High Throughput, Multiplexed Detection of Inflammatory Cytokines in an Astrocyte and Monocyte Co-culture Model

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Abstract

Inflammatory cytokine levels have been reported to undergo significant increase in multiple neurodegenerative disorders, including Alzheimer's disease (AD) and multiple sclerosis (Mrak, *et al.*, 1995). It has been repeatedly documented that astrocytes are a critical source of IL-6, while glial cells are an important producer of IL-1 and TNFα (Chao, *et al.*, 1995). Research has also revealed that under certain conditions, astrocytes and neurons can express multiple different cytokines which are normally undetectable. These three cell types commonly interact to produce the inflammatory response. For example, in AD activated astrocytes and microglia are characteristically found in abundance near neurons and plaques (Griffin, *et al.*, 1989). Therefore it is imperative that assessment of pertinent cytokine levels be performed with relevant cell types in isolation as well as in a co-culture model.

In the past, cytokine levels were assessed using assays that measured protein concentrations on an individual basis. As cytokines are secreted into the surrounding media, multiple analyses are still able to be performed from a single well. However, the procedure is labor intensive and performing individual assessments in separate wells can increase variability within the collective data set. By incorporating an assay technology capable of providing assessment of multiple cytokines in the same well, variability can be eliminated, creating a robust detection method.

Here we describe a homogeneous, bead-based immunoassay technology which offers multiplexed detection of several cytokines in a single well. Donor beads are bound to antibodies specific for one of the target analytes being assessed. Acceptor beads, providing disparate emission profiles upon excitation, are also directly conjugated to a specific anti-analyte antibody. In the presence of the individual analyte, the beads come into close proximity to each other. When excited using the 680 nm laser on a novel high throughput multi-mode microplate reader, donor beads convert dissolved oxygen to singlet oxygen molecules, causing a cascade of energy transfer in the acceptor beads and an increase of light emission at the appropriate wavelength. Therefore high signal values are indicative of greater cytokine emission within the well. Cryopreserved primary astrocytes and peripheral blood CD14+ monocytes were incorporated independently and in co-culture to increase the relevance of generated results. The combination of multiplexed assay technology, cell model and laser-based detection provide a rapid, yet highly sensitive method to assess levels of important inflammatory cytokines.

BioTek Instrumentation

Synergy™ Neo2 Multi-Mode Reader. Synergy Neo2 Multi-Mode Reader is designed for speed and ultra high performance, incorporating BioTek's patented Hybrid Technology™. Independent optical paths accommodate diverse assay requirements with variable bandwidth quadruple monochromators, sensitive filter-based optics, laser-based excitation for Alpha assays and up to 4 PMTs for ultra-fast measurements. Advanced environment controls, including CO_2 /O_2 control, incubation to 65 °C and variable shaking are ideal for live cell assays and cell based detection is optimized with direct bottom illumination. Barcode-labeled filter cubes help streamline workflows and limit errors.

AlphaPlex™ Assay Technology

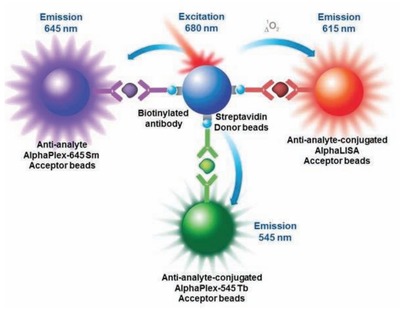

Figure 1. AlphaPlex Assay Principle. Biotinylated anti-analyte antibodies bind to the Streptavidin-coated Alpha Donor beads, while other anti-analyte antibodies are conjugated directly to AlphaLISA, AlphaPlex 545, or AlphaPlex 645 Acceptor beads. In the presence of the specific analyte(s), the beads come into close proximity.

AlphaPlex™ is a homogeneous, all-in-one-well multiplexing reagent technology that provides highly sensitive detection of a wide range of analytes from large proteins to small molecules and scarce biological samples such as primary cells and stem cells, in a simple no-wash assay format based on Alpha Technology. When the Donor and Acceptor beads are brought together, a cascade of chemical reactions is set in motion, causing a greatly amplified signal. By using multiple Acceptor beads which emit different wavelengths (AlphaLISA Europium (Eu):615 nm; AlphaPlex Terbium (Tb):545 nm; AlphaPlex Samarium (Sm):645 nm), multiple analytes can be detected.

Materials and Methods

Assay and Experimental Components: AlphaPlex 545 (Tb) Human IL-6 Detection Kit (Catalog No. AP223TB-C), AlphaPlex 645 (Sm) Human IL1β Kit (Catalog No. AP220SM-C), and Human TNFα Immunoasssay Kit (Catalog No. AL208C) were donated by PerkinElmer (Waltham, MA).

