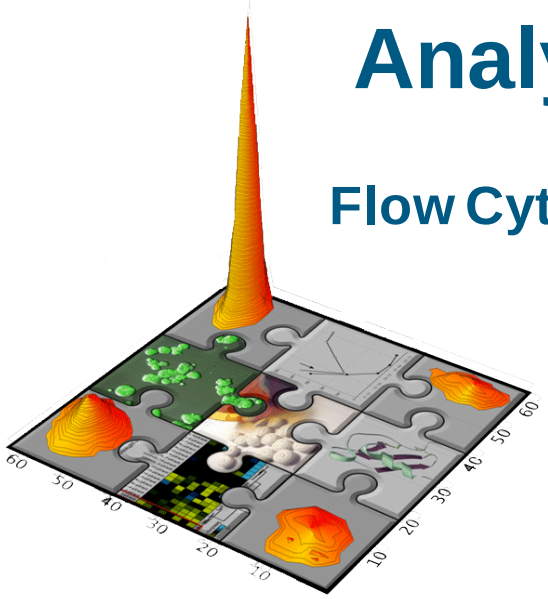

Analytical Method Validation:

Perspectives from the AAPS Flow Cytometry Action Programming Committee

Virginia Litwin, Ph.D.,
Principal Scientist,
Hematology Department, Covance
Founder and Chair
Flow Cytometry Action Program Committee, AAPS

European Bioanalysis Forum
Barcelona, Spain
November 20-22, 2013

Virginia.Litwin@covance.com

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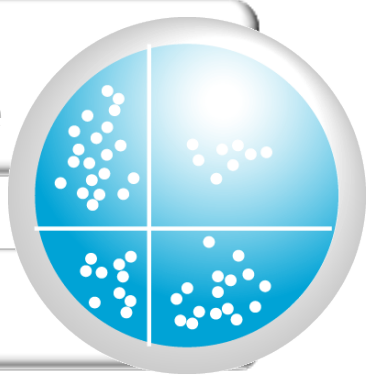
Presentation Overview

Introduction to AAPS Flow Cytometry Action Programming Committee

Flow Cytometry Method Validation Perspective from AAPS Flow Cytometry Action Programming Committee



The Path Towards Flow-Specific Guidelines

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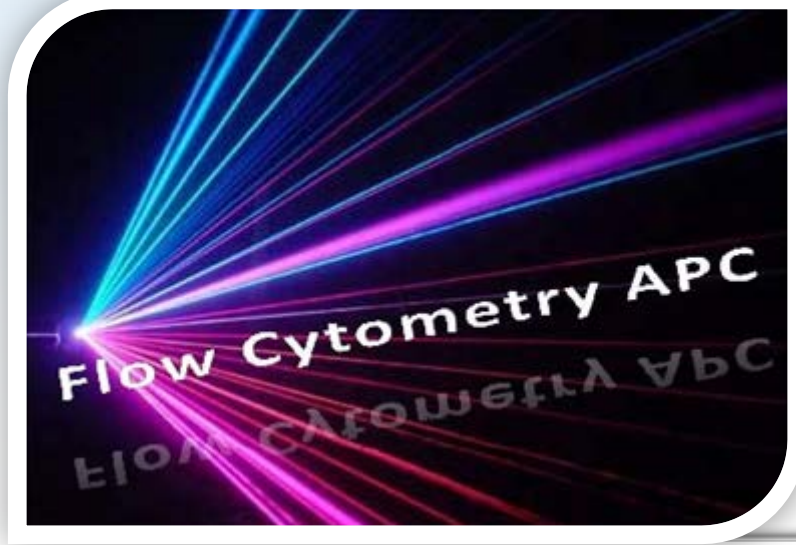
Flow Cytometry APC

AAPS, Bio-Tec Section, Ligand Binding Assay Bioanalytical Focus Group, Flow Cytometry Action Program Committee



Mission Statement

To promote discussion regarding the proper application of flow cytometry in drug development with an emphasis on establishing best practices regarding assay and instrument validation.

Steering Committee Past and Present

- **Virginia Litwin**
- **Cherie Green**
- **John Ferbas**
- **Peter O'Brien**
- Lynette Brown
- Sophie Corneau
- Kathy Howell
- Nicholas Jones
- Murli Krishna
- Zhyian (Eric) Lianz
- Thomas McCloskey
- Denise O'Hara
- Manjula Reddy
- John Sloan
- Jennifer Stewart
- Wendy White
- Christopher Wiwi
- Dianna Wu
- Timothy Wyant
- Yuanxin Xu
- Maxime Moulard

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Programming at AAPS

- Hot Topics Session, Annual Meeting 2006
- Roundtable Session, NBC Meeting, 2007
- Presentation, Fit-for-Purpose Symposium, Annual Meeting 2011
- Symposium Session –Intracellular Signaling by Flow Cytometry, Annual Meeting 2012
- Symposium Session –Receptor Occupancy Flow Cytometry, AAPS NBC 2013
- Roundtable Session–Validation for Regulatory Environments AAPS NBC 2014

http://www.aaps.org

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Programming at CYTO 2013

ISAC CYTO 2013 Innovation, Discovery, Translation

San Diego, CA May 19-22, 2013

Workshop: Navigating the Labyrinth of Regulated Flow Cytometry in Drug Development

Workshop: Design and application of receptor occupancy assays used to measure pharmacodynamic response to treatment with biologic

http://www.isac-net.org/

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Presentations at the FDA

Public Workshop - Clinical Flow Cytometry in Hematologic Malignancies, February 25-26, 2013

The Role of Biomarkers in Clinical Trials and The Fit-for-Purpose Method Validation Approach

