Development and Validation of in Vitro Flow Cytometry-based Assays for Preclinical Immunology.

Kurt Sales

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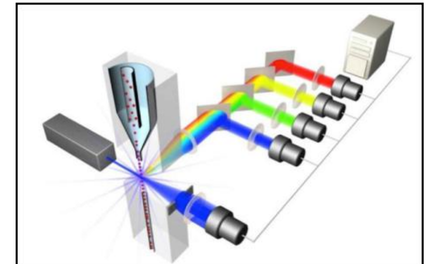
Outline

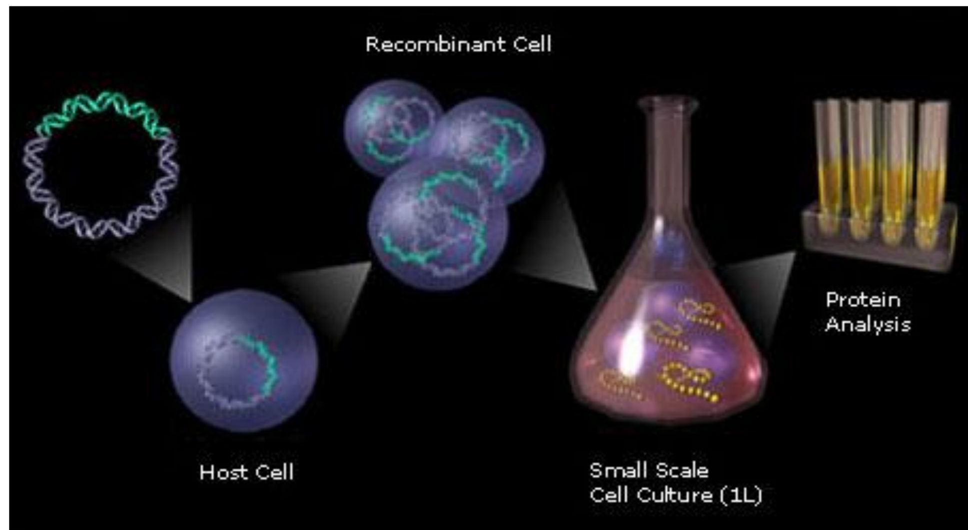
- Introduction
  - Edinburgh site, Study design and endpoint analysis
  - Flow Cytometry – the basics
- Development and Validation of Flow Cytometry methods – discussion points
- Summary



Introduction

What is a Biological Product?

- Estimated that > 30% of new pharmaceuticals in development are biological products
- Biological product – defined as: Substances derived from living organisms – including humans, animals, plants and microorganisms or produced by biotechnology methods



Industry Metrics

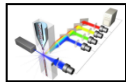
- ~ 25% to 40% of biologicals in development are monoclonal antibodies
- World-wide sales of monoclonal antibody products to >$94 billion by 2017 and nearly $125 billion by 2020
- 47 monoclonal antibody products on the market in the US and Europe (Nov 2014)
- In 2013, recorded sales of nearly $11 billion, the highest sales figure ever recorded for a biopharmaceutical product
- Increasing complexity in biomolecule development – bivalent molecules etc

Basic Study Design - Multi-species

Key Features – Flow used throughout the life-cycle of the product

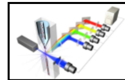
Pilot study (~2 weeks)

- Few Animals
- Dose selection
- Dosing schedule and duration



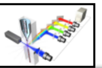
Main Study (typical 4-6 weeks)

- Dosing: determined by clinical regimen
- Sample collection and immunobiology analysis etc
- in vitro/ex vivo analysis



Recovery/observation period

- Examine reversibility of pharmacological or toxicological effects



Study Design

Key Features – Types of Flow Analysis

- Flow Cytometry:
  - Immunophenotyping
  - Receptor Occupancy
  - Oxidative Burst/Phagocytosis
  - CD34 Stem cell analysis
  - Other functional assays
- Cytometric Bead Array (Flow)/Luminex Multiplex:
  - Immune biomarkers
  - Cytokine release assays: measure possible cytokine storm
- In Vitro Cell-based assays:
  - AAV1/AAV5 Neutralising antibody assays

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Development of a Cell-based Flow Cytometric Assay to Detect the Presence of AAV5 Neutralising Antibodies in Human Matrix

