monocyte-macrophage model to identify the surface marker expression profiles of undifferentiated (monocytic) and differentiated (macrophagocytic) THP-1 cells.

The human monocytic cell line THP-1 is a widely used cell line with properties similar to human monocyte-derived macrophages. Suspension THP-1 cells can be easily induced to differentiate or mature into adherent macrophages when stimulated with phorbol esters such as phorbolmyristate acetate (PMA). This is a well established model that closely resembles native monocyte-derived macrophages, yet very little information is available on the expression of surface proteins and receptors during differentiation. Understanding the differential expression of surface markers can be applied to various aspects of macrophage biology such as role of macrophages in host defense, tissue homeostasis, and in immunological and inflammatory responses.

Using the THP-1 monocyte and macrophage model, screening was performed on suspension THP-1 monocytes using flow cytometry, and on adherent differentiated THP-1 macrophages using high-content imaging. The goal of the experiments outlined in this application note is to demonstrate how flow cytometry and high-content imaging can be used as synergistic technologies to obtain quantitative results from a high-content screen of cells at multiple states of differentiation.

Objective

The objective of the proof-of-principle experiments described in this application note was to determine the surface protein expression profile of the THP-1 cell line model with following specific goals:

- Screen suspension THP-1 cells (monocytes) stained with the BD Lyoplate human cell surface marker screening panel using flow cytometry.
- Screen PMA-differentiated THP-1 cells (adherent macrophages) stained with the BD Lyoplate human cell surface marker screening panel using high-content imaging.
- Demonstrate a time course of expression of a subset of markers identified in the BD Lyoplate screen during the differentiation of THP-1 cells using PMA.
- Demonstrate the effects of macrophage activation on the expression of CD54 and multiplex with nuclear translocation and lysosomal tracking assays.

Table 2. List of monoclonal antibodies in the BD Lyoplate human cell surface marker screening panel

Plate 1

Specificity	Clone	Isotype
CD1a	HI149	Ms IgG$_1$, κ
CD1b	M-T101	Ms IgG$_1$, κ
CD1d	CD1d42	Ms IgG$_1$, κ
CD2	RPA-2.10	Ms IgG$_1$, κ
CD3	HIT3a	Ms IgG$_{2a}$, κ
CD4	RPA-T4	Ms IgG$_1$, κ
CD4v4	L120	Ms IgG$_1$, κ
CD5	L17F12	Ms IgG$_{2a}$, κ
CD6	M-T605	Ms IgG$_1$, κ
CD7	M-T701	Ms IgG$_1$, κ
CD8a	SK1	Ms IgG$_1$, κ
CD8b	2ST8.5H7	Ms IgG$_{2a}$, κ
CD9	M-L13	Ms IgG$_1$, κ
CD10	HI10a	Ms IgG$_{2a}$, κ
CD11a	G43-25B	Ms IgG$_{2a}$, κ
CD11b	D12	Ms IgG$_{2a}$, κ
CD11c	B-ly6	Ms IgG$_1$, κ
CD13	WM15	Ms IgG$_1$, κ
CD14	M5E2	Ms IgG$_{2a}$, κ
CD15	HI98	Ms IgM, κ
CD15s	CSLEX1	Ms IgM, κ
CD16	3G8	Ms IgG$_1$, κ
CD18	6.7	Ms IgG$_1$, κ
CD19	HIB19	Ms IgG$_1$, κ
CD20	2H7	Ms IgG$_{2b}$, κ
CD21	B-ly4	Ms IgG$_1$, κ
CD22	HIB22	Ms IgG$_1$, κ
CD23	EBVCS-5	Ms IgG$_1$, κ
CD24	ML5	Ms IgG$_{2a}$, κ
CD25	M-A251	Ms IgG$_1$, κ
CD26	M-A261	Ms IgG$_1$, κ
CD27	M-T271	Ms IgG$_1$, κ

Specificity	Clone	Isotype
CD28	L293	Ms IgG$_1$, κ
CD29	HUTS-21	Ms IgG$_{2a}$, κ
CD30	BerH8	Ms IgG$_1$, κ
CD31	WM59	Ms IgG$_1$, κ
CD32	FL18.26	Ms IgG$_{2b}$, κ
CD33	HIM3-4	Ms IgG$_1$, κ
CD34	581	Ms IgG$_1$, κ
CD35	E11	Ms IgG$_1$, κ
CD36	CB38 (NL07)	Ms IgM, κ
CD37	M-B371	Ms IgG$_1$, κ
CD38	HIT2	Ms IgG$_1$, κ
CD39	TU66	Ms IgG$_{2b}$, κ
CD40	5C3	Ms IgG$_1$, κ
CD41a	HIP8	Ms IgG$_1$, κ
CD41b	HIP2	Ms IgG$_3$, κ
CD42a	ALMA.16	Ms IgG$_1$, κ
CD42b	HIP1	Ms IgG$_1$, κ
CD43	1G10	Ms IgG$_1$, κ
CD44	G44-26	Ms IgG$_{2b}$, κ
CD45	HI30	Ms IgG$_1$, κ
CD45RA	HI100	Ms IgG$_{2b}$, κ
CD45RB	MT4	Ms IgG$_1$, κ
CD45RO	UCHL1	Ms IgG$_{2a}$, κ
CD46	E4.3	Ms IgG$_{2a}$, κ
CD47	B6H12	Ms IgG$_1$, κ
CD48	TU145	Ms IgM, κ
CD49a	SR84	Ms IgG$_1$, κ
CD49b	AK-7	Ms IgG$_1$, κ
CD49c	C3 II.1	Ms IgG$_1$, κ
CD49d	9F10	Ms IgG$_1$, κ
CD49e	VC5	Ms IgG$_1$, κ
CD50	TU41	Ms IgG$_{2b}$, κ

Specificity	Clone	Isotype
CD51/61	23C6	Ms IgG$_1$, κ
CD53	HI29	Ms IgG$_1$, κ
CD54	LB-2	Ms IgG$_{2b}$, κ
CD55	IA10	Ms IgG$_{2a}$, κ
CD56	B159	Ms IgG$_1$, κ
CD57	NK-1	Ms IgM, κ
CD58	1C3	Ms IgG$_{2a}$, κ
CD59	p282 (H19)	Ms IgG$_{2a}$, κ
CD61	VI-PL2	Ms IgG$_1$, κ
CD62E	68-5H11	Ms IgG$_1$, κ
CD62L	Dreg 56	Ms IgG$_1$, κ
CD62P	AK-4	Ms IgG$_1$, κ
CD63	H5C6	Ms IgG$_1$, κ
CD64	10.1	Ms IgG$_1$, κ
CD66 (a,c,d,e)	B1.1/CD66	Ms IgG$_{2a}$, κ
CD66b	G10F5	Ms IgM, κ
CD66f	IID10	Ms IgG$_1$, κ
CD69	FN50	Ms IgG$_1$, κ
CD70	Ki-24	Ms IgG$_3$, κ
CD71	M-A712	Ms IgG$_{2a}$, κ
CD72	J4-117	Ms IgG$_{2b}$, κ
CD73	AD2	Ms IgG$_1$, κ
CD74	M-B741	Ms IgG$_{2a}$, κ
CD75	LN1	Ms IgM, κ
CD77	5B5	Ms IgM, κ
CD79b	CB3-1	Ms IgG$_1$, κ
CD80	L307.4	Ms IgG$_1$, κ
CD81	JS-81	Ms IgG$_1$, κ
CD83	HB15e	Ms IgG$_1$, κ
CD84	2G7	Ms IgG$_1$, κ
CD85	GHI/75	Ms IgG$_{2b}$, κ