Virginia Litwin, Covance and Cherie Green, Amgen

http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm334772.htm

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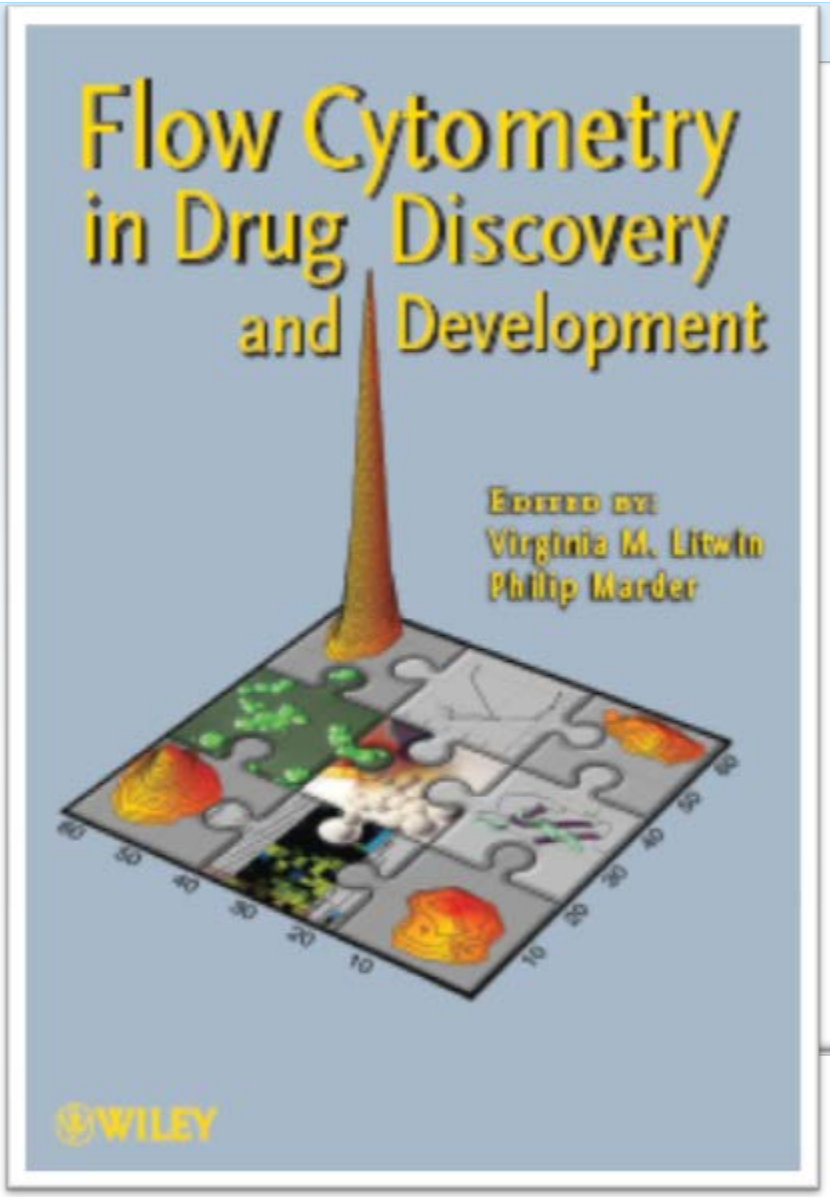
Publications



Litwin, V., and Andahazy, J.
A Translational Medicine Approach to Monitoring the Immune System during Clinical Trials

O'Hara, D.M., and Theobold, V.
Immunogenicity Testing Using Flow Cytometry

Xu, Y., and Richards, S.M.
Pharmacokinetics by Flow Cytometry - Recommendations for Development and Validation of Flow Cytometric Method for Pharmacokinetic Studies

Hill, C., Wu, D., Ferbas, J., Litwin, V., and Reddy, M.
Regulatory Compliance and Method Validation

Ferbas, J., and Schroeder, M.
Instrument Validation for Regulated Studies

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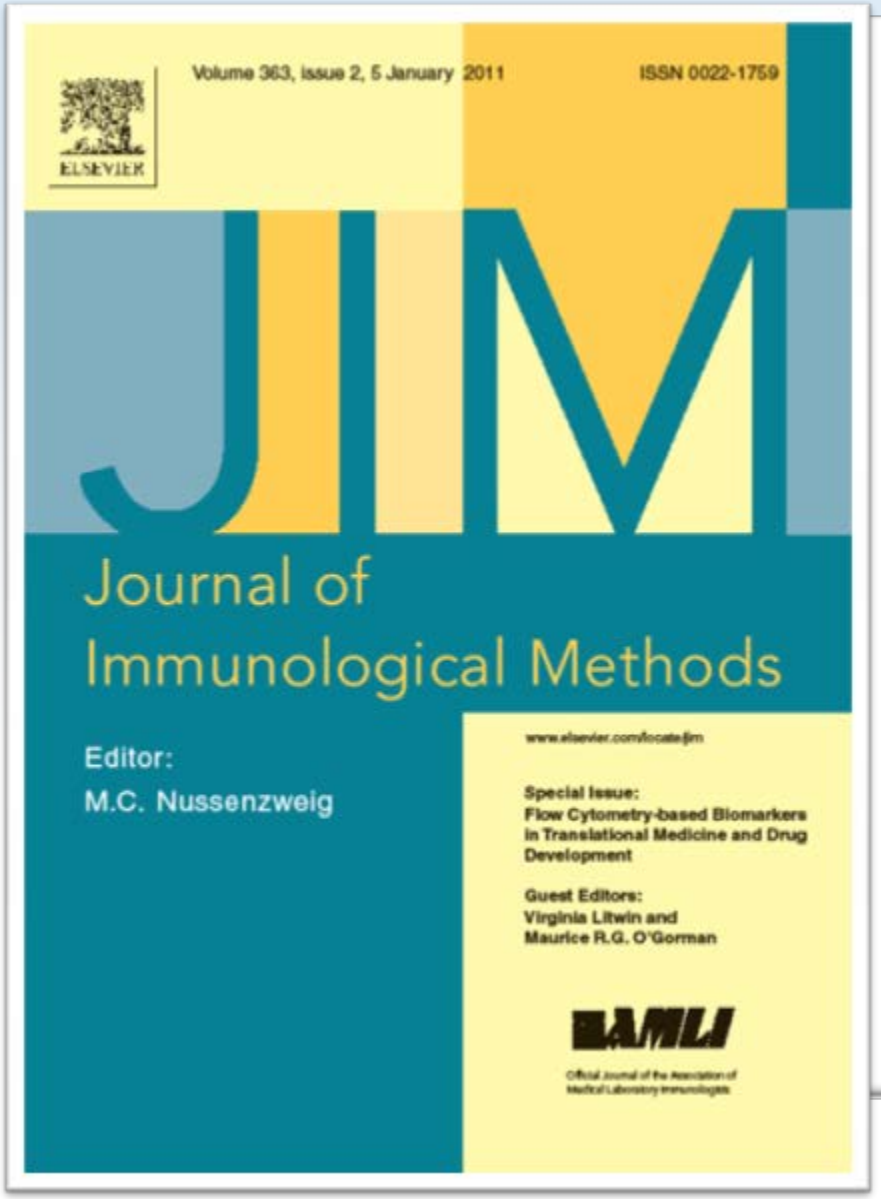
Publications