Lipopolysaccharides from E. coli 055:B5 (LPS) (Catalog No. L2880) and Human Interferon-γ (IFNγ) (Catalog No. 11040596001) were purchased from Sigma-Aldrich (Saint Louis, MO).

Cells: Cryopreserved Normal Human Astrocytes (Catalog No. CC-2565), ABM Basal Medium (Catalog No. CC-3187), AGM Bullet Kit (CC-3186), and Cryopreserved Peripheral Blood CD14+ Monocytes (2W-400C) were donated by Lonza Group, Ltd. (Basel, Switzerland).

Assay Procedure: Cell Plating: Cryopreserved astrocytes were thawed and added to 96-well plates upon vendor recommendations at a concentration of $1.5x10^4$ cells/cm^2 in a volume of 100 µL. For the 0.32 cm^2 wells, a total of 5000 cells were added per well and incubated at 37 °C/5% CO^2 for 72 hours. Media exchanges were performed after 24 hours and every 48 hours thereafter. Astrocyte doubling time was predetermined to be ~24 hours, creating ~40,000 cells/well after 72 hours. Media was removed and an equivalent number of monocytes was then added, in astrocyte complete media, to empty wells or wells containing astrocytes in a 50 µL volume. Media was added to other wells containing astrocytes creating astrocyte, monocyte, or co-cultured wells.

LPS+ IFNγ Addition: LPS and IFNγ were then diluted in media to create 2X LPS concentrations ranging from 2000-0 ng/mL in a constant 300 U/mL IFNγ concentration. 50 µL of the titration was then added to designated wells containing each cell model.

AlphaPlex Assay Performance: 5 µL aliquots were then removed from each well after 8, 24, and 48 hour incubations and run with either the AlphaPlex Tb IL-6/AlphaLISA TNFα or AlphaPlex Sm IL-1β:AlphaLISA TNFα duplex assays. Laser-based microplate reading was then performed to detect and quantify the level of secreted cytokines from each well.

Duplex Assay Signal Optimization

Prior to performing the cell-based assay, optimization of reader settings and assay component concentrations were performed to limit signal crossover when performing multiplex detection. Narrow filter bandwidths (Figure 2A) were used to minimize the capture of unwanted signal from each read. Variable concentrations of acceptor beads were then tested to further minimize crosstalk (Figure 2B and C).

A.

Synergy Neo2 AlphaPlex Assay Setup		
	Filters	**Dichroic Mirror**
Excitation Cube (All Acceptor Beads)	None	100% Mirror
Terbium/Europium Emission Cube	545/7 nm (Tb) / 620/10 nm (Eu)	570 nm
Samarium Emission Cube	Plug /645/7 nm (Sm)	None

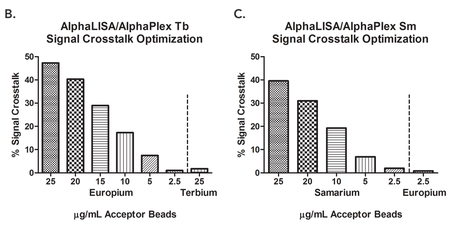

Figure 2. (A) Optimized filters and mirrors for AlphaPlex reads using Synergy Neo2. Gen5™ reader protocol includes initial simultaneous reading of terbium and europium signals using universal Alpha excitation and Tb/Eu emission cube. Tb/Eu cube then replaced with Sm emission cube to complete the protocol. Using reader settings and protocol, % signal crosstalk then shown for **(B)** Eu/Tb duplex assay, and **(C)** Eu/Sm duplex assay optimization.

Two duplexes were created to demonstrate the ability to combine and effectively detect the signals from multiplexed Tb/Eu and Sm/Eu assays. Upon testing, the starting Tb concentration of 25 µg/mL was shown to minimize crosstalk to an acceptable level of 1.8%, while a concentration of 2.5 µg/mL of Eu AlphaLISA beads was necessary to minimize crosstalk to the same level (1%) (Figure 2B). This same Eu bead concentration also yielded minimal crosstalk in the Sm channel (0.85%), whereas a 2.5 µg/mL concentration of Sm beads was again necessary to minimize crosstalk in the Eu channel (1.95%) (Figure 2C).