Plate 2

Specificity	Clone	Isotype
CD86	2331 (FUN-1)	Ms IgG$_1$, κ
CD87	VIM5	Ms IgG$_1$, κ
CD88	D53-1473	Ms IgG$_1$, κ
CD89	A59	Ms IgG$_1$, κ
CD90	5E10	Ms IgG$_1$, κ
CD91	A2MR-alpha 2	Ms IgG$_1$, κ
CDw93	R139	Ms IgG$_{2b}$, κ
CD94	HP-3D9	Ms IgG$_1$, κ
CD95	DX2	Ms IgG$_1$, κ
CD97	VIM3b	Ms IgG$_1$, κ
CD98	UM7F8	Ms IgG$_1$, κ
CD99	TU12	Ms IgG$_{2a}$, κ
CD99R	HIT4	Ms IgM, κ
CD100	A8	Ms IgG$_1$, κ
CD102	CBR-1C2/2.1	Ms IgG$_{2a}$, κ
CD103	Ber-ACT8	Ms IgG$_1$, κ
CD105	266	Ms IgG$_1$, κ
CD106	51-10C9	Ms IgG$_1$, κ
CD107a	H4A3	Ms IgG$_1$, κ
CD107b	H4B4	Ms IgG$_1$, κ
CD108	KS-2	Ms IgG$_{2a}$, κ
CD109	TEA 2/16	Ms IgG$_1$, κ
CD112	R2.525	Ms IgG$_1$, κ
CD114	LMM741	Ms IgG$_1$, κ
CD116	M5D12	Ms IgM, κ
CD117	YB5.B8	Ms IgG$_1$, κ
CD118	12D3	Ms IgG$_1$, κ
CD119	GIR-208	Ms IgG$_1$, κ
CD120a	MABTNFR1-A1	Ms IgG1
CD121a	HIL1R-M1	Ms IgG$_1$, κ
CD121b	MNC2	Ms IgG$_1$, κ
CD122	Mik-beta 3	Ms IgG$_1$, κ

Specificity	Clone	Isotype
CD123	9F5	Ms IgG$_1$, κ
CD124	hIL4R-M57	Ms IgG$_1$, κ
CD126	M5	Ms IgG$_1$, κ
CD127	hIL-7R-M21	Ms IgG$_1$, κ
CD128b	6C6	Ms IgG$_1$, κ
CD130	AM64	Ms IgG$_1$, κ
CD134	ACT35	Ms IgG$_1$, κ
CD135	4G8	Ms IgG$_1$, κ
CD137	4B4-1	Ms IgG$_1$, κ
CD137 Ligand	C65-485	Ms IgG$_1$, κ
CD138	Mi15	Ms IgG$_1$, κ
CD140a	alpha R1	Ms IgG$_{2a}$, κ
CD140b	28D4	Ms IgG$_{2a}$, κ
CD141	1A4	Ms IgG$_1$, κ
CD142	HTF-1	Ms IgG$_1$, κ
CD144	55-7H1	Ms IgG$_1$, κ
CD146	P1H12	Ms IgG$_1$, κ
CD147	HIM6	Ms IgG$_1$, κ
CD150	A12	Ms IgG$_1$, κ
CD151	14A2.H1	Ms IgG$_1$, κ
CD152	BNI3	Ms IgG$_{2a}$, κ
CD153	D2-1173	Ms IgG$_1$, κ
CD154	TRAP1	Ms IgG$_1$, κ
CD158a	HP-3E4	Ms IgM, κ
CD158b	CH-L	Ms IgG$_{2b}$, κ
CD161	DX12	Ms IgG$_1$, κ
CD162	KPL-1	Ms IgG$_1$, κ
CD163	GHI/61	Ms IgG$_1$, κ
CD164	N6B6	Ms IgG$_{2a}$, κ
CD165	SN2	Ms IgG$_1$, κ
CD166	3A6	Ms IgG$_1$, κ
CD171	5G3	Ms IgG2a

Specificity	Clone	Isotype
CD172b	B4B6	Ms IgG$_1$, κ
CD177	MEM-166	Ms IgG$_1$, κ
CD178	NOK-1	Ms IgG 1
CD180	G28-8	Ms IgG$_1$, κ
CD181	5A12	Ms IgG$_{2b}$, κ
CD183	1C6/CXCR3	Ms IgG$_1$, κ
CD184	12G5	Ms IgG$_{2a}$, κ
CD193	5E8	Ms IgG$_{2b}$, κ
CD195	2D7/CCR5	Ms IgG$_{2a}$, κ
CD196	11A9	Ms IgG$_1$, κ
CD197	2H4	Ms IgM, κ
CD200	MRC OX-104	Ms IgG$_1$, κ
CD205	MG38	Ms IgG$_{2b}$, κ
CD206	19.2	Ms IgG$_1$, κ
CD209	DCN46	Ms IgG$_{2b}$, κ
CD220	3B6/IR	Ms IgG$_1$, κ
CD221	3B7	Ms IgG$_1$, κ
CD226	DX11	Ms IgG$_1$, κ
CD227	HMPV	Ms IgG$_1$, κ
CD229	HLy9.1.25	Ms IgG$_1$, κ
CD231	M3-3D9 (SN1a)	Ms IgG$_1$, κ
CD235a	GA-R2 (HIR2)	Ms IgG$_{2b}$, κ
CD243	17F9	Ms IgG$_{2b}$, κ
CD244	2-69	Ms IgG$_{2a}$, κ
CD255	CARL-1	Ms IgG3
CD268	11C1	Ms IgG$_1$, κ
CD271	C40-1457	Ms IgG$_1$, κ
CD273	MIH18	Ms IgG$_1$, κ
CD274	MIH1	Ms IgG$_1$, κ
CD275	2D3/B7-H2	Ms IgG$_{2b}$, κ
CD278	DX29	Ms IgG1

Plate 3

Specificity	Clone	Isotype
CD279	MIH4	Ms IgG$_1$, κ
CD282	11G7	Ms IgG$_1$, κ
CD305	DX26	Ms IgG$_1$, κ
CD309	89106	Ms IgG$_1$, κ
CD314	1D11	Ms IgG$_1$, κ
CD321	M.AB.F11	Ms IgG$_1$, κ
CDw327	E20-1232	Ms IgG$_1$, κ
CDw328	F023-420	Ms IgG$_1$, κ
CDw329	E10-286	Ms IgG$_1$, κ
CD335	9E2/NKp46	Ms IgG$_1$, κ
CD336	P44-8.1	Ms IgG$_1$, κ
CD337	P30-15	Ms IgG$_1$, κ
CD338	5D3	Ms IgG$_{2b}$, κ
CD340	Neu24.7	Ms IgG1
αβTCR	T10B9.1A-31	Ms IgM, κ
β2-microglobulin	TU99	Ms IgM, κ
BLTR-1	203/14F11	Ms IgG$_1$, κ
CLIP	CerCLIP	Ms IgG$_1$, κ
CMRF-44	CMRF44	Ms IgM, κ
CMRF-56	CMRF56	Ms IgG$_1$, κ
EGF Receptor	EGFR1	Ms IgG$_{2b}$, κ

Specificity	Clone	Isotype
fMLP receptor	5F1	Ms IgG$_1$, κ
γδTCR	B1	Ms IgG$_1$, κ
HPC	BB9	Ms IgG1
HLA-A,B,C	G46-2.6	Ms IgG$_1$, κ
HLA-A2	BB7.2	Ms IgG$_{2b}$, κ
HLA-DQ	TU169	Ms IgG$_{2a}$, κ
HLA-DR	G46-6 (L243)	Ms IgG$_{2a}$, κ
HLA-DR, DP, DQ	TU39	Ms IgG$_{2a}$, κ
Invariant NK T	6B11	Ms IgG$_1$, κ
Disialoganglioside GD2	14.G2a	Ms IgG2a
MIC A/B	6D4	Ms IgG2a
NKB1	DX9	Ms IgG$_1$, κ
SSEA-1	MC480	Ms IgM, κ
SSEA-4	MC813-70	Ms IgG3
TRA-1-60	TRA-1-60	Ms IgM
TRA-1-81	TRA-1-81	Ms IgM, κ
Vβ 23	AHUT7	Ms IgG$_1$, κ
Vβ 8	JR2	Ms IgG$_{2b}$, κ
Ms IgM IC	G155-228	Ms IgM
Ms IgG1 IC	MOPC-21	Ms IgG1

