Multiplex Immunoassay Validations

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6-6-2016

Multiplex Assay Validations

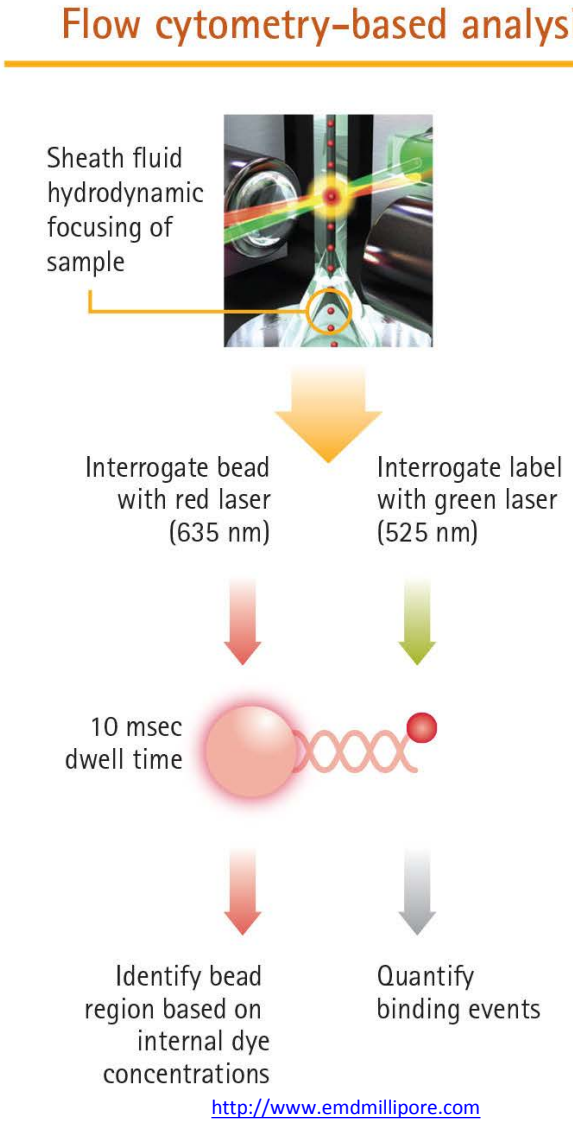
- Over the past 10-15 years, a number of different companies, and a few different technologies have been developed with the goal of maximizing data output (number of analytes measured), while minimizing the sample volume and cost required for each data point.
- This document will give a general overview of the technologies that have been utilized and analyzed by the University of Vermont – LCBR. This document will also highlight the benefits as well as limitations of each of these measurement platforms.

Multiplex Assay Validations

- Gold Standard Assay
  - Large volume of clinical and epidemiological data that demonstrates known relationships
  - Frequently referenced in literature and/or data generated by other facilities
  - Large volume of historical data with good reproducibility and accuracy/precision.
  - However, there is still the question of how “gold” are the gold standards.

Flow-Cytometry Based Multiplexing

- Open System: Many Different Vendors
- Up to 80 Analytes in single sample
- Many Different Assay Configurations
- Low Sample Volume required (Typically ≤ 25uL per replicate)
- Large Dynamic Ranges
- Require specialized plate washing equipment



Flow Cytometry Based Multiplexing

- Vendors:
  - EMD Millipore
  - Bio-Rad
  - RnD Systems
  - Invitrogen (Thermo-Fisher)
  - Affymetrix
  - & More

Flow-Cytometry Based Multiplexing

- Challenges
  - Many different assay configuration
    - Even within the same vendor, there are many different assay combinations. With each of these various combinations, there comes an increase source of variation. This makes validating all configurations extremely difficult for a research laboratory. Sensitivities can also be effected by the different assay configuration/panel selections.
    - Cross vendor validation can be even more challenging due to the various sources of antibodies available. Not only do antibodies manufactured at different facilities have the potential for targeting different epitopes, but the biological source of the antibodies (mouse, goat, sheep etc..) can also have an impact on comparative data.
    - Our research facility attempts to address and mitigate these issues by comparing back to “gold standard” assays.

Flow-Cytometry Based Multiplexing

- Challenges
  - Per plate throughput is slower than other methods of multiplexing
  - Read time per 96 well plates is ~45 minutes
    - The greater the number of analytes per plate, the less of an impact the 45 minute read time has on the overall speed of data output.
    - Currently UVM has 2 Bio-Plex instruments, with a max capacity of eight 96 well plates per run day.