Special Issue: Flow Cytometry-base Biomarkers in Translational Medicine and Drug Development

Litwin, V., and O'Gorman, MRG.
Flow Cytometry-based Biomarkers in Translational Medicine and Drug Development.
JIM, 363:101-102, 2011.

Green, C., Brown, L., Stewart, J., Litwin, V., and McClosky. T.
Recommendations for the Validation of Flow Cytometric Testing During Drug Development: I Instruments.
JIM, 363:104-119, 2011.

O'Hara, D., Xu, Y. Lianz, E., Reddy, M., Wu, D., and Litwin, V.
Recommendations for the Validation of Flow Cytometric Testing During Drug Development: II Assays.
JIM, 363:120-134, 2011.

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Webinars

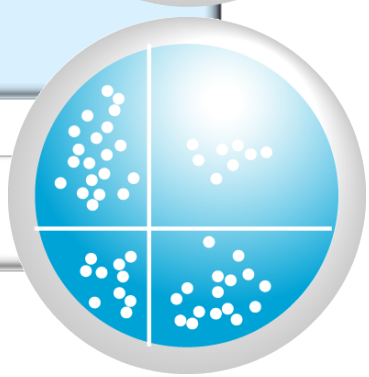
http://www.aaps.org/Meetings_and_Professional_Development/eLearning_Repository

Presentation Overview

Introduction to AAPS Flow Cytometry APC

Flow Cytometry Method Validation Perspective from AAPS Flow Cytometry APC

The Path Towards Flow-Specific Guidelines



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Fit-for-Purpose Approach in Flow Cytometry

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. McCloskey[e]*

Recommendations for the validation of flow cytometric testing during drug development: II assays

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

JIM, 363:104-119, 2011
JIM, 363:120-134, 2011

The Role of Biomarkers in Clinical Trials and The Fit-for-Purpose Method Validation Approach, V. Litwin and C. Green
http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm334772.htm

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Fit-for-Purpose Method Validation

WHO

Lee, et al. Pharm. Res. 22:499, 2005

AAPS, Bio-Tec Section, Ligand Binding Assay Bioanalytical Focus

WHY

Usage of biomarker data was impeded by a lack of understanding on how to interpret the data

Application of existing validation paradigms to were not appropriate to biomarker research

Can't apply one set of rules to all technologies

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Fit-For-Purpose Method Validation