Specificity	Clone	Isotype
Ms IgG2a IC	G155-178	Ms IgG2a
Ms IgG2b IC	27-35	Ms IgG2b
Ms IgG3 IC	J606	Ms IgG3
CD49f	GoH3	Rt IgG$_{2a}$, κ
CD104	439-9B	Rt IgG$_{2b}$, κ
CD120b	hTNFR-M1	Rt IgG$_{2b}$, κ
CD132	TUGh4	Rt IgG$_{2b}$, κ
CD201	RCR-252	Rt IgG$_1$, κ
CD210	3F9	Rt IgG$_{2a}$, κ
CD212	2B6/12beta 2	Rt IgG$_{2a}$, κ
CD267	1A1-K21-M22	Rt IgG$_{2a}$, κ
CD294	BM16	Rt IgG$_{2a}$, κ
CD326	EBA-1	Ms IgG$_1$, κ
CLA	HECA-452	Rt IgM, κ
Integrin β7	FIB504	Rt IgG$_{2a}$, κ
SSEA-3	MC631	Rt IgM
Rt IgM IC	R4-22	Rt IgM
Rt IgG1 IC	R3-34	Rt IgG1
Rt IgG2a IC	R35-95	Rt IgG2a
Rt IgG2b IC	A95-1	Rt IgG2b

Methods

Reagents and Materials

Product Description	Vendor	Catalog Number
THP-1 cell line	ATCC	TIB-202
BD™ Cytometer Setup and Tracking (CS&T) beads	BD Biosciences	641319/642412
BD Falcon™ 96-well microplates, black/clear with lid, for high-content imaging assays (imaging plates)	BD Biosciences	353219
BD Falcon 96-well microplates, round bottom, no lid, for high-throughput flow cytometry analysis (HTS plates)	BD Biosciences	353910
BD Cytofix™ fixation buffer	BD Biosciences	554655
BD Pharmingen™ stain buffer (FBS)	BD Biosciences	554656
BD Perm/Wash™ buffer I	BD Biosciences	557885
BD Lyoplate human cell surface marker screening panel	BD Biosciences	560747
HCS CellMask™ Red stain	Invitrogen	H32712
Alexa Fluor® 647 goat anti-mouse IgG	Invitrogen	A-21236
Alexa Fluor® 488 goat anti-rat IgG	Invitrogen	A-11006
Alexa Fluor® 488 goat anti-rabbit IgG	Invitrogen	A-11008
Alexa Fluor® 488 goat anti-mouse IgG	Invitrogen	A-11029
Phosphate Buffered Saline (PBS)	Invitrogen	14190-144
L-Glutamine	Invitrogen	25030
NFκb p65 (C-20)	Santa Cruz Biotechnology	sc-372
Phorbol-12-myristate-13-acetate (PMA)	Sigma	P8139
Dimethyl Sulfoxide (DMSO)	Sigma	D2650
4', 6'-diamidino-2-phenylindole (DAPI)	Sigma	D9564
IgG from human serum	Sigma	I4506
Lipopolysaccharide (LPS)	Sigma	L2630
Fetal bovine serum (FBS) (HyClone, SH30088.03)	VWR	16777-534
RPMI 1640 Medium (HyClone, SH30096.02)	VWR	16777-180
Penicillin-streptomycin mixture (Pen-Strep) (Lonza, 17-602F)	VWR	120001-694

Instruments and Software

Flow cytometry data was acquired using a BD FACSCanto II flow cytometer. The flow cytometer was set up using BD Cytometer Setup and Tracking (CS&T) beads. BD FACSDiva™ software (v6.1.3) was used for acquisition and analysis, and FCS Express was used for additional analysis.

High-content imaging data was acquired on a BD Pathway 435 system using BD AttoVision™ v1.7 software in the form of a 3 x 3 montage using a 20x (0.75NA) objective in non-confocal mode.

Cell Culture and Differentiation

The THP-1 cell line was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mmol/L of L-glutamine, and 1% Pen-Strep (complete medium). Suspension THP-1 cells (18,000 cells per well) were differentiated into adherent cells in imaging plates using 100 nmol/L of PMA for 3 days followed by 1 day in PMA-free medium and incubated at 37°C in 5% CO_2 in air.

BD Lyoplate Screening Panels

BD Lyoplate human cell surface marker screening panel plates, containing lyophilized antibodies, were reconstituted by adding 110 µL of 1X sterile PBS to each well as outlined in the technical data sheet of the kit.[2] The reconstituted antibodies were stored at 4°C and used for screening within 10 days.

Screening Strategy

Figure 1 summarizes the screening strategy used with the THP-1 cell line. To perform high-throughput flow cytometry screening, cells were dispensed into HTS plates, and stained following the BD Lyoplate flow cytometry screening protocol.[2] Data from stained cells was acquired on the BD FACSCanto II system with the HTS. For macrophage screening, THP-1 cells were directly differentiated in imaging plates and stained following the BD Lyoplate image screening protocol.[2] The data was acquired using a BD Pathway 435 high-content cell analyzer.

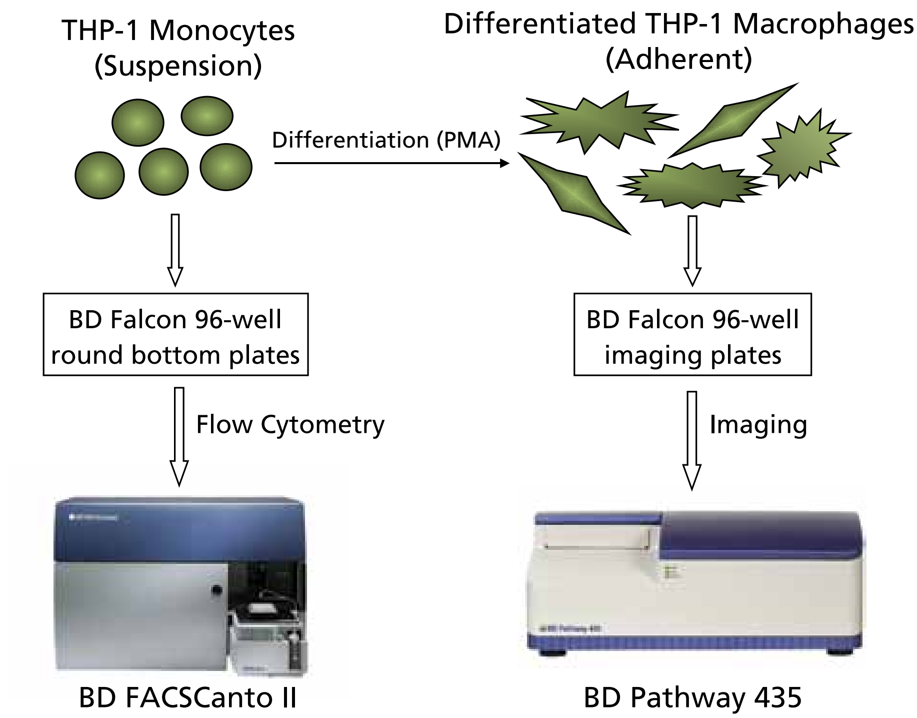


Figure 1. Screening strategy.

THP-1 monocytes were suspended in HTS plates, stained with the BD Lyoplate human cell surface marker screening panel, and screened using the BD FACSCanto II system with a BD HTS. For high-content imaging, THP-1 monocytes were differentiated into macrophages using PMA in imaging plates. The differentiated macrophages were then stained with the BD Lyoplate human cell surface marker screening panel and screened on a BD Pathway 435 system.