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Introduction

Gene therapy, the use of nucleic acid polymers as a drug to treat disease by genetic transfer to patient cells, has showed massive promise in the treatment of a variety of genetic disorders in recent years. The adeno-associated virus (AAV) family has emerged as a promising vehicle in the development of gene therapies as they can be engineered to contain any nucleic acid cargo, targeted to specific genes and can infect both quiescent and dividing cells without inducing disease in humans. Several considerations are needed in preclinical development of such gene therapies. Firstly, the emergence of immunogenic response and development of neutralising antibodies towards the vector is a concern. Secondly, natural AAV infections, as highlighted in AAV2 trials, leading to naturally occurring seropositive AAV antibodies can limit the type of vector used as well as the treatment efficiency of the gene

Results and Discussion

FIGURE 1: Multiplicity of Infection (MOI)

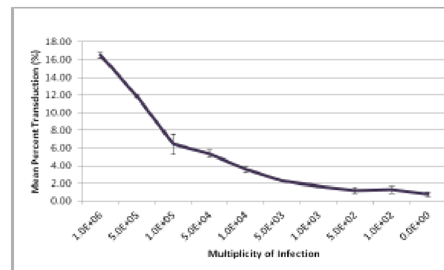
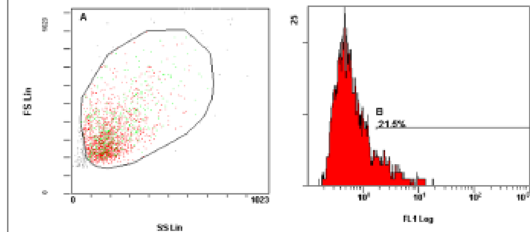


FIGURE 2. Flow Cytometric assessment of transduction



In addition to complex multiplex analysis, we can also support in vitro Assays for immunogenicity or other immunological proagrams

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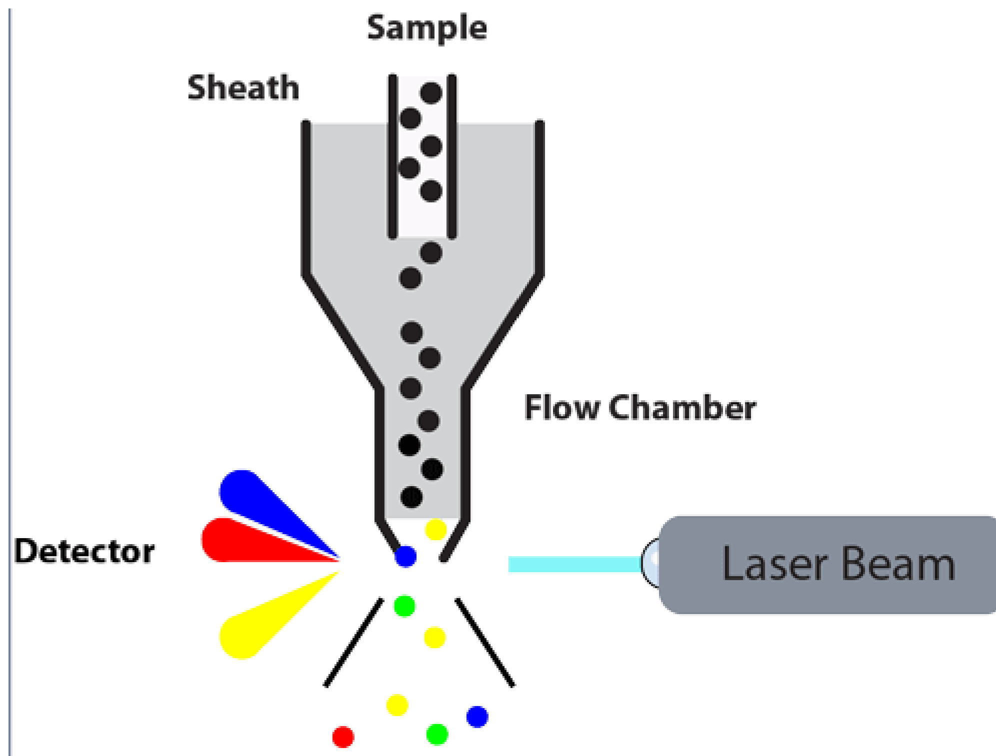
Flow Cytometry