Fit

Biomarker data must be reliable and accurate data

Purpose

Decision making during drug development

Fit-for-Purpose

Analytical validation requirements are specific to the stage of drug development

Consideration to the **intended use** of the biomarker data

Consideration to the regulatory requirements associated with that use

Practical, iterative approach

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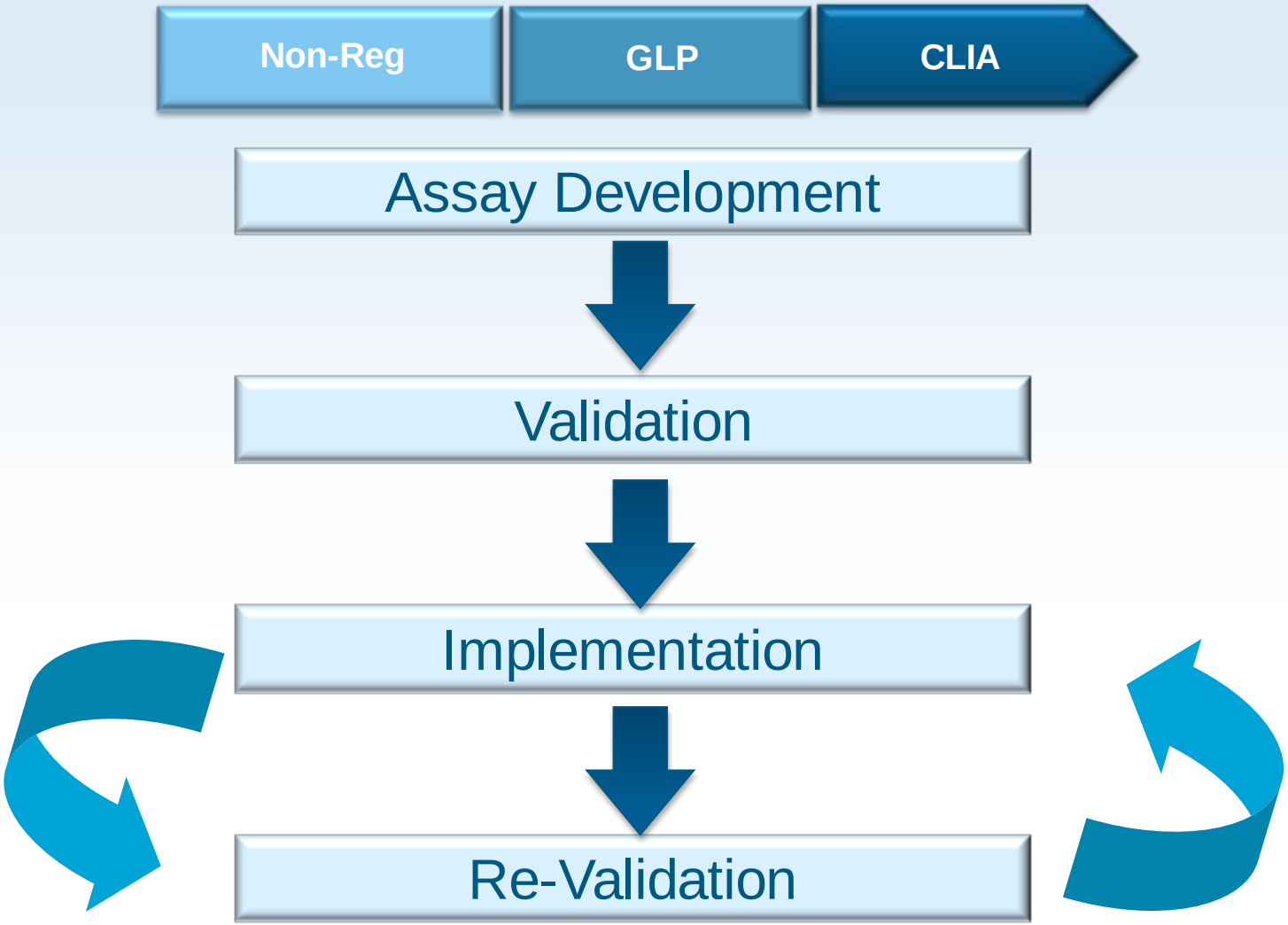
Iterative Approach



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Fit-for-Purpose Approach in Flow Cytometry

Why

Flow cytometric methods can be more challenging to validate that other technologies

Analytical Issues

- Cellular measurands
- Lack of cellular reference material
- Highly complex reagents
  - mAb, fluorescent tags, tandem dyes
- Highly complex instrumentation

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Validation Considerations Flow Cytometry

Methodology

- Flow Cytometry

Sample Type

- Whole blood, bone marrow, other
- Isolated cells, PBMC, tissue, cell lines

Data Type

- Bioanalytical Category
- Assay format/readout

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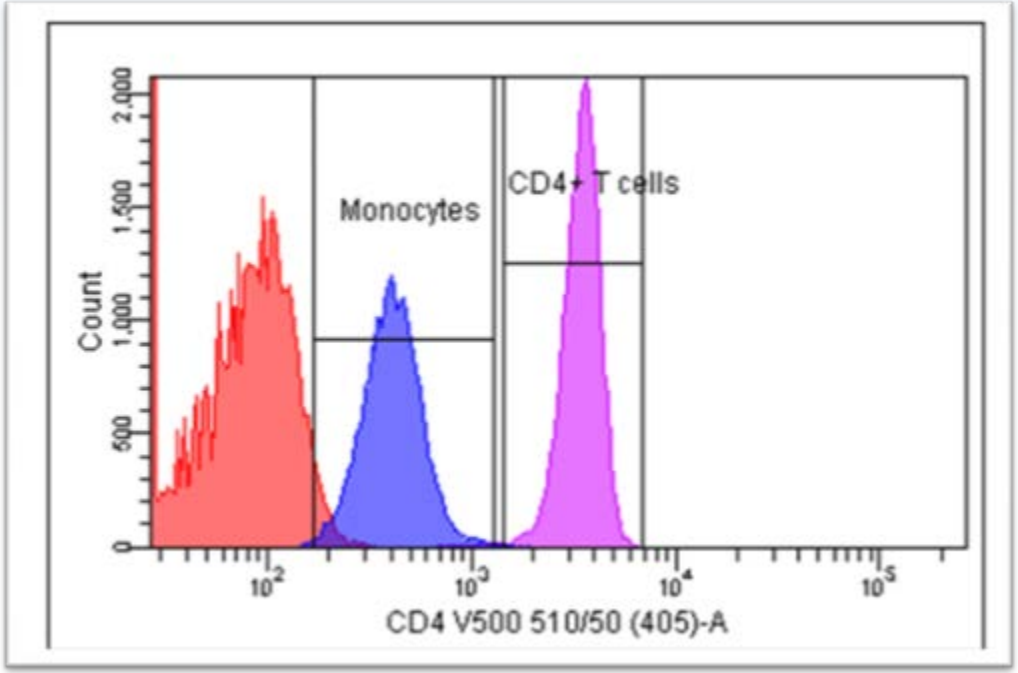
Bioanalytical Category

Quasi-Quantitative

- Quasi--Possess certain attributes
- Results are numeric and expressed in terms of a characteristic of the test sample
- No reference standard



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Readout/Assay Complexity

Surface phenotyping

- Simple/complex
- Quantitative antigen expression

Intracellular

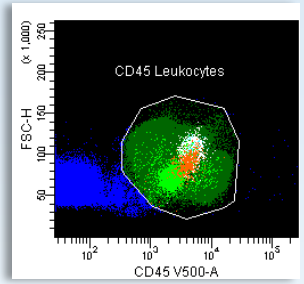
- Cytokines
- Nuclear proteins
- Phosphorylated antigen detection