Flow Cytometry Assay Optimization and Screening

THP-1 suspension cells were harvested, washed in stain buffer, counted using the Trypan Blue exclusion method, and resuspended in stain buffer at a concentration of 2.5×10^6/mL. Nonspecific antibody binding sites were blocked with 10 µg/mL of human IgG for 15 minutes at room temperature (RT). Cells were washed (300g, 5 min, RT), resuspended in stain buffer, and dispensed in three HTS plates at 2.5×10^5 cells/100 µL per well. The staining protocol recommended in the technical data sheet of the BD Lyoplate human cell surface marker screening panel was followed.[2]

Imaging Assay Optimization and Screening

THP-1 cells were differentiated in three imaging plates as outlined. To prevent nonspecific binding of antibodies, differentiated THP-1 cells were incubated with 10 µg/mL of human IgG in stain buffer for 15 minutes at room temperature. The staining protocol recommended in the TDS of the BD Lyoplate human cell surface marker screening panel was followed except that, in the last step, cells were stained with Alexa Fluor® 488 (2.5 µg/mL) conjugated secondary antibody and DAPI (0.2 µg/mL) to facilitate multiplexing with other fluorochromes.[2]

Flow Cytometry Data Analysis

Flow cytometry data was analyzed using BD FACSDiva batch analysis followed by the export of FCS files into FCS Express to create histogram overlays for surface markers with the appropriate controls (Figure 2). Microsoft® Excel® 2007 (Excel 2007) was used to organize output data, and heat maps based upon the percent positive values of each surface marker were created using the conditional formatting feature.

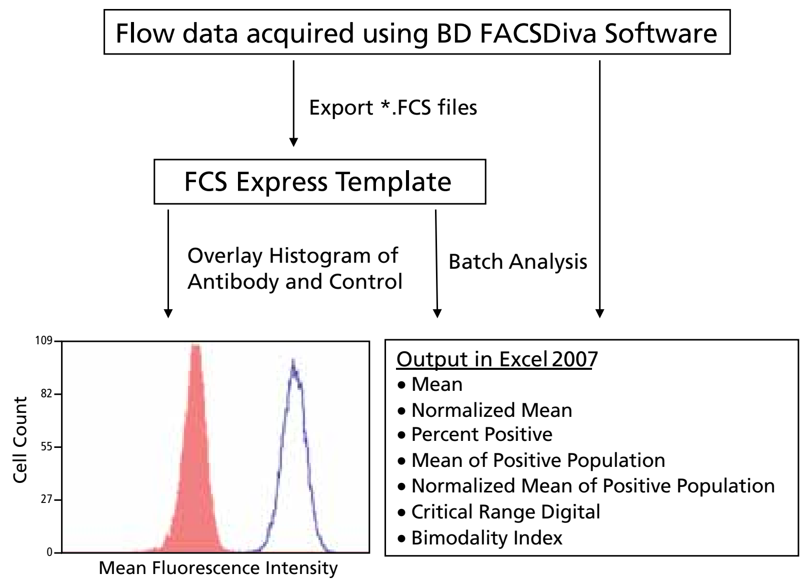


Figure 2. Flow cytometry screen data analysis.

The flow cytometry data was acquired using BD FACSDiva software. The data analysis was performed using batch analysis in BD FACSDiva software, and the histogram overlays for surface markers (blue) and control antibodies (pink) were created using an FCS Express template. After the batch analysis, the data was automatically exported to Excel 2007 in the column and plate format.

Image Data Analysis

Image analysis was performed using dual-channel segmentation using DAPI and whole cell dye channels to create regions of interest (ROIs) around the whole cells. Image pre-processing in the form of shading was used in both channels (Figure 3). In addition, smoothing filters such as gaussian_4096 and median 3x3 from BD AttoVision software were used to smooth the boundaries around the cells. After segmentation, the intensity of the antibodies was measured within the ROIs and the percent positive cells was calculated using intensity of secondary antibody controls as a threshold.

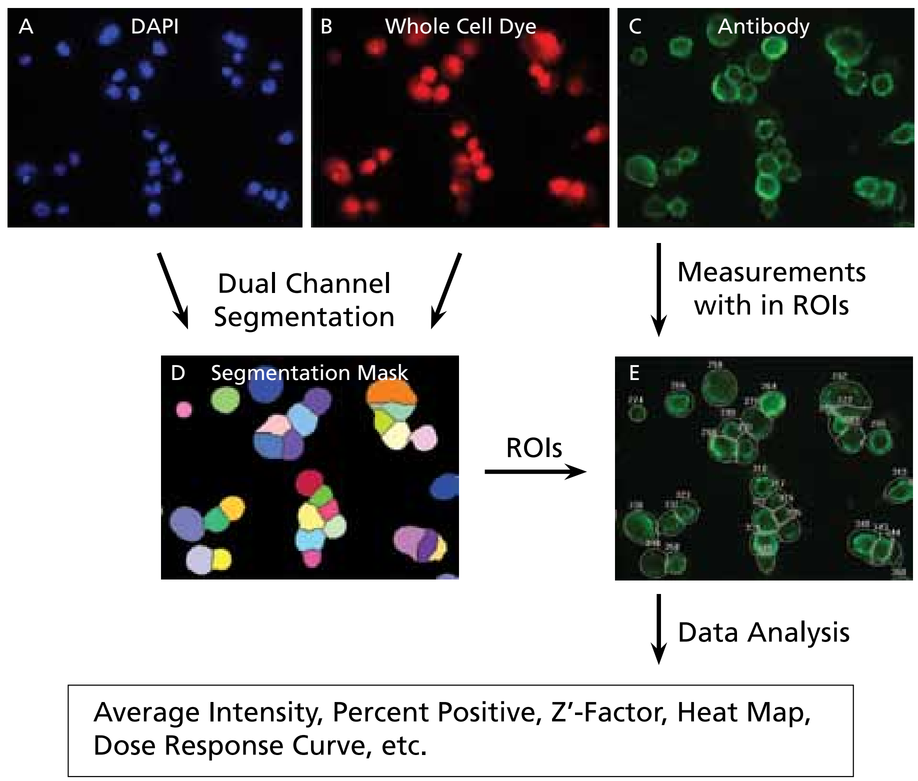


Figure 3. High-content screen data analysis.

Representative pseudocolor merged images: panel A, DAPI, panel B, whole cell dye, and panel C, antibody channels. DAPI and whole cell dye channels were used to segment the cells to create ROIs using dual-channel segmentation. Panel D: segmentation mask. Panel E: antibody channel displaying ROIs.

Data analysis was performed using BD™ Image Data Explorer (IDE) software to calculate the Z'-factor and to create dose response curves. The heat maps were created in Excel 2007 from percent positive values of each surface marker using conditional formatting.

Time Course of Differentiation of Monocytes into Macrophages

THP-1 cells were differentiated in imaging plates as outlined in the preceding sections. Cells were fixed at various time points using 100 µL per well of BD Cytofix fixation buffer and stained with selected antibodies (CD11b, CD15s, CD18, CD44, CD49e, CD81, and CD85) following the staining protocol described in the Imaging Assay Optimization and Screening section. The images were acquired on a BD Pathway 435 system, and the average intensity of antibody was determined at each time point as described in the Image and Data Analysis section.

Multiplexing Assays

Differentiated THP-1 cells in 96-well imaging plates were treated with LPS in a dose-dependent manner using a range from 0 to 1,000 ng/mL of LPS for 6 hours. After treatment, cells were incubated with LysoTracker® Red at 37°C for 30 minutes. Cells were then fixed using BD Cytofix fixation buffer and permeabilized with BD Perm/Wash buffer I. Cells were stained with mouse anti-human CD54 antibody and rabbit NFκB antibody for 1 hour at room temperature in stain buffer. After two washes with PBS (200 µL per well), cells were then stained with a cocktail of Alexa Fluor® 647 goat anti-mouse antibody (2.5 µg/mL), Alexa Fluor® 488 anti-rabbit antibody (2.5 µg/mL), and DAPI (0.2 µg/mL). Images were acquired on a BD Pathway 435 system, and the average intensity of antibody and LysoTracker was determined as described in the Image and Data Analysis section. For NFκB analysis, the ROIs for nuclei and cytoplasmic rings around nuclei were created using segmentation, and the intensity of NFκB antibody was measured in the ROIs. A ratio of nuclear to cytoplasmic intensity was calculated for generating the dose response curve of LPS.

Results

Immunophenotyping is a cellular analysis method for the identification of protein markers and their expression and co-expression profiles using directly or indirectly fluorochrome conjugated antibodies on an analyzer such as a flow cytometer or imaging system. These methods facilitate the identification, characterization, and isolation of cells of interest for downstream applications. Flow cytometry and imaging recently have been used to identify markers on monocytes and macrophages.[3,4] In the present study we used a BD Lyoplate screening panel to demonstrate a rapid and easy screening method for the cell surface signatures of monocytic and macrophage cell populations.