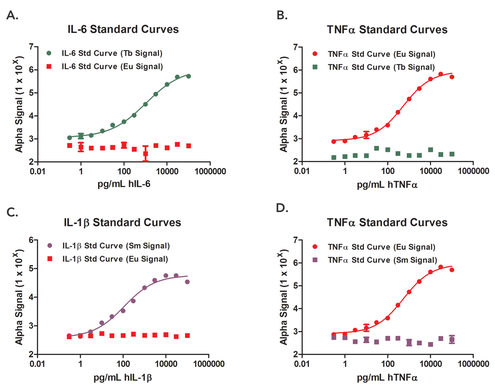

Figure 3. Analyte standard curve detection using target and duplex assay reader channels. (A) IL-6 Tb assay read using Tb and Eu channels; **(B)** TNFα Eu assay read using Eu and Tb channels; **(C)** IL-1β Sm assay read using Sm and Eu channels; **(D)** TNFα Eu assay read using Eu and Sm channels.

Complete analyte standard curves were then created and tested with assay acceptor bead concentrations of 25 µg/mL Tb:IL-6; 2.5 µg/mL Eu:TNFα; and 2.5 µg/mL Sm:IL-1β. The signal was captured using the two reader channels for the multiplexed assays (IL-6 and TNFα or IL-1β and TNFα). The graphs in Figure 3 illustrate that broad assay windows and accurate standard curves can be maintained while effectively minimizing crosstalk using optimized settings.

Final Duplex Assay Validation

Comparisons were then made between analyte standard curves added to assay wells containing either single assay or dual assay acceptor beads to assess whether assay quality was affected in the multiplexed setting.

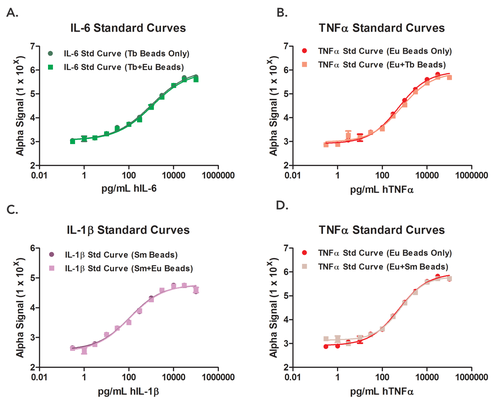

Figure 4. Single and duplex assay analyte standard curves. Analyte titrations ranging from 100,000-0 pg/mL assayed in the presence of target assay acceptor beads only or duplex assay acceptor beads. **(A)** IL-6 plus IL-6 Tb acceptor beads only or plus IL-6 Tb acceptor beads and TNFα Eu acceptor beads; **(B)** TNFα plus TNFα Eu acceptor beads only or plus TNFα Eu acceptor beads and IL-6 Tb acceptor beads; **(C)** IL-1β plus IL-1β Sm acceptor beads only or plus IL-1β Sm acceptor beads and TNFα Eu acceptor beads; **(D)** TNFα plus TNFα Eu acceptor beads only or plus TNFα Eu acceptor beads and IL-1β Sm acceptor beads.

The similarity in alpha signal and curve shape from analyte standard curves run in a single or duplex assay format (Figure 4) validates that there is no negative impact on assay quality by the addition of a second set of acceptor beads to assay wells.

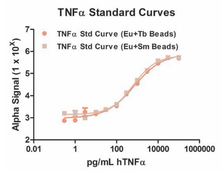

Figure 5. hTNFα analyte titrations assayed in the presence of TNFα Eu acceptor beads duplexed with IL-6 Tb or IL-1β Sm acceptor beads.

Finally, the signal from the hTNFα analyte standard curve, when assayed with the TNFα Eu assay and duplexed with either the IL-6 Tb or IL-1β Sm assays, was compared. The plot of the two curves in Figure 5 demonstrates that equivalent results can be attained when the AlphaLISA assay is multiplexed with either terbium or samarium AlphaPlex assays.

Inflammatory Cytokine Detection

Following optimization and validation of the two multiplexed assays, a cell-based experiment was set up to detect inflammatory cytokine secretion from glial and mononuclear cells. Cryopreserved primary astrocytes and monocytes were added to wells independently (Figure 6A and B) or in co-culture (Figure 6C) as previously described. Variable concentrations of the known stimulant LPS combined with IFNγ were then added to the wells. Aliquots from the supernatant were removed at regular intervals and run with either the IL-6 Tb AlphaPlex/TNFα AlphaLISA or IL-1β Sm AlphaPlex/TNFα AlphaLISA duplex assays to monitor potential changes in cytokine secretion between basal and stimulated cell conditions.

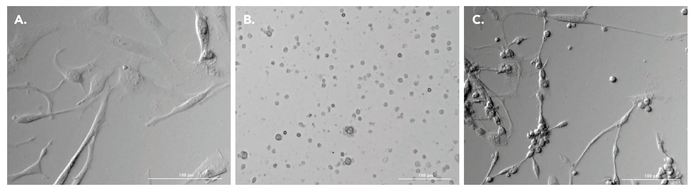

Figure 6. Brightfield images of glial and monocytic cell cultures. Brightfield images captured by the Cytation 5 (BioTek Instruments, Inc.) using a 20x objective showing **(A)** astrocytes; **(B)** monocytes; or **(C)** co-cultured cells in 96-well format.