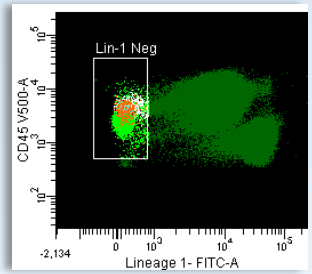
Neutralizing or Anti-drug Antibody

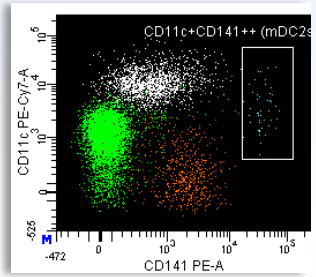
Receptor Occupancy

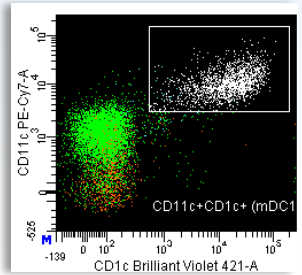
Nucleic acid detection

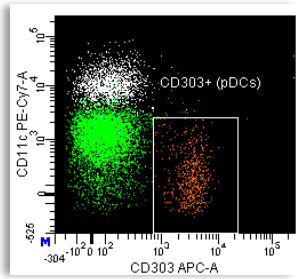
- Cell cycle
- Apoptosis











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Validation Stringency & Regulatory Requirements

Non-regulated, GLP, CLIA, GMP

Establish the intended use of the data

- Exploratory biomarker
- PD biomarker
- Safety
- Enrollment biomarker
- Companion Diagnostic

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Validation Parameters

Achievable in Flow Cytometry Validations?

Always

- Specificity
- Precision/Robustness
- Sensitivity
  - Limit of Detection
  - Limit of Quantitation
- Stability
- Reference Intervals

Sometimes

- Linearity
- Standard Calibrators

No

- Accuracy
- Selectivity
- Range of Quantification
- Incurred Sample Reanalysis
- Interference (Matrix, Drug)
- Normal Signal Distribution
- Prozone Effect

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Flow Biomarker Assay

B cell Panel-4 Configuration

	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8
	BV421	V500	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
Gating Control	CD19	CD3/CD14/ CD56			CD20			CD45
EXP1	CD19	CD3/CD14/ CD56	IgD	CD27	CD20		CD69	CD45
EXP2	CD19	CD3/CD14/ CD56	CD138	CD24	CD20	CD38		CD45
EXP3	CD19	CD3/CD14/ CD56	IgD	CD27	CD20		CD10	CD45

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Flow Biomarker Assay

Reportable Results	
Population	**Phenotype**
CD19 B cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19+
CD20 B cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD20+
Activated B cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19+, CD20+, CD69+
Naïve B cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19+, CD20+, CD27–
Memory B cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19+, CD20+, CD27+
Short-lived plasma cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19dim, CD20-, CD27bright
Breg	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19+, CD24bright, CD38bright
Plasma cells	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19dim, CD20-, CD138+
Plasmacytoid	CD45bright, SSClow, CD3-, CD14-, CD56-, CD20+, CD138+
Transitional	CD45bright, SSClow, CD3-, CD14-, CD56-, CD19+, IgD+, CD10+, CD27–

Specificity

Assay Development

- Fluorochrome selection
  - Most abundant antigens in dimmer fluorochromes
  - Antigens-of-interest in brighter fluorochromes
  - Population auto-fluorescence and spectral overlap from all fluorochromes must also be considered
- Clone evaluation
- Reagent titration
- Matrix- cell lines, whole blood, PBMC
- Lysis, fixation, permeablization buffer selection
- Acquisition and analysis templates/gating strategy

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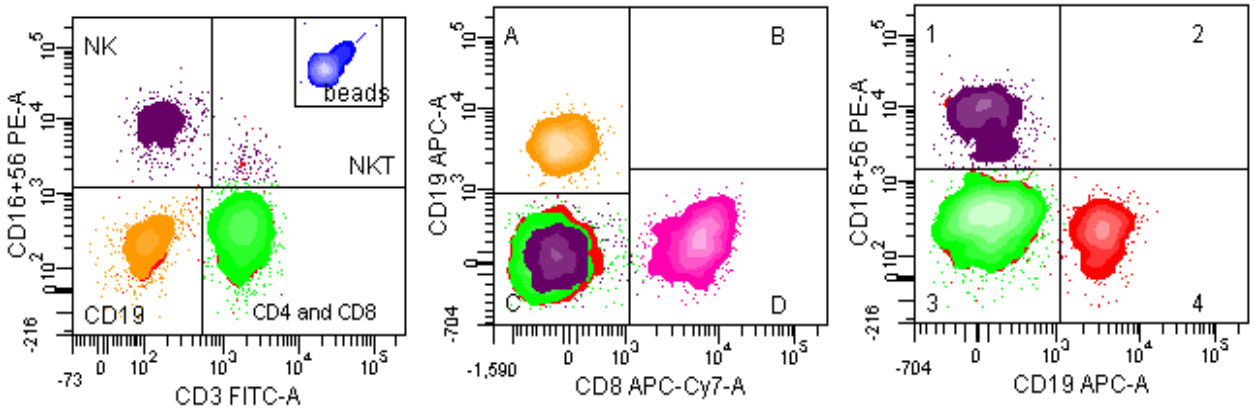
Specificity

Assay Development

- CD Marker Selection
  - CD markers used to define the cellular population or antigens of interest must be justified from the literature
  - Monoclonal antibody specificity should be verified by the Leucocyte Differentiation Antigens Workshops or peer reviewed publication
  - Gating strategies must be verified to establish the cell subset of interest is included while other cell subsets and non-specific events are excluded



