Multiplexed Microarray Assessments of Cellular and Molecular Toxicity Biomarkers

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Introduction

• Toxicological insults, infections, and cellular stress can result in complex alterations to cellular function and levels of circulating biomarkers

• A thorough assessment of potential toxicity requires quantitative analysis of…
  - molecular biomarkers
  - levels of particular cell types
  - immune and other cell functions
  - circulating levels of potential toxicants, toxins, and/or pathogens

• As the field of toxicology continues to evolve, it is becoming clear that a combination of cellular and molecular biomarkers can provide a unique “biomarker signature” of toxicity, disease, or infection
The Problem...

- Traditional methods for assessing biomarkers of toxicity ...
  - limited sensitivity
  - limited scope
  - labor and reagent intensive
  - require extensive sample preparation
The Needs...

- An ideal platform for multiplexed analysis of both cellular and molecular biomarkers of toxicity would permit...
  - sensitive detection
  - quantitative detection
  - rapid detection
  - label-free detection
  - detection of multiple analytes
  - detection from untreated clinical or environmental samples

The Solution...

Grating Coupled Surface Plasmon Resonance Imaging
Grating Coupled Surface Plasmon Resonance Imaging (GCSPRI)

• Surface plasmon resonance (SPR) measurements are based on the physical phenomenon of energy transfer at a metal-dielectric interface (e.g. gold and water)
  - Under specific optical conditions, the energy of incident light excites propagating electron oscillations (plasmons) within the metal. This energy transfer reduces the intensity of the reflected light.

• SPR technologies must match the wave vector of the illuminating beam of light with the plasmon wave vector
  1. Using a prism (Kretschman configuration)
  2. Using an optical diffraction grating (GCSPRI)

• There is a critical angle of incidence, the SPR angle, at which this vector matching is best, and at which the energy transfer into the metal is largest.
  – The SPR angle is the angle at which maximum plasmons are produced (and subsequently minimum light is reflected)
  – This transfer is very sensitive to the refractive index of the dielectric/metal interface
  – The capture of analytes by molecules immobilized on the chip surface changes the refractive index at that interface and will increase the angle at which maximum coupling occurs.
How we do it...

1. Immobilize capture molecules onto chip surface using a spotter

2. Assemble 40 μl flow cell by attaching window with self-adhesive gasket

3. Flow cell is inserted into the analyzer for spot definition
   - Blue = Region of Interest (ROI)
   - Red = Bare gold reference

Representative GCSPR Trace

Measure simultaneous real-time binding kinetics by flowing analyte(s) over up to 1000+ antibody (or other capture molecule) spots
GCSPRI: Measuring the SPR Minimum Angle

Collimated light source

CCD Camera and Detection Optics

θ

Data capture and real-time display

Intensity

Interrogation angle
GCSPRI: Measuring the SPR Minimum Angle
Collimated light source

θ

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Measuring the SPR Minimum Angle
GCSPRI: Measuring the SPR Minimum Angle

Collimated light source

CCD Camera and Detection Optics

θ

Data capture and real-time display

Intensity

Interrogation angle

SPR angle

Measuring the SPR Minimum Angle
GCSPRI: Measuring the SPR Minimum Angle after analyte capture
Software processes all ROIs simultaneously.

Affinity trace displays one data point for each complete SPR curve for each ROI.

Proteins, cells, or other molecules binding at the sensor surface change the refractive index and thus cause a shift in the SPR angle.
Representative Analytes

• **Mammalian Cells:**
  • Primary Immune Cells
  • Cell Lines

• **Pathogens:**
  • **Bacteria:** viable, spores, heat-killed
  • **Viruses**

• **Proteins:**
  • **Immune regulators:**
    • Cytokines, Antibodies, Transcription Factors, Enzymes (and activity)
  • **Toxins**
  • **Toxicant-adducted proteins**
    • **7C4-BSA:** Benzo(a)pyrene-adducted bovine serum albumin
    • **NNK-Ova:** NNK-adducted ovalbumin

• **Toxicants***:
  • Benzo(a)pyrene (BAP)
  • Pyrene
  • **NNK:** 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
Spots of BSA and mIgG (500 μg/ml) were immobilized in a 32x32 array using a SpotBot2 robotic microarrayer. Instrument software was used to draw blue regions of interest around visible spots of immobilized protein (blue target ROIs), and red reference ROIs were interspersed in checkerboard fashion between target ROIs and used to reference-correct data. **Spot diameter ~ 110 um; Spot-to-spot spacing ~ 285 um (center-to-center) ==> ~ 175 um (edge-to-edge)**
Target ROIs: mIgG or BSA, each at (500 ug/ml)
Sample: anti-mouse IgG (10 ug/ml)
Large SPR angle shifts are observed at mIgG ROIs but **NOT** at nearby BSA ROIs
Antibodies (500 ug/mL) were each immobilized in triplicate on three separate chips.