Assay Optimization

To perform any high-content screening experiment, assay optimization using known negative and positive controls is an essential step. Flow-based screening does not involve complex image analysis algorithms, whereas for imaging-based assays, a number of parameters such as image acquisition and image analysis need to be optimized during assay development (see the BD application note *Navigating the High-Content Imaging Process*).[5]

Evaluating an image-based assay's robustness in terms of assay window and variability is a prerequisite to performing a screening campaign. These quality control measurements are usually derived from analysis of minimum and maximum (min/max) assays in which half of the wells in a multiwell plate are negative controls (min) and the other half are positive controls (max). The Z′-factor, presented by Zhang et al, provides a useful summary of assay quality and is a widely accepted standard. Assays with a Z′-factor value of greater than 0.5 are generally accepted as having sufficient robustness for screening.[6]

To test the robustness of this screening assay on differentiated macrophages using high-content imaging, we performed a min/max assay using known surface markers that downregulate (min) or upregulate (max) during differentiation of THP-1 cells. CD11b surface expression has been reported to be induced during differentiation of monocytes into macrophages, whereas CD14 is a monocyte marker that is downregulated during differentiation.[7] CD11b and CD14 were used along with an isotype control to run a min/max assay using 32 replicates of each antibody followed by an Alexa Fluor® 488 conjugated secondary antibody. The images were analyzed and the average intensity of the surface markers from each well was used to calculate the Z′-factor (Figure 4). Z′-factor values of >0.5 were obtained, which clearly showed that there was very low well-to-well variability in the assay along with a very good separation of isotype control vs positive antibody (Z′-factor = 0.9). Results were similar for CD11b vs CD14, for which a Z′-factor of 0.7 was obtained (Figure 4, panel D). The representative pseudocolored images in panels A, B, and C also show the strong antibody staining (green) with CD11b compared to CD14 and control.

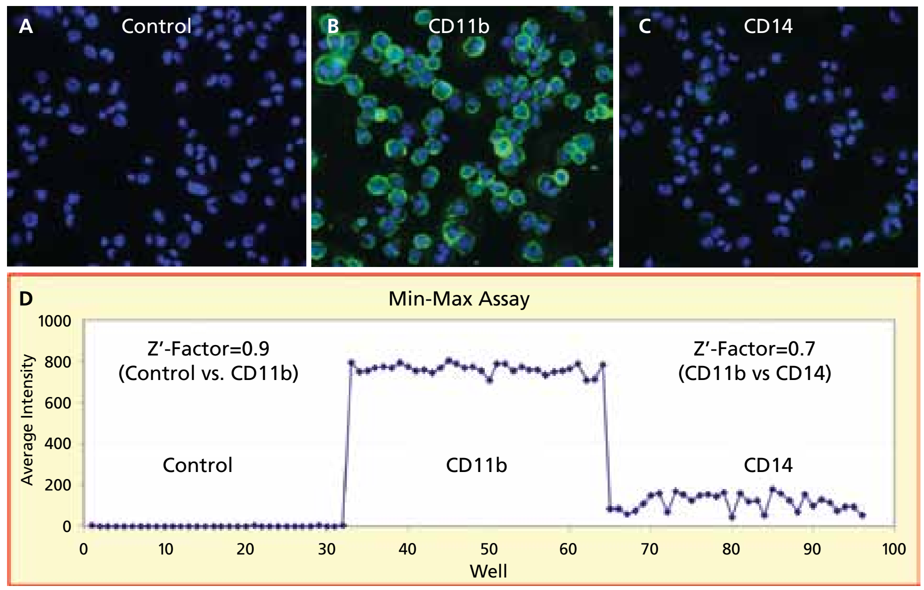


Figure 4. Min/max data for assay optimization.

Panels A–C: Representative pseudocolor merged images from antibody (green) and DAPI (blue) channels of differentiated THP-1 macrophages for control (min), CD11b (max), and CD14 (min) antibodies respectively. Panel D: Average intensity quantified from antibody channels from control, CD11b, and CD14 wells (n = 32 wells each).

A similar optimization test was performed with suspension THP-1 monocytes using flow cytometry and known surface markers. CD1a is a dendritic cell marker that is abundant in monocyte-derived dendritic cells, but is not expressed on monocytic cells such as THP-1 (negative control).[8] In contrast, CD4 is an abundantly expressed membrane glycoprotein on human THP-1 cells (positive control).[9] CD1a (negative control) and CD4 (positive control) along with isotype control antibodies were used to stain suspension THP-1 cells, which were analyzed by flow cytometry. Data in Figure 5, panels A and B, shows the histograms of CD1a and CD4 respectively, overlaid with isotype controls displaying cell count versus the fluorescence intensity. A significant separation of negative and positive controls was observed.

The data from known negative and positive controls on both macrophages and monocytes using imaging and flow cytometry respectively clearly reveals that, despite the differences in the analysis methods, these tools are robust enough to discriminate between the negative and positive population of cells for the chosen markers.

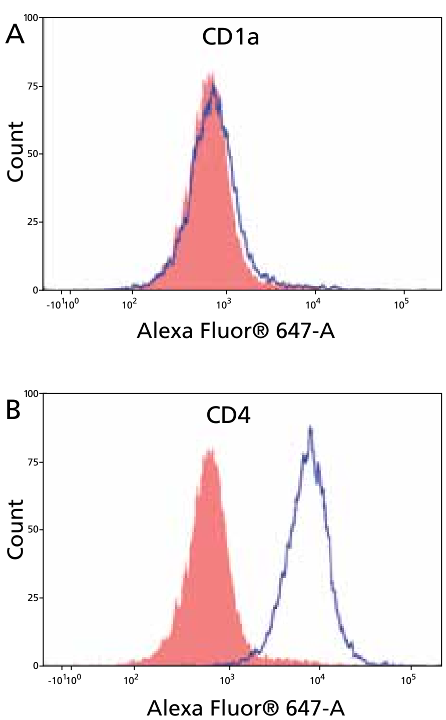


Figure 5. Flow assay optimization.

Flow cytometry data from THP-1 cells presented as histograms (blue) overlaid with control antibody (pink). Panel A: CD1a (negative control). Panel B: CD4 (positive control).

THP-1 Monocyte Screening Using Flow Cytometry

After assay optimization, a screen of 242 surface antibodies was conducted on THP-1 monocytes (undifferentiated) using flow cytometry. Staining and screening were carried out in HTS plates as outlined in the methods section, and data was acquired using a BD FACSCanto II system with an HTS. The data was analyzed in FCS Express, and heat maps created in Excel. Using automated templates, a comprehensive list of analysis parameters such as Mean, Normalized Mean, Percent Positive, Mean of Positive Population, Normalized Mean of Positive Population, Critical Range Digital, and Bimodality Index were obtained in an Excel worksheet in column and table format. Figure 6 shows a heat map of percent positive cells for each antibody in THP-1 monocytes (first column) using flow cytometry. A threshold of 85% positive and higher was used to score hits, and 44 CD markers expressed on THP-1 monocytes were identified.