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Specificity

Clone Evaluation

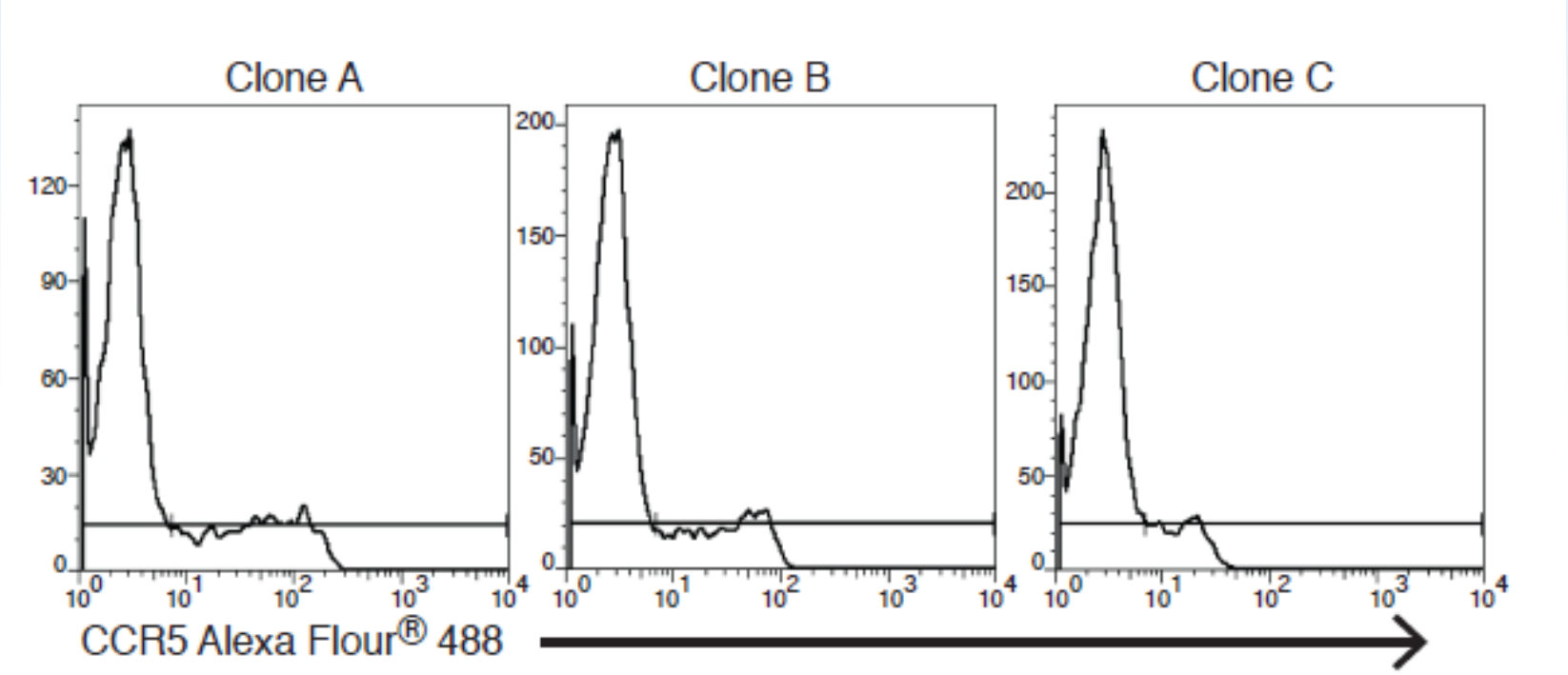

Figure courtesy of Anagha Divekar, BioLegend

Specificity

Fluorochrome Evaluation

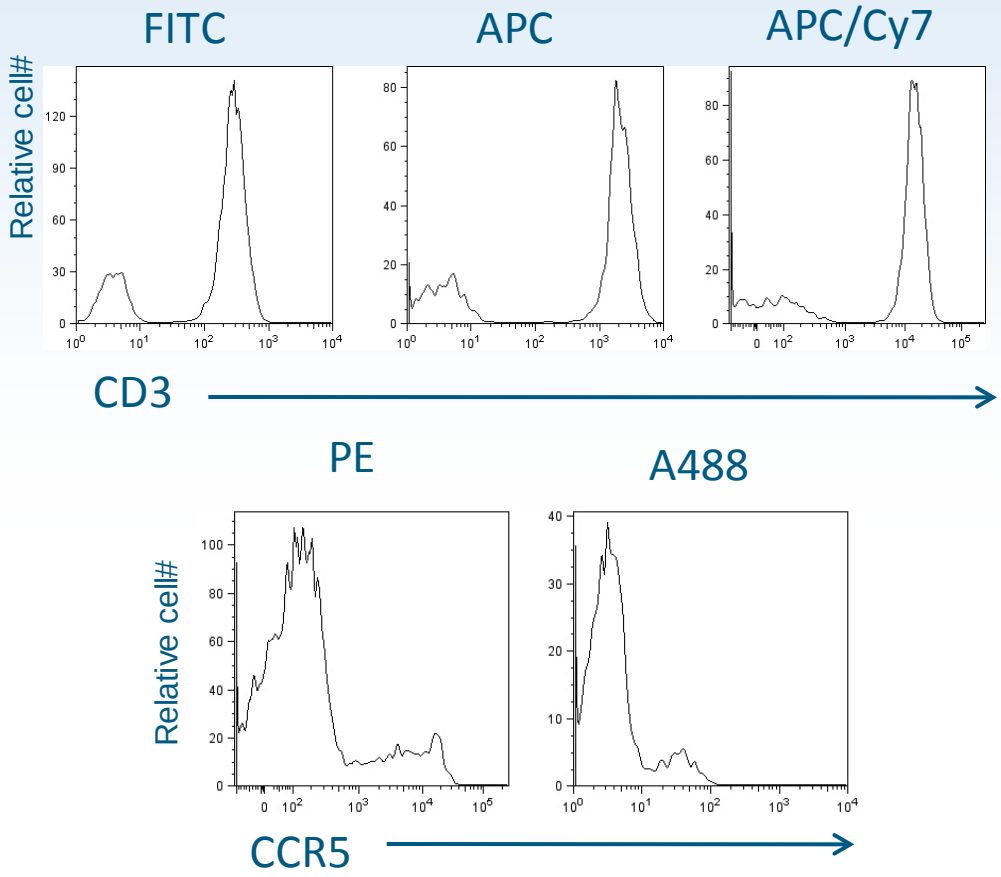

Figure courtesy of Anagha Divekar, BioLegend

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Accuracy

Standard Definition

- Closeness of the result compared to the true value of the analyte

GLP

- Determined by the mean bias determined in spiked recovery experiments

CAP/CLIA

- Comparison to “gold standard” method
- Measured concentrations in an official reference sample
- Measuring a concentration in comparison to an official standard

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Accuracy for Flow Biomarker Assays

IVD

- Proficiency Testing Surveys are available
- QC material with target values are available
- Inter-laboratory comparison

RUO/LDT

- Lack of proficiency testing programs
- Lack of cellular reference/QC material with target values for the populations of interest
- For novel or proprietary methods, sample exchange in not possible