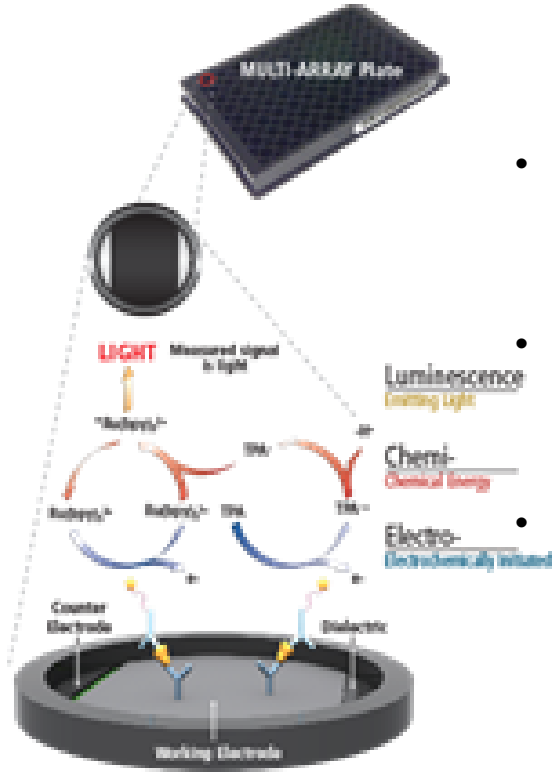
Flow-Cytometry Based Multiplexing

- Challenges
  - Signal Loss
    - Capture antibodies are bound to small (6.45uM) magnetic beads.
    - These beads are not “fixed” to a solid surface similar to a traditional ELISA.
    - Because of this, bead loss can occur at multiple stages in the assay. This can occur due to:
      - Over vigorous plate shaking
      - Incorrect washing equipment (needs to be magnetic based)
      - Incorrect wash settings (nozzles pressure to high, height adjustments incorrect)
      - Accidental plate inversion
      - Photo-bleaching due to long term light exposure, or intense light exposure (i.e. halogen lamp)
      - Laser failure (Red or Green: 635nm/525nm)
      - Bead Aggregation due to incorrect preparation (insufficient sonication, insufficient mixing)
      - Bead Settling due to prolonged period of time from preparation to utilization.
        - » This results in uneven distribution of beads across the 96 well plate.

Flow-Cytometry Based Multiplexing

- Sample Condition:
  - Sample quality is extremely important for Flow-Cytometry based assays. Incomplete processing that results in cellular debris can have a highly negative impact on results. This is due to wash equipment clogs, as well as instrument fluidics clogs.
  - Hemolysis, Lipemia and other pre-analytical variable can also impact data results. However, these subtle variables are difficult to analyze for their overall individual and combined effect on data results.

Electrochemiluminescence Based Multiplexing



- High binding carbon electrodes in the bottom of MULTI-ARRAY and MULTI-SPOT microplates allow for easy attachment of biological reagents (10X greater binding capacity than polystyrene).
- MSD assays use electrochemiluminescent labels that are conjugated to detection antibodies. The labels are called SULFO-TAG, and allow for ultra-sensitive detection.
- Electricity is applied to the plate electrodes by an MSD instrument leading to light emission by SULFO-TAG labels. Light intensity is then measured to quantify analytes in the sample.

- Closed System:
  - https://www.mesoscale.com
- 10 Different Analytes Per Well
- Many Different Assay Configurations Available
- Custom Multiplexing
- Low Sample Volume Required (Typically ≤ 25uL per replicate)
- Large Dynamic Ranges
- Highly Reproducible Results
- Manufacturer Validated Assay

Electrochemiluminescence Based Multiplexing

- Number of Analytes Per Plate is limited by physical space inside each of the 96 wells. Currently the maximum Configuration is 10 analytes per plate.
- Antibodies are fixed, chance of signal loss due to particulate loss is greatly diminished compared to flow-cytometry based
- Different assay combinations seem to have minimal impact on analyte reproducibility.
  - Wash Buffer and Calibrator source have greater impact than panel configurations
  - Antibody sources appear to be consistent across various panel configurations

Analyte Comparisons

- To date, the number of analytes run using a multiplex platform include:

Adiponectin	**IFNg**	**IL-1b**	**IL-2**
IL-4	**IL-6**	**IL-8**	**IL-10**
Insulin	**Leptin**	**MCP-1**	**PAI-1**
Resistin	**TNF-a**	**TNF-R1**	**TNF-R2**
VEGF	**D-Dimer**	**GM-CSF**	**Galectin-3**
ST2	**Amylin**	**C-Peptide**	**CRP**
Cystatin C	**E-Selectin**	**GLP-1**	**ICAM-1**
RANKL	**SAA**	**SAP**	**VEGF**
VCAM	**IL-6sR**	**sCD30**	

This list will continue to grow as budget and kit requests permit

Validation Materials

- N=20 Serum Sets
- N=20 EDTA Sets
  - Various lot numbers
    - Each change in lot number made evident in comparative analysis
  - Sets purchased from biovendor as 1.00L pooled human serum or human plasma.
  - Each of the 20 pooled sources were aliquoted into 0.5mL cryovials and stored at -80 until assay utilization
  - Utmost care to preserve individual integrity of each specimen including attention to: temperature, environmental exposure, cross contamination and other variables.
  - Serum pools DO NOT match EDTA pools
  - Sets run for both evaluative/validation purposes as well as at defined intervals.