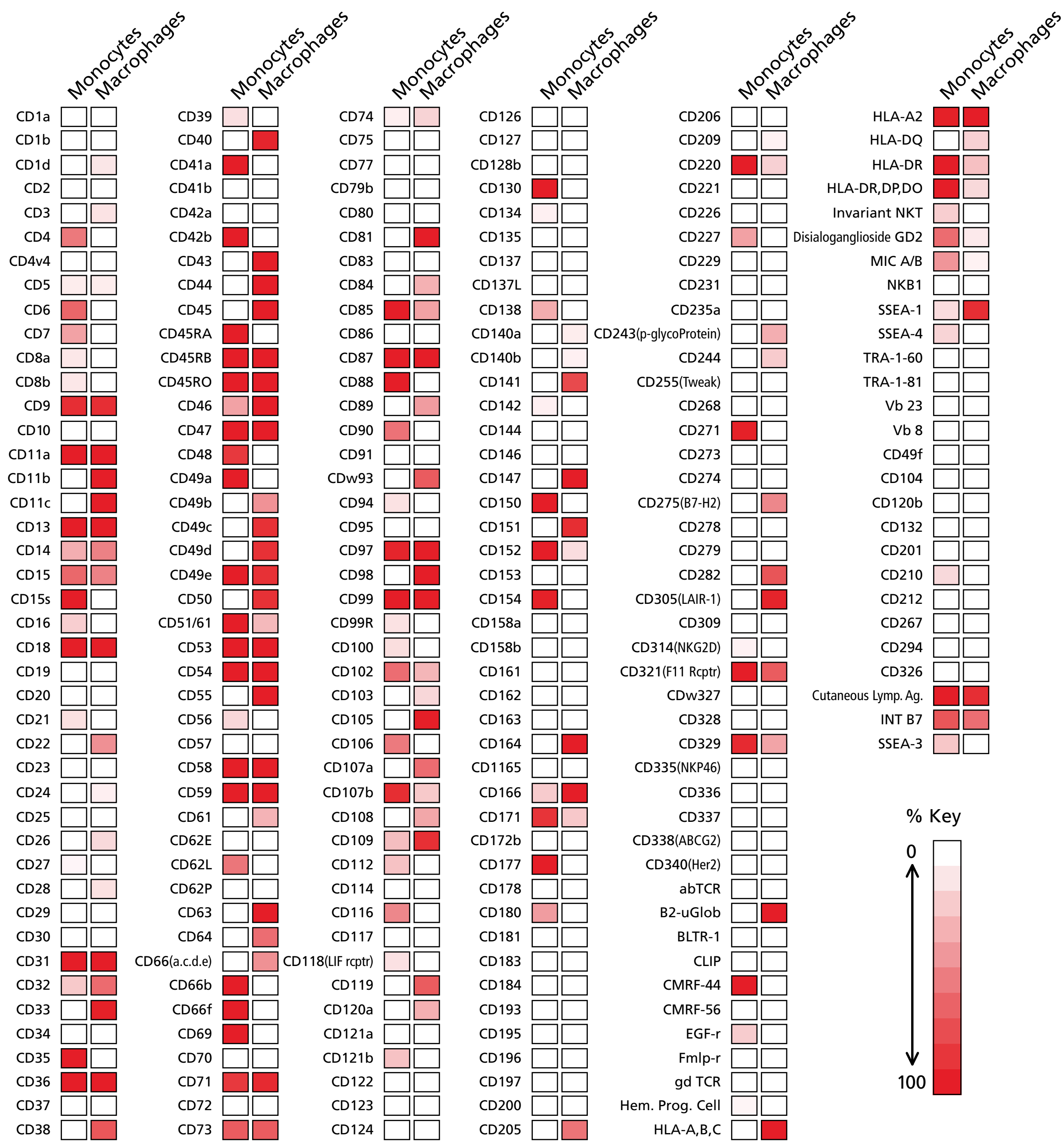


Figure 6. Expression of BD Lyoplate panel markers on THP-1 monocytes and macrophages.

The data from a flow cytometry screen on THP-1 monocytes and high-content imaging screen on THP-1 macrophages compiled as percent positive cells.

Flow cytometry is an excellent technique that provides sensitivity, statistical precision, and simultaneous examination of multiple parameters. It has been less popular in high-throughput screening assays due to its conventional tube format and slow throughput compared to other screening technologies. However, the HTS addresses these limitations. It can acquire samples with most BD flow cytometers in a 96-well microtiter plate in less than 15 minutes. High-throughput screening by flow cytometry has recently been used to identify unique markers in fibrocytic lesions that distinguish monocytes, macrophages, fibrocytes, and fibroblasts.[4]

Differentiated THP-1 Macrophage Screening Using High-Content Imaging

After image analysis assay optimization, a screen of 242 surface antibodies was conducted on adherent THP-1 monocytes that were differentiated in imaging plates using PMA, as outlined in the methods section. To perform optimal segmentation for image analysis, cells were also stained with DAPI and a whole cell red dye. The data calculated in the form of percent positive cells based on average intensity of antibody compared to control is presented in Figure 6 as heat maps (macrophage column).

A hit criterion of more than 85% positive population was also used to identify hits from the imaging screen to identify markers that were expressed in macrophages. The imaging screen identified 40 surface markers expressed on THP-1 macrophages.

Comparison of Flow Cytometry and Imaging Data

The data from both the flow cytometry and imaging screens was further compared to identify unique hits that were present on monocytes or macrophages and on both cell types. To identify unique markers, a hit criterion of 85% positive cells was used in both the screens. Further, to score markers expressed both on monocytes and macrophages, a dual criterion of 85% positive in one screen and 50% positive in the other screen was used. Using these hit criteria, the data was organized into three categories (Table 3).

Positive hits of surface markers expressed both on THP-1 monocytes and macrophages detected by flow cytometry and imaging respectively
A representative example of CD18 antibody from this category is shown in the form of a histogram from flow cytometry data and a pseudocolored merged image from imaging data. Both the flow cytometry histogram and imaging data clearly show the expression of CD18 on both THP-1 monocytes and macrophages. A similar trend of CD18 expression in PMA differentiated THP-1 cells has been reported by Spano et al.[10]

Positive hits of surface markers uniquely expressed on THP-1 monocytes as identified by flow cytometry
Flow cytometry and imaging data from THP-1 monocytes and macrophages, respectively, from a representative CD15s antibody is shown. The histogram from the flow cytometry data clearly shows an increased MFI over the control. On the other hand, expression of CD15s antibody on THP-1 macrophages was not detected, as shown in the images from antibody channel (green). While cells clearly were present, as seen from nuclei staining with DAPI, no antibody staining (green color) was detected. This result is in line with existing literature on CD15s, which suggests that the prevalent expression of CD15s on monocytes diminishes when monocyte cell lines such as HL60 are differentiated into macrophages after treatment with PMA.[11]

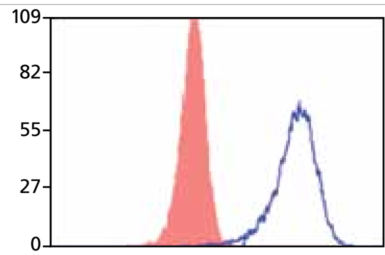
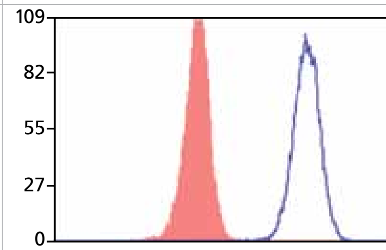
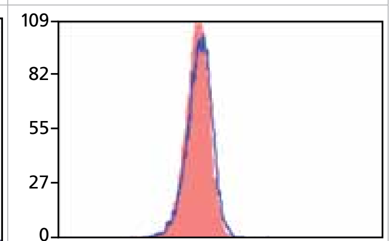
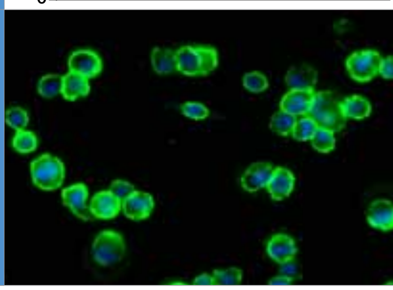
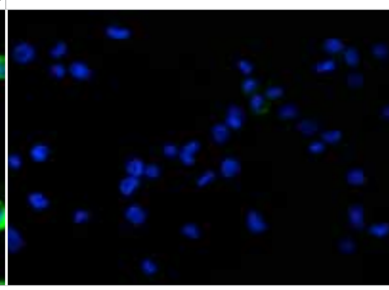
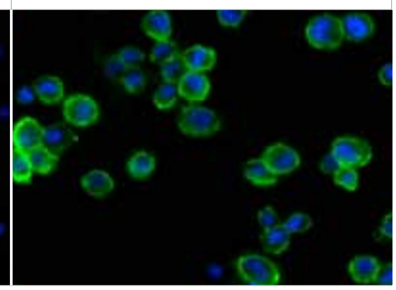
Markers expressed	Expressed on both THP-1 monocytes and differentiated THP-1 macrophages	Expressed only on THP-1 monocytes	Expressed only on (differentiated) THP-1 macrophages
Technology used	Flow cytometry and imaging	Flow cytometry	Imaging
Positive hits	CD9, CD11a, CD13, CD18, CD31, CD36, CD45RB, CD45RO, CD47, CD49e, CD53, CD54, CD58, CD59, CD71, CD87, CD97, CD99, CD321(F11 Rcptr), HLA-A2, Cutaneous Lymph Antigen	CD15s, CD35, CD41a, CD42b, CD45RA, CD49a, CD51/61, CD66b, CD66f, CD69, CD85, CD88, CD130, CD150, CD152, CD154, CD177, CD220, CD271, CDw329, CMRF-44, HLA-DR, HLA-DR DP DO	CD11b, CD11c, CD33, CD43, CD44, CD45, CD46, CD49d, CD50, CD55, CD63, CD81, CD98, CD105, CD147, CD164, CD166, CD305 (LAIR-1), B2-μGlob, HLA-A B C
Representative example	CD18	CD15s	CD11c
Flow cytometry data			
Imaging data			