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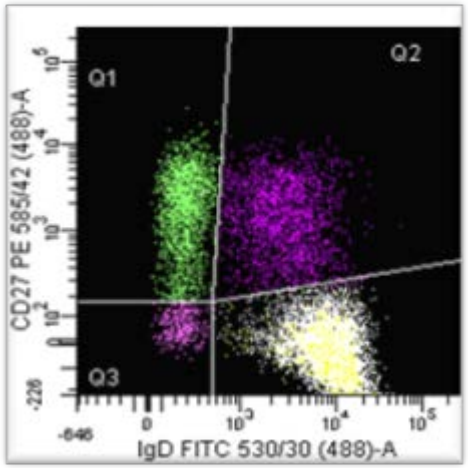
Precision for Flow Biomarker Assays

Precision

- Difficult to find samples with varying levels of each reportable result
- Weighted importance for biomarker data
  - Intended use of the data
  - Longitudinal, multicenter studies
  - Monitor responses due to treatment



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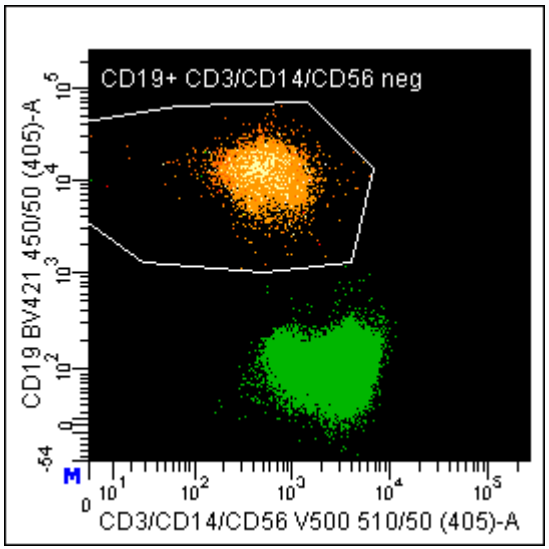
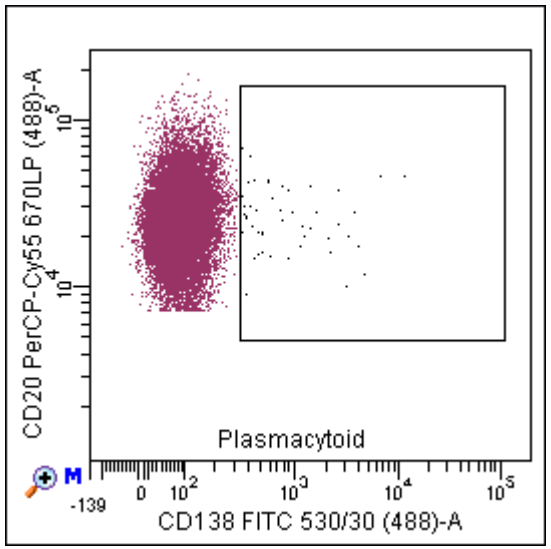
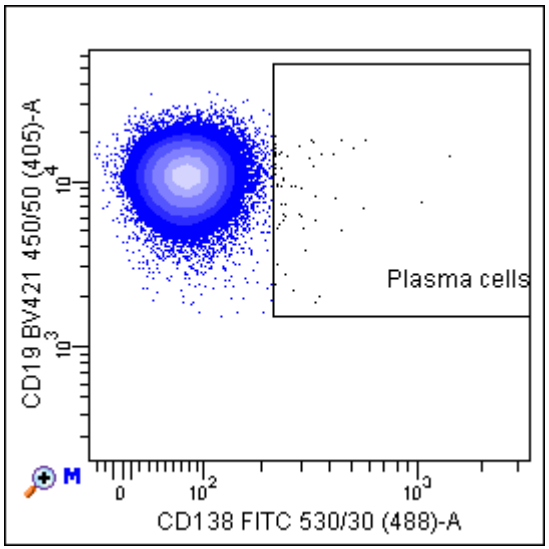
Precision for Flow Biomarker Assays

Acceptance Criterion

- <10 %CV desirable for all methods
- <20-25 %CV acceptable for immunoassays per Fit-for-Purpose paper
- <30 %CV may be acceptable for rare event detection use as exploratory biomarkers
  - With poor precision, more replicates and samples are required **(iterative approach!)**







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Sensitivity

Standard Definition

- The lowest reportable result

GLP

- Lower limit of quantification (LLOQ) as the lowest concentration that can be measured with acceptable accuracy and precision (e.g., ± 20% CV)

CAP/CLIA

- Response above the limit of detection (LOD)

