Multi-parametric Cell-Based Inflammation Assays Detecting Cytokines and Cell Surface Antigens

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INTRODUCTION

Inflammation is a complex event in which cells respond to various endogenous and exogenous stimuli. Macrophages can initiate inflammation by releasing cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ into the blood stream. Vascular cells are then activated by these factors and express additional cytokines and cell-surface antigens. The ability to monitor up-regulation of these molecules provides an important physiological read-out for cell-based models of inflammation. We present here two models for multi-parametric assays measuring expression of secreted cytokines and cell surface markers with a novel low-volume ELISA and automated imaging systems to evaluate the effect of different mediators on inflammatory response. First, human monocytic THP-1 cells were used to monitor effects of compounds on monocyte activation. Macrophages originate from blood monocytes that leave the circulation to differentiate into various tissues. They can initiate inflammation by releasing cytokines that activate vascular cells and facilitate adhesion of cytokines to blood vessels and migration into the tissues. THP-1 cells were differentiated to macrophages by phorbol 12-myristate 13-acetate (PMA) and activated by LPS. The differentiation process was monitored by phenotypic imaging and secretion of IL-8, IL-1β and TNF-α by low-volume ELISA. Second, primary human umbilical vein endothelial cells (HUVEC) were stimulated by proinflammatory factors (IFN-γ, TNF-α, and IL-1b) overnight. Differences were assessed in signaling pathways, adhesion molecule expression, and cytokine secretion induced by those factors. The combination of imaging and low volume ELISAs provides an efficient multiparametric assay system that can be used to test the efficacy of anti-inflammatory compounds and provide insight into mechanisms of action.

ASSAY BACKGROUND

THP-1 Assay

- Differentiation of THP-1 cells into macrophages was quantified using transmitted light imaging with an ImageXpress® Pico Automated Cell Imaging System. Adherent cells were classified using CellReporterXpress™ Automated Analysis Software
- The amount of IL-8, IL-1β and TNF-α in cell supernatants was quantified using a low volume, microfluidic-based Pu·MA System ELISA. The ELISAs were run with sample and antibody reagent volumes of 20 µl. Antibodies were obtained from BioLegend.

HUVEC Assay

- The Pu·MA System was used to quantify MCP-1, IL-8, and IL-6 in cell supernatants. The ELISAs were run with sample and antibody reagent volumes of 20 µl. Antibodies were obtained from BioLegend.
- The ImageXpress® Nano Automated Imaging System was used to monitor cells surface expression of VCAM and HLADR. Positive cells were classified using CellReporterXpress™ Automated Analysis Software

INSTRUMENTATION

The Pu·MA System is a practical and affordable benchtop instrument that runs ELISA assays using your own antibodies, or precoated flowchips in a streamlined workflow. The Pu·MA System has been designed to fit seamlessly into your current laboratory workflow.

- Runs complete ELISA in < 3 hours with "hands-off" processing
- Reduces sample and reagent volumes to 10 - 20 µl
- Works with your existing ELISA kits and antibody pairs

SpectraMax® iD5 Multi-Mode Microplate Reader was used for absorbance measurements, calculation of concentrations and EC_{50} values

The ImageXpress Pico system includes:

- Four colors + transmitted light
- Environmental control
- Automated data analysis

The ImageXpress® Nano Automated Imaging System includes:

- Four colors + transmitted light
- Environmental control
- Automated data analysis

Both systems are controlled by CellReporterXpress™ Automated Imaging Acquisition and Analysis Software

References:
[1]Optimized THP-1 differentiation is required for…; Park et al, Inflamm Res. 2007, **56,** 45.
[2]p38α MAP kinase serves cell type-specific inflammatory functions…; Kim et al, Nat Immunol. 2008, **9**, 1019.
[3]PDTC is a potent antioxidant…; Zhu et al, FEBS Letters 2002, **532,** 80.
[4]Anti-Inflammatory Effects of Moxifloxacin…; Weiss et al, Antimicrob Agents Chemother. 2004, **48,** 1974.