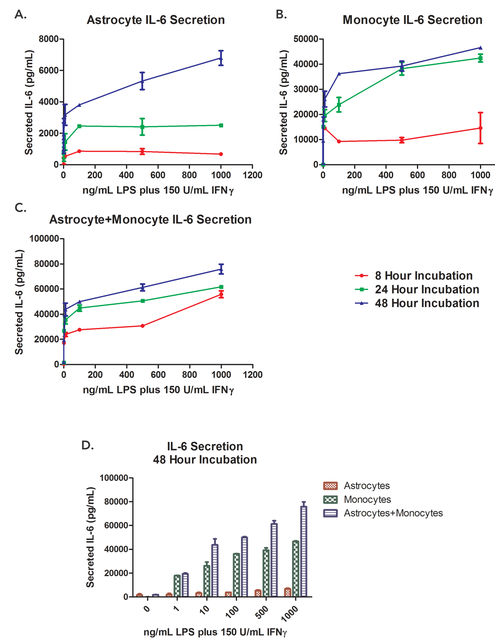

Figure 7. IL-6 secretion from astrocyte and monocyte cell cultures. Secreted pg/mL IL-6 concentrations in media following 8, 24, and 48 hour LPS and IFNγ stimulation of **(A)** astrocytes; **(B)** monocytes; or **(C)** co-cultured cells. **(D)** 48 hour timepoint IL-6 concentrations in media per cell model in basal and stimulated conditions. IL-6 pg/mL concentrations determined through interpolation of Alpha signal from unknown samples based upon purified analyte standard curves using Graph Pad Prism V. 5.01.

Upon examination of the graphs in Figure 7A-C, it is apparent that the combination of LPS and IFNγ stimulate secretion of IL-6 with all cell models tested. Furthermore, cytokine secretion is both time and concentration dependent. Figure 7D also illustrates that astrocytes and monocytes maintain consistent IL-6 secretion ability when co-cultured, creating proportionately higher cytokine concentrations when combined, compared to independent cell cultures. This agrees with the findings of Klegeris, *et al.*, 2001, when using astrocytic and monocytic cell lines.

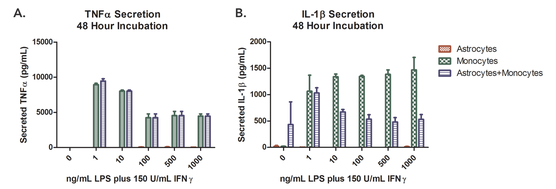

Figure 8. 48 hour LPS/IFNγ stimulation of TNFα and IL-1β cytokine secretion. Secreted concentrations of **(A)** TNFα; or **(B)** IL-1β from astrocytes, monocytes, or co-cultured cells following 48 hour LPS/IFNγ stimulation. pg/mL concentrations interpolated as previously described.

Assayed levels of TNFα secretion into media, upon completion of a 48 hour incubation with LPS and IFNγ (Figure 8A), show little change in wells containing astrocytes only. In comparison, stimulation is seen in wells containing monocytes or co-cultured cells, peaking at 1 ng/mL LPS. However, concentrations do not vary from monocyte only wells, indicating that secretion is due to monocytes in the co-culture. These findings are equivalent to previous literature publications (Sawada *et al.*, 1989) reporting higher TNFα production in LPS stimulated microglia compared to astrocytes. IL-1β follows a similar pattern (Figure 8B), with higher levels of cytokine secretion seen from monocytes, compared to astrocytes. An interesting phenomenon is the fact that IL-1β secretion decreases in the co-cultured cell model in a dose dependent manner, indicating a potential suppressive affect between the two cell types on specific inflammatory pathways, again agreeing with the literature (Klegeris, *et al.*, 2001), and underscoring the importance of incorporating correct cell models into experimental procedures.

Conclusions

1. The AlphaPlex assays from PerkinElmer represent a simple, sensitive, homogeneous high throughput method to monitor secretion of duplex analytes from the same assay well.
2. Incorporation of cryopreserved primary cells from Lonza simplify cell-based assay procedures and provide cell models that increase the relevancy of generated results.
3. The laser-based excitation and dual PMT capabilities of the Synergy Neo2 allow high density, multiplexed AlphaPlex assays to be easily performed with a single protocol.
4. The combination of multiplexed assay methods, cell models, and microplate reader create robust *in vitro* methods to easily and accurately measure secretion of inflammatory cytokines.