Table 3. Comparison of hits from flow cytometry and high-content imaging screens.

Flow data is presented as histograms from the surface marker (blue) overlaid with control (pink) from THP-1 monocytes. Imaging data is presented as pseudocolored merged images of surface marker (green) and DAPI-stained nuclei (blue) from differentiated THP-1 macrophages.

Positive hits of surface markers uniquely expressed on THP-1 macrophages as identified by imaging

Flow cytometry and imaging data from a representative unique hit (CD11c) from this category is shown. CD11c was not detected on THP-1 monocytes as shown in the overlaid histograms of CD11c vs control. High expression of CD11c was detected on THP-1 macrophages, which is clear from fluorescence imaging data from the bright antibody staining. CD11c is an adhesion molecule and it has been reported to be expressed during maturation of monocytes into macrophages.[12]

Overall, the results from this proof-of-principle study are in agreement with the limited literature available on THP-1 cell differentiation. Using the same sets of primary antibodies from a surface marker panel for both flow cytometry and fluorescence imaging screens, it was possible to generate data for surface markers that are constitutively expressed throughout differentiation as well as unique markers present on either THP-1 monocytes or macrophages.

It is generally accepted that cellular screening should be performed on cells in their native state. Flow cytometry can be used to analyze adherent cells, but first the cells must be removed from their substrate. This process is typically accomplished by scraping or enzymatic treatment. However, such manipulations might alter surface marker expression or cell function, and might damage fragile cells, leading to fewer or a non-representative population of cells available for analysis. Using fluorescence imaging techniques to evaluate adherent cells has the added advantage that fewer cells are lost during preparation, and cell morphology is also maintained.

Time course of differentiation and validation of hits

To further validate the results identified on THP-1 monocytes and macrophages, a time course of differentiation of THP-1 monocytes into macrophages using PMA was performed using imaging. Based on existing literature, a subset of surface CD markers commonly and differentially expressed on monocytes and macrophages was used in the time course. At each time point, cells were fixed and imaged using the protocol described in the methods section. Imaging data from the following surface CD markers was quantified at each time point, and results from a proof of principle experiment are shown in Figure 8.

We obtained the following results from the time course of these CD markers, all shown in Figure 7.

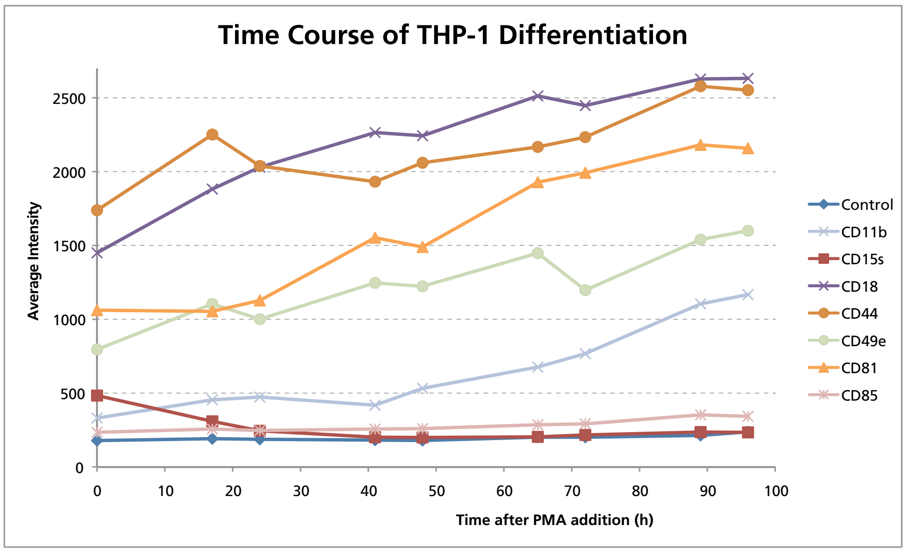


Figure 7. Time course of THP-1 differentiation.

Effect of treatment time of PMA on the average intensity of selected CD markers during differentiating of THP-1 cells.

CD11b/CD18: CD11b is also called integrin αM subunit/complement receptor CR3, which forms a heterodimer with the β2 integrin receptor CD18. CD11b/CD18 complexes mediate the adhesion of macrophages to the endothelial lining of blood vessels as well as to extracellular matrix components. The expression of these receptors has been reported to be upregulated after treatment of THP-1 cells with PMA.[13] In the time course experiment, expression of CD11b started increasing after 40 hours of PMA treatment, and a significant expression was found after 100 hours of differentiation. Similarly, expression of CD18 was enhanced after PMA treatment. A similar trend of CD18 expression in PMA differentiated THP-1 cells has been reported by Spano et al.[10]

CD15s: Expression of CD15s, leucocyte cell surface carbohydrate (Sialyl Lewis x), has been reported to decrease when monocytes are treated with TPA.[11] As evident from Figure 7, treatment of THP-1 monocytes with PMA led to a 50% reduction in intensity of CD15s within 24 hours.

CD44: The CD44 antigen is a cell-surface glycoprotein that plays a role in cell-cell interactions, cell adhesion, and cell migration. PMA has been reported to cause a dose-dependent increase in CD44 expression.[14] A similar increase