THP-1 MACROPHAGE ASSAY

- THP-1 cells were plated 20,000 cells per well (96-well plate) and incubated for 48 hr. Next they were stimulated with a mix of PMA & LPS for 24 hr (0-5 pg/mL of PMA, and 0-100 pg/mL LPS; all from Sigma).
- Anti-inflammatory compounds were added 2 hr prior to cytokine stimulation
- After incubation, 60 µl of supernatant was taken for ELISA analysis from each well. The samples were analyzed fresh or stored at -70C for subsequent analysis.
- Cells were imaged using transmitted light (TL) using ImageXpress Pico system. Before imaging, non-adherent cells were washed 2x with media. Cells were counted in TL.
- Supernatants were diluted 3:1 in assay buffer and analyzed for IL-8, TNFα, and IL-1β using the Pu·MA System flowchips and reagents (all Ab pairs from BioLegend).

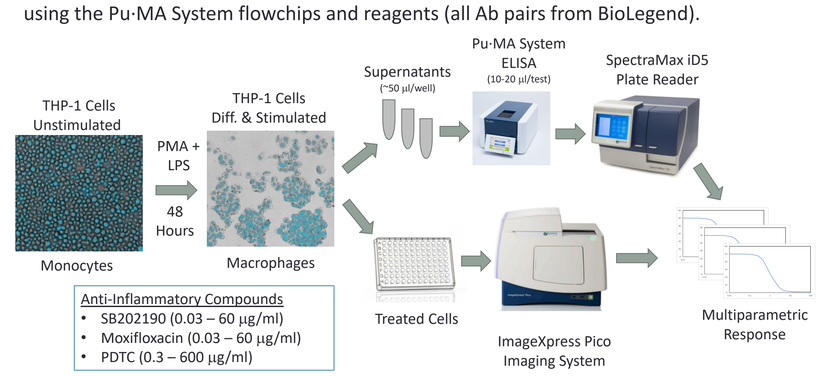


Figure 1. Schematic of THP-1 cell Multiparametric Inflammation assay workflow.

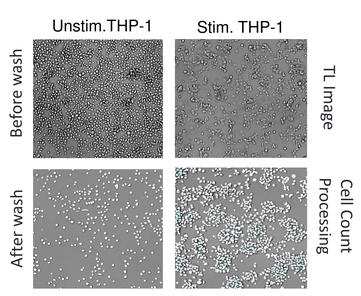


Figure 2. Images of stimulated or un-stimulated THP-1 cells before and after removal of non-adherent cells. Top: Unstimulated (Left) and stimulated (Right) THP-1 cells. Transmitted light images. Bottom: Cell cultures after wash. Analysis masks for cell count shown in white.

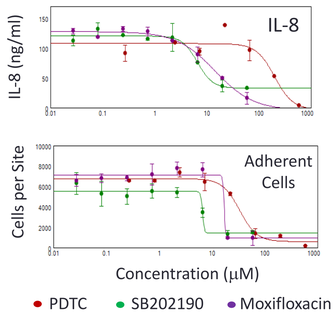


Figure 3. Concentration dependent effects on IL-8 and number of adherent cells by three anti-inflammatory compounds. EC_{50} values derived from a 4-P fit are given in the table below.

EC_{50}, µM	IL-8	IL-1β	TNFα	Adhesion
PDTC	201	42	63	33
SB202190	6.6	9.8	6.4	6.6
Moxifloxacin	17.4	9.9	17.9	17.1

LOW-VOLUME AUTOMATED ELISA

The benefits of microfluidic assays include reduced reagent use and faster time to results. In the Pu·MA System workflow all reagents are pre-loaded into flowchips and then automatically sent through an assay channel where the immunoassay complex is formed. All wash steps are integrated into the protocol. After the protocol is finished quantification is done by reading absorbance on a plate reader.

Pu·MA System Workflow

Assay Steps

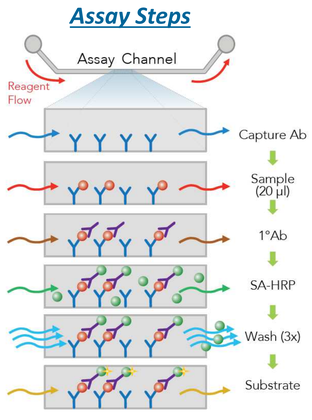


Figure 4. ELISA workflow for automated Pu·MA System and cartoon of automated assay steps in an ELISA protocol. The microfluidic assay channel provides solid support for the capture antibody.