**Chip 1 sample**: M13 at $10^6$ PFU/mL in PBS, then ovalbumin (Ova) at 20 ug/mL

**Chip 2 sample**: M13 at $10^6$ PFU/mL and Ova at 20 ug/mL together in PBS

**Chip 3 sample**: M13 at $10^6$ PFU/mL and Ova at 20 ug/mL together in aquarium water
Cell Surface Marker Expression by GCSPRI

RAW264.7 cell capture by antibodies specific for immune cell surface markers. Experiment was performed to compare binding affinities of various labeled antibodies.
NNK [4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone] is a carcinogenic nitrosamine in tobacco. 7F mAb is an NNK-Ova specific antibody from a murine hybridoma. NNK-Ova was immobilized on three separate chips and 7F mAb was flowed over each chip at a different concentration.
**GCSPRI Kinetic Analysis of 7F mAb Binding to Triplicate NNK-Ova ROIs**

### Local Kinetic Analysis With Mass Transport Effects

<table>
<thead>
<tr>
<th>ROI</th>
<th>Spot Content</th>
<th>Concentration</th>
<th>kon</th>
<th>koff</th>
<th>Ka (kon/koff)</th>
<th>Kd (koff/kon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s3</td>
<td>Ova-NNK</td>
<td>500 ug/ml</td>
<td>1.94E+06</td>
<td>7.38E-04</td>
<td>2.63E+09</td>
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<td>s12</td>
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<tr>
<td>s21</td>
<td>Ova-NNK</td>
<td>500 ug/ml</td>
<td>3.76E+06</td>
<td>1.01E-03</td>
<td>3.63E+09</td>
<td>2.75E-10</td>
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### Global Kinetic Analysis With Mass Transport Effects

<table>
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<th>ROI</th>
<th>Spot Content</th>
<th>Concentration</th>
<th>kon</th>
<th>koff</th>
<th>Ka (kon/koff)</th>
<th>Kd (koff/kon)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ova-NNK</td>
<td>500 ug/ml</td>
<td>2.87E+06</td>
<td>8.41E-04</td>
<td>3.41E+09</td>
<td>2.93E-10</td>
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</table>
Uses same gold-coated sensor chips with sinusoidal grating used for GCSPRI
Excitation of fluorophores at specific distance from gold grating creates plasmons
Plasmons further excite fluorophore, leading to enhanced emission
Grating contributes to cone-shaped directional lobes of emission
New instrument allows both GCSPRI and SPEF measurements in a single assay
Primary peritoneal macrophages from WT, MTKO, and MTTG mice were stimulated with LPS for 24 hrs and cell supernatant was analyzed to identify components of an inflammatory biomarker signature for these cells.
Dilutions of anti-CD3 antibody were mixed with anti-IL2 or anti-IFN-gamma antibody and spotted on a SPEF sensor chip. Jurkat T cells were passed over the chip and captured by anti-CD3 ROIs. Cells were incubated on the chip for 24 hr in media (unstimulated) or media + 5 ug/ml ConA (stimulated) then removed with a lysis buffer. Subsequently a biotinylated detection antibodies and streptavidin-Alexafluor 647 were passed over the chip and cytokine levels were assessed by SPEF.

On-chip functional analysis of captured immune cells:
ConA + anti-CD3 stimulates Jurkat T cells more than anti-CD3 alone
**Limits of Detection**: SPEF Limits of Detection (LOD) defined by meeting the following two criteria: Lowest observed concentration where (1) signal was $>$3std above blank, negative and reference controls and (2) ratio of signal to positive control for sample was $>$3std above blank, negative and reference controls. ELISA experimental LOD defined as concentration where signal was $>$2std above blank. ELISA published LOD data from Shao, et al., *J. Biomedicine and Biotechnology*, 2003: 5 299-307.
Stressors
1. Cold Restraint (CR, 1 hr)
2. \textit{Listeria monocytogenes} (LM, $10^3$ cfu)
3. CR + LM

Lymphoid Subsets
- CD4+ T cells
- CD8+ T cells
- CD19+ B cells
- CD16/56+ NK cells

Immunodeficient mice:
NOD.Cg-\textit{Prkd}^{scid}/\textit{Il2rg}^{tm1Wjl}/SzJ

Generation of humanized mice
(Giassi LJ et al., Exp Biol Med 233:997-1012, 2008)

Immunodeficient mice:
NOD.Cg-\textit{Prkd}^{scid}/\textit{Il2rg}^{tm1Wjl}/SzJ
Biomarker Profiles of Stress. Serum from humanized mice was collected after mice were challenged with Cold Restraint (CR), *Listeria Monocytogenes* infection (LM), or both (CR + LM). Human and mouse cytokine biomarker levels were measured by SPEF and are the average of six measurements. Fluorescent Intensity Signal is normalized to the positive control.
Summary

- Advantages of GCSPRI and SPEF
  - Detection of wide range of analytes in a single assay:
    - Bacteria
    - Viruses
    - Proteins
    - DNA
    - Mammalian cells
  - Detection of analytes from microliter volume samples:
    - Cell supernatants
    - Cells cultured on-chip
    - Serum
    - Saliva
    - Interstitial Fluid
    - Complex environmental samples
Conclusions

- **GCSPRI**
  - Good for large analytes (cells) as well as higher abundance analytes (pg/mL --> ng/mL LOD)
  - Real-time, label-free detection
  - Kinetic analysis permits affinity determinations

- **SPEF**
  - Very sensitive (fg/mL --> pg/mL LOD)

- **Dual-mode instrument ==> Best of both worlds!**
  - Highly multiplexed analysis (1000+)
  - Small sample requirements (uLs)
  - Very wide dynamic range (~8 orders of magnitude)
  - Allows detection of molecular biomarkers, phenotypic analysis of cells and on-chip functional assessments of cell activation states

- These unique capabilities allow a more thorough analysis of a large set of biomarker signatures from complex samples than currently available technologies
THANK YOU!!!

NESOT

Genzyme

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