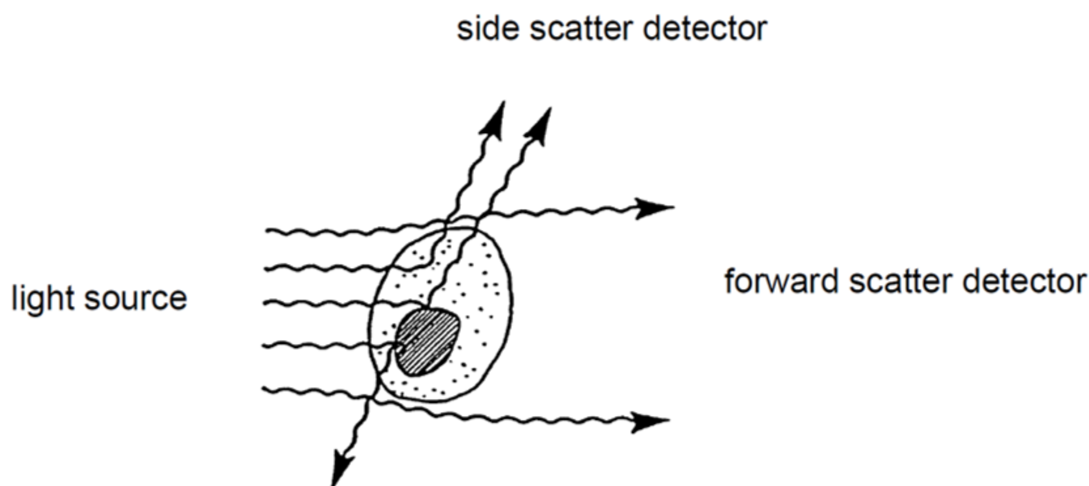
Sample Flow – the basics!

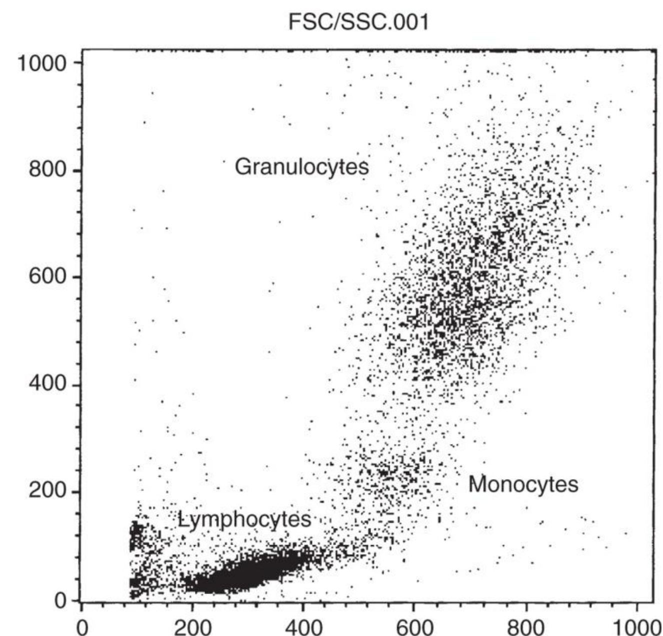
- Particles in suspension pass through a source of light in single file
- Laser beams illuminate sample – generates light scatter (forward and sideways scatter)



Light Scatter

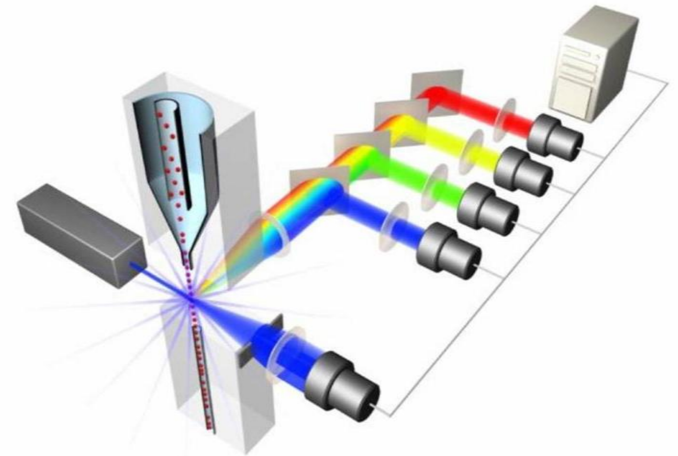
- Measures relative size and complexity (granularity) using light scatter characteristics