HUVEC INFLAMMATION ASSAY

- HUVEC were plated 5,000 cells per well (96-well plate) and incubated for 48 hr. Next they were stimulated with a mix of inflammatory cytokines for 24 hr (5 ng/mL of TNFα, 1 ng/mL IL-1β, 50 ng/mL of IFNγ; all from R&D Systems).
- Anti-inflammatory compounds were added 1 hr prior to cytokine stimulation
- After incubation, 60 µl of supernatant was taken for ELISA analysis from each well. The samples were analyzed fresh or stored at -80C for subsequent analysis.
- Cells were then fixed with 4% formaldehyde and stained with antibodies (Ab) for 1 hr and imaged using an ImageXpress Nano Automated Imaging System.
  - Ab's: FITC-mouse anti-human VCAM and PE-mouse anti-human HLA-DR
- Supernatants were diluted 3:1 in assay buffer and analyzed for MCP-1, IL-6 and IL-8 using PuMA Flowchip system and reagents (all Ab pairs from BioLegend)

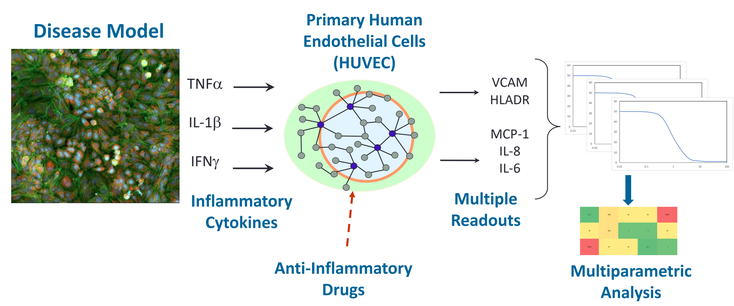


Figure 5. Cartoon of endothelial cell inflammation assay workflow.

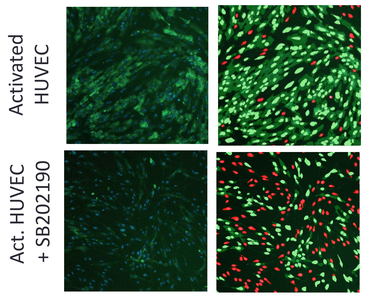


Figure 6. Images of activated HUVEC cells stimulated with CK mix with or without treatment of 10µM SB202190. Left: Cells stained with anti-VCAM antibodies (green) and Hoechst (blue). Right: Analysis masks for nuclei of VCAM-positive cells (light green), cytoplasm of VCAM positive cells (dark green), nuclei of VCAM negative cells (red).

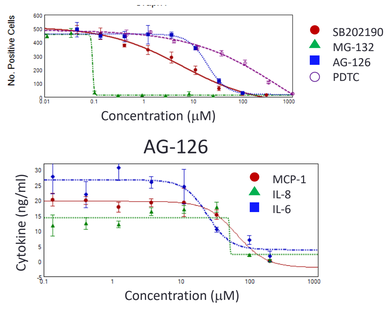
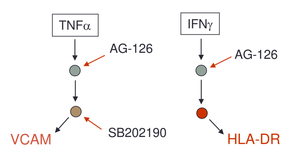


Figure 7. **Top**: Concentration dependent effect on up-regulation of adhesion molecules by four anti-inflammatory compounds. **Bottom:** Concentration dependent effect on IL-8, IL-6, and MCP-1 by three anti-inflammatory compounds as measured by PuMA Flowchip immunoassay.



	SB202190	MG-132	AG-126	PDTC
VCAM	9.9	0.09	19	344
HLA-DR	48	0.09	51	>1000
MCP-1	29	0.22	66	
IL-6	3.1	3.7	24	
IL-8	26	>1000	52	

Color Scale (µM): 0.1, 1, 10, 100, 1000

Figure 8. Left: Cartoon showing effect of AG-126 and SB202190 on signaling pathways leading to differential expression. **Right:** Heat map of the multiparametric immunoassay EC_{50} results.

CONCLUSIONS

- We have demonstrated a multiparametric inflammation assay using two cell models (THP-1 & HUVEC) with automated cellular imaging and an automated low-volume ELISA system.
- The Pu·MA System performs immunoassays with existing ELISA antibody pairs using microfluidic flowchips that reduce reagent use and improve time-to-results
- Multiparametric responses of different inflammation markers to anti-inflammatory compounds were characterized. Observed differences in behavior were consistent with published mechanisms of action.

MOLECULAR DEVICES

PROTEIN FLUIDICS