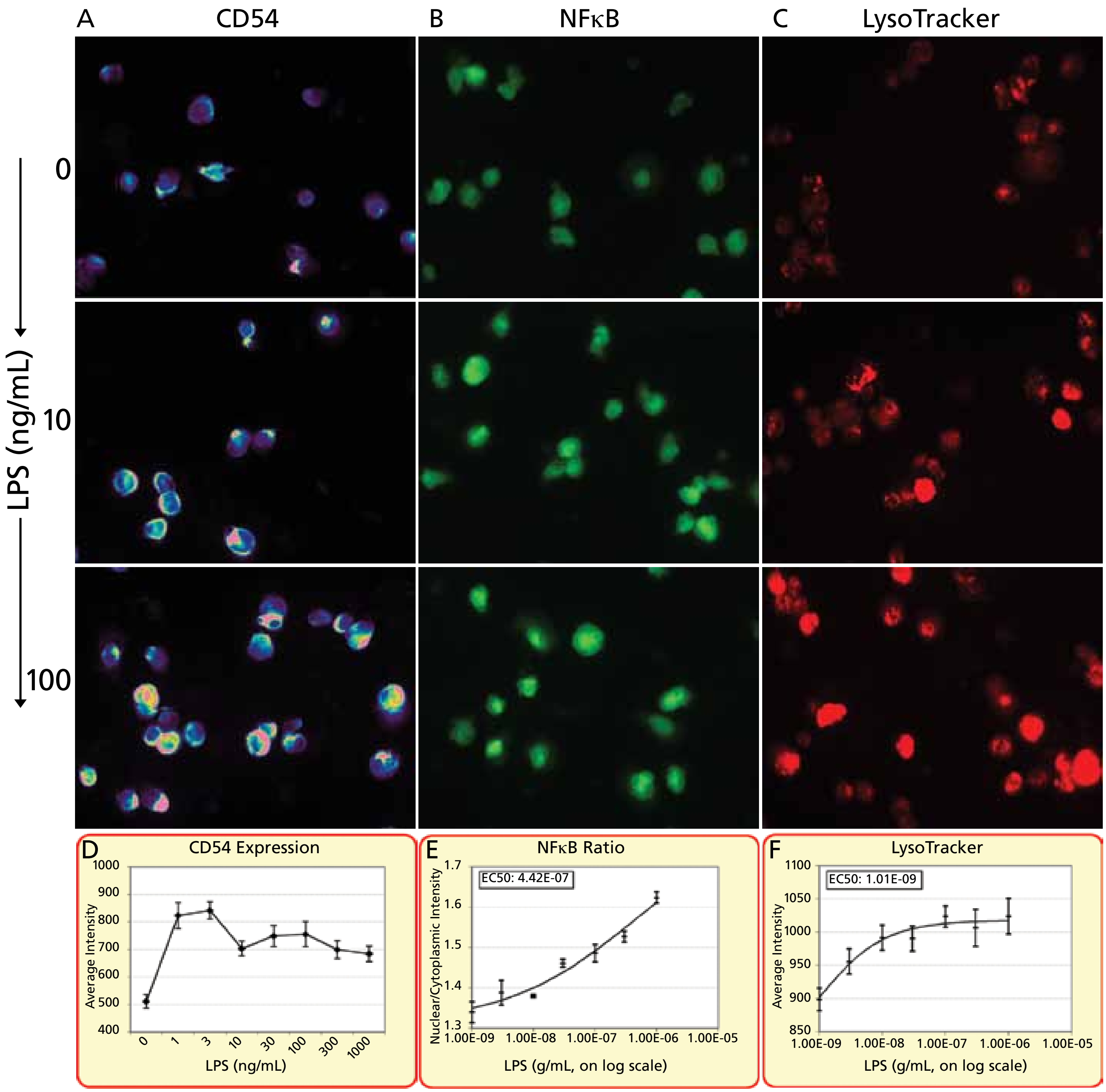


Figure 8. Multiplexed assays.

Panel A. Intensity based psuedocolored merged images of the CD54 channel from differentiated THP-1 cells treated with 10 and 100 ng/mL of LPS along with control. Panel B. Pseudocolored merged images of the NFκB channel with the same treatment as in panel A. Panel C. Pseudocolored merged images of the Lyostracker channel with the same treatments as in panel A. Panel D. Effect of increasing concentrations of LPS on the average intensity of CD54 antibody. Panel E. Dose-dependent effect of LPS on the NFκB ratio in differentiated THP-1 macrophages. Panel F. Dose-dependent effect of LPS on LysoTracker intensity in differentiated THP-1 macrophages.

occurred in expression of CD44 during PMA treatment in the time course experiment.

CD49e: Macrophages express various integrins that play a role in clearance of microbial pathogens during immune responses to infection. CD49e is one of the integrins reported to be upregulated when monocytes differentiate into macrophages.[15] Data presented shows an increase in expression of CD49e during the differentiation of THP-1 cells using PMA.

CD81: CD81 is an integral membrane protein belonging to the tetraspanin superfamily and is expressed in all cell types, except for red blood cells and platelets.[16] Its role in macrophage differentiation is not defined. In this proof of principle experiment, upregulation of CD81 after PMA treatment was observed.

CD85: The CD85/LIR-1/ILT2 molecule belongs to the leucocyte Ig-like receptor (LIR)/Ig-like transcript (ILT) family. A slight upregulation of this surface marker was seen after 72 hours of treatment with PMA.

Overall, the time course results of these markers clearly validate the screen data, which corroborates the existing literature.

Multiplexing: Effect of Activation of Macrophages on Expression of CD54, LysoTracker, and NFκB Translocation

LPS is a potent and well established activator of macrophages. Stimulation of differentiated THP-1 cells with LPS has been shown to produce a high level of TNF-α, which is mediated via several nuclear transcription factors. NFκB is one of the nuclear factors that play an important role in regulation of TNF-α gene expression.[17]

Intracellular adhesion molecule-1 (ICAM-1), also known as CD54, is a cell surface glycoprotein that is expressed on various cell types including macrophages and is known to be upregulated by stimulation with LPS. The upregulation has also been reported to be mediated by NFκB.[18]

Furthermore, activation of macrophages with LPS and TNF-α release has been reported to be associated with activity of lysosomal enzymes.[19]

To simultaneously monitor all the effects of LPS on THP-1 macrophages, three markers were multiplexed in a single proof of principle experiment. Differentiated THP-1 cells were treated with LPS in a dose-dependent manner for 6 hours as outlined in the methods section. After treatment, cells were fixed and stained with the following:

1. CD54 followed by anti-mouse Alexa Fluor® 647 (secondary)
2. anti-NFκB rabbit polyclonal antibody followed by anti-rabbit Alexa Fluor® 488 (secondary)
3. LysoTracker Red
4. DAPI for staining of macrophage nuclei

Using this 4-color multiplexed assay, dual-channel segmentation was performed to create ROIs and measure the intensity within the ROIs. Figure 8, panel A shows the differentiated THP-1 macrophages stained with CD54 in control and LPS-treated cells. It is evident from the intensity-based pseudocolor and the line graph (panel D) that expression of CD54 was higher compared to untreated control, although a dose-dependent effect could not be seen. This might be due to a heterogeneous response that has been reported to be influenced by the differentiation state of macrophages.[3]

NFκB translocation analysis was performed by segmentation of nuclei and creating a cytoplasmic ring around the nucleus. For details about segmentation and image analysis for translocation assays, see the BD application note *Navigating the High-Content Imaging Process*.[5] The translocation of the NFκB was expressed as ratio of nuclear vs cytoplasmic intensity for creating a dose response curve (Figure 8, panel E). The representative images from the NFκB channel (panel B) also clearly show the increased intensity of NFκB in the nuclei with the increasing dose of LPS.

The analysis of LysoTracker red was performed by measuring intensity within ROIs created as described in the methods section. As shown in Figure 8, the average intensity of LysoTracker increased with the increasing dose of LPS, which is evident from the dose response curve (panel F) and representative images of LysoTracker-stained cells at 0, 10, and 100 ng/mL of LPS (panel C).

Multiplexing assays is one of the biggest advantages of high-content imaging and flow cytometry. Using four different colors, several assays can be easily combined in a single high-content imaging experiment. This proof of principle experiment demonstrates the simultaneous measurement of the effect of activation of LPS on THP-1 macrophages on a surface marker, an intracellular nuclear factor translocation event, and tracking of acidic organelles using LysoTracker, a fluorescent acidotropic probe.

Conclusions

BD Lyoplate screening panels, combined with BD flow cytometers with HTS options and BD Pathway high-content imaging systems, provide valuable tools for researchers to develop novel strategies to isolate and characterize cells in various states of differentiation and culture conditions. This application note demonstrates the rapid, easy, and efficient characterization of cell surface markers of the THP-1 cell differentiation model by using the BD Lyoplate screening panel on suspension THP-1 cells (monocytes) using flow cytometry and on PMA-differentiated THP-1 cells (macrophages) using high-content imaging. The results of both screens revealed comprehensive information about markers uniquely and commonly expressed on both types of cells. A time course of selected CD marker expression in response to PMA further confirmed expression in monocytes and differentiated macrophages. In addition, a proof of principle experiment demonstrated the effect of LPS activation of THP-1 macrophages on the expression of CD54, NFκB translocation, and lysosome acidification. The experiments described in this application note demonstrate that both flow cytometry and high-content imaging can be employed for the characterization of cell surface markers. For any given application, one platform might be better suited than the other because its particular strengths better address the experimental question. When these two powerful techniques are used in combination, they can provide complementary information, enabling a more comprehensive cellular analysis.

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