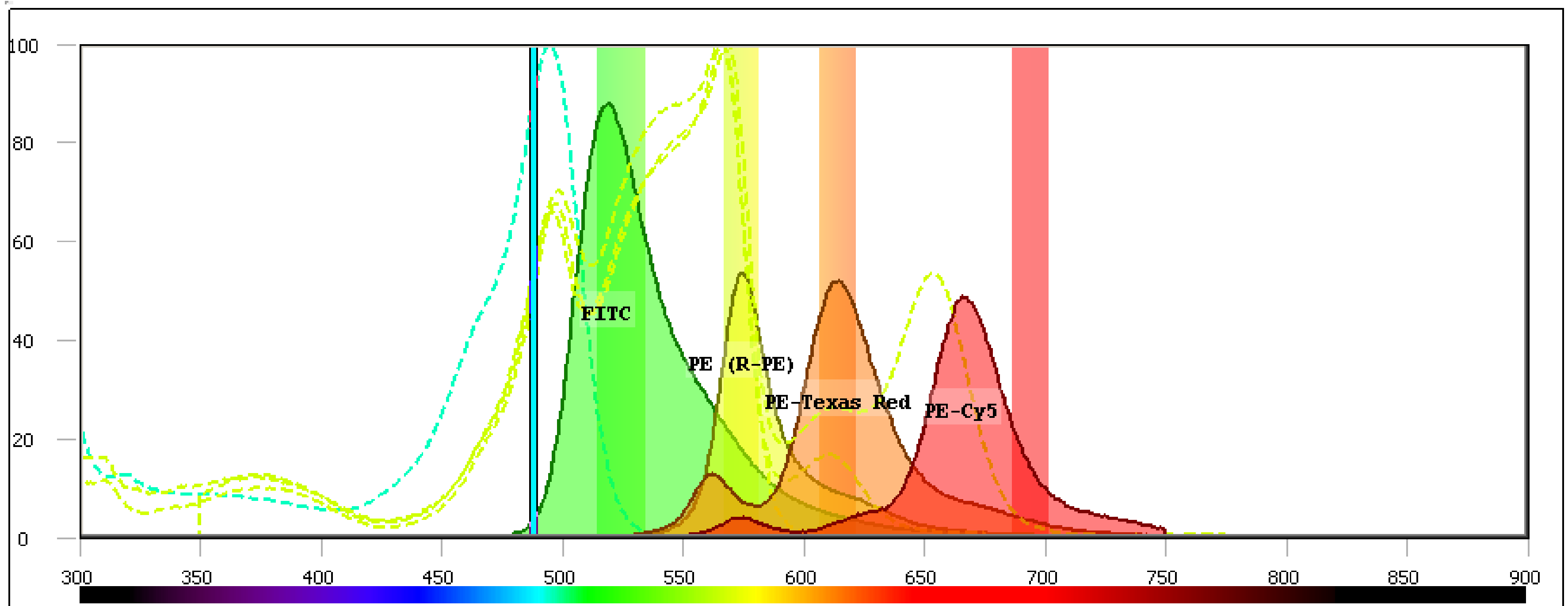
Fluorescence

- Fluorochromes (fluorescent dyes) are used to measure presence of antibodies, peptides, nucleic acids …..
- Fluorochromes absorb energy from a laser
  - electrons excited to a higher energy level
  - electron decays to ground state emitting a photon of light
  - Photons detected by photomultiplier tubes (PMTs)



Multicolour Analysis

- Can use more than one fluorochrome at a time provided they have different emission spectra



Flow Cytometry Method Development and Validation – discussion points

Validation Parameters

Outlined below is the proposed life-cycle of an assay

Assay Development **Assay Qualification** **Assay Validation**

Validation is defined as: an evaluation of the method on its fitness for the intended applications (FDA; FaDA, 1996 Guidance for industry: Validation of Analytical Procedures)

Current draft guidance (FDA 2013) – recommends a fit-for –purpose approach

-The testing process must ensure that the assay meets predetermined standards and performs reliably and is fit for its intended use

-recognise that there is no one-size-fits-all regulation

There is currently a lack of guidelines for the validation of flow cytometric assays

- guidelines are taken from white papers, guidance documents and scientific publications

Journal of Immunological Methods 363 (2011) 104–119

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Recommendations for the validation of flow cytometric testing during drug development: I instrumentation

Cherie L. Green [a,*], Lynette Brown [b], Jennifer J. Stewart [b], Yuanxin Xu [c], Virginia Litwin [d], Thomas W. Mc Closkey [e]

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Denise M. O'Hara [a], Yuanxin Xu [b], Zhiyan Liang [c], Manjula P. Reddy [d], Dianna Y. Wu [e], Virginia Litwin [f,*]

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Typical Assay Performance Parameters

Typical Bio-Assay parameters as defined by FDA and ICH Q2R1 include:

- Accuracy
- Selectivity/Specificity
- Assay range/Limit of detection
- Reproducibility/Robustness
- Precision: Inter and intra-assay coefficient of variation (CV)
- Linearity
- Stability

These can be used as a starting point for assay development, qualification and validation – many parameters might not be relevant or practical to consider.

Achievable Assay Performance Parameters for Flow Cytometry

YES	MAYBE	NO
•Specificity •Precision/Robustness •Sensitivity •Stability	•Linearity •Standard Calibrators •Interference (Matrix or drug) •Limit of Detection •Limit of Quantitation	•Accuracy •Selectivity •Range of Quantification •Prozone Effect

Assay Development/Optimisation

Where to start?

For Multicolour analysis:

- Panel design: Deciding on the panel
- Fluorochrome selection and spacing to avoid spectral overlap
- Sourcing the antibodies: mAb clone Selection
- Optimising antibody titration
- Matrix
  - Cell lines, whole blood, PBMC
  - Wash/lyse/fix sequence evaluation
- Gating strategy

Challenges of Flow Cytometry Method Validation

Accuracy:

For Flow Cytometry - Establishing **accuracy** in an assay is often impractical – as the true value of the biologic is unknown.

Limited reference materials and QC's available

- reagents may be available for human but limited for other species
- limited to a few well characterised proteins only, So of limited use for rare events

Challenges of Flow Cytometry Method Validation

Selectivity/Specificity:

Deciding on a **gating strategy, PMT and compensation settings**

- Gating strategies must be verified to establish the cell subset of interest is included while other cell subsets/non-specific events are excluded

Fluorochrome selection

- Abundantly expressed antigens in dimmer fluorochromes
- Antigens-of-interest or dimmer antigen on brighter fluorochromes
- Population auto-fluorescence and spectral overlap from all fluorochromes must also be considered

Challenges of Flow Cytometry Method Validation

Precision:

Inter and intra-assay precision – limited blood stability is an issue for interassay precision tests.

- **Interassay precision** – a sample from three individuals is tested in three different experiments (one sample is prepared and analysed by 3 Analysts in one day).
- **Intra-assay precision** – a sample from three individuals is tested at least three times within the same experiment (one batch)

<10 %CV desirable (and generally achievable for Standard markers) for all methods

- <20-25 %CV acceptable for immunoassays - achievable for flow
- <30 %CV may be acceptable for rare event detection and use as exploratory biomarkers
  - With poor precision, more replicates and samples are required

Challenges of Flow Cytometry Method Validation

Linearity:

- Optimise antibody dilutions
- Need to define the minimum number of events needed to get reliable data.
  - Linearity could be addressed by diluting stimulated cells with unstimulated cells and performing intracellular cytokine staining for cytokines present in stimulated cells only.

Challenges of Flow Cytometry Method Validation

Stability:

Definition: The capability of a sample material to retain the initial property of a measured constituent for a period of time within specified limits when the sample is stored under defined conditions (ISO Guide 30/92-2.7)

Sample stability, prepared (labelled) sample stability, Instrument stability

Factors influencing stability

Largely depends on :

- Assay type
- Specimen Type
- Panel Design
- Sample Collection Methods; Matrix present; Processing Procedure

Challenges of Flow Cytometry Method Validation

Reproducibility/Robustness:

•Instrumentation setup – eg: using calibrated fluorescent beads; BD CS&T beads.

•SOP for sample preparation.

•single-operator assays.

•assessing lot-to lot variation.

•Gating strategy.

Inter-equipment variation

Analysis of the same sample on 2-3 cytometers

Ideally should be <20% variation (generally achieved

Challenges of Flow Cytometry Method Validation

Additional considerations:

Background range

Analysis of a standard panel in 5M +5F animals over a 3 week period to get a baseline value

Summary

Currently a lack of guidelines for the validation of flow cytometric assays

Unlike drug assays, novel biomarker assays are accompanied by unique analytical issues, in many cases ruling out the use of universal, strict validation guidelines

These issues include the common absence of suitable reference standards, the employment of unique analytical reagents and assay platforms, the presence of endogenous biomarkers in a sample, analyte heterogeneity, and a variety of disease-specific effects