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Sensitivity for Flow Biomarker Assays

Lower Limit of Detection (LOD)

- FMO controls

Gating Control	**CD19**	**CD3/CD14 /CD56**			**CD20**			**CD45**
EXP1	**CD19**	**CD3/CD14 /CD56**	**IgD**	**CD27**	**CD20**		**CD69**	**CD45**

Lower Limit of Quantitation (LLOQ)

- Difficult to find samples
- Mix stained and unstained samples
- Targeted cell depletion followed by re-spiking

Weighted importance for biomarker data

- Need to know at what point are the results are imprecise

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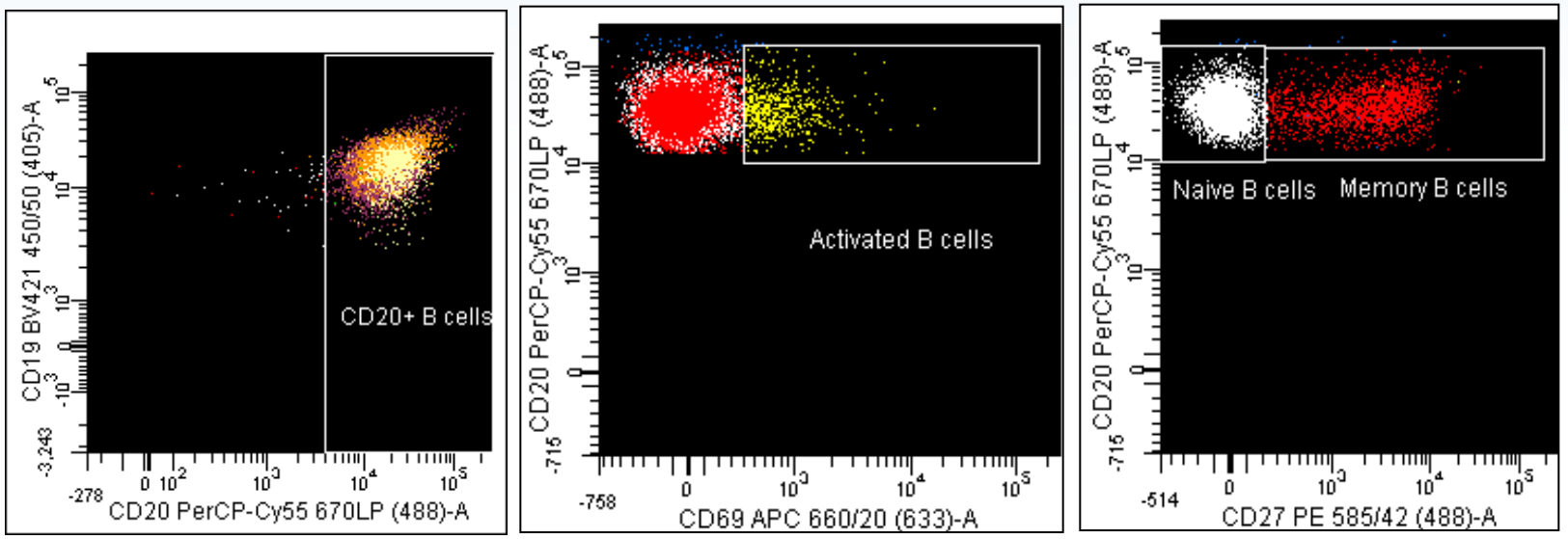
Reference Intervals for Flow Biomarker Assays

Reference Intervals

- Not required for first usage exploratory biomarkers, PD biomarkers
- Required for safety, diagnostic/disease biomarkers or companion diagnostics **(iterative approach!)**



Presentation Overview

Introduction to AAPS Flow Cytometry APC

Flow Cytometry APC Method Perspective

The Path Towards Flow Cytometry-Specific Validation Guidelines

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Path Towards Flow-Specific Guidelines

Three Important First Steps

2011

- AAPS
- Recommendations for the Validation of Flow Cytometric Testing During Drug Development: II Assays (JIM 363:120, 2011).

2011 - 2013

- International Council for Standardization in Haematology ICSH/ International Clinical Cytometry Society ICCS Workgroup
- Cytometry Part B: Clinical Cytometry- Special Issue: Validation of Cell Based Fluorescence Assays: Practice Guideline
- http://onlinelibrary.wiley.com/doi/10.1002/cyto.b.v84.5/issuetoc

2013

- FDA Public Workshop - Clinical Flow Cytometry
  - www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm334772.htm

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ICSH/ICCS Workgroup

Two day Think-tank

- Dedham, Maine, March, 2011

Chairs

- Bruce H. Davis (ICSH; CLSI)
- Brent Wood (ICCS/ICSH)
- David Barnett (UK NEQAS)
- Teri Oldaker (ICCS)

Participants

- 36 international experts
- 10 observers of corporate sponsors
- Experience in the development and/or standardization of cell-based fluorescence assays
- USA, UK, France, Spain, Canada, Germany, S Korea, China, Japan, The Netherlands, Australia

Stakeholders

- ICSH, ICCS, NEQAS, CLSI, EuroFlow, AAPS, FDA, flow diagnostic companies, reference labs

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ICSH/ICCS Workgroup

Why

Controversy and confusion about regulation of laboratory-developed tests (LDT) in diagnostic laboratories in the USA

- Testing in diagnostic laboratories is regulated by Centers for Medicare and Medicaid Services (CMS)
- Approval for In Vitro Diagnostic Tests is regulated by the FDA
- In 2009, the College of American Pathologists (CAP) recommended that FDA play a role in the oversight of LDT

The FDA has not issued guidance documents regarding LDT

- Most current guidance documents were designed for clinical chemistry methods
- There is a need for flow-specific guidelines
- Flow-specific guidelines should be prepared by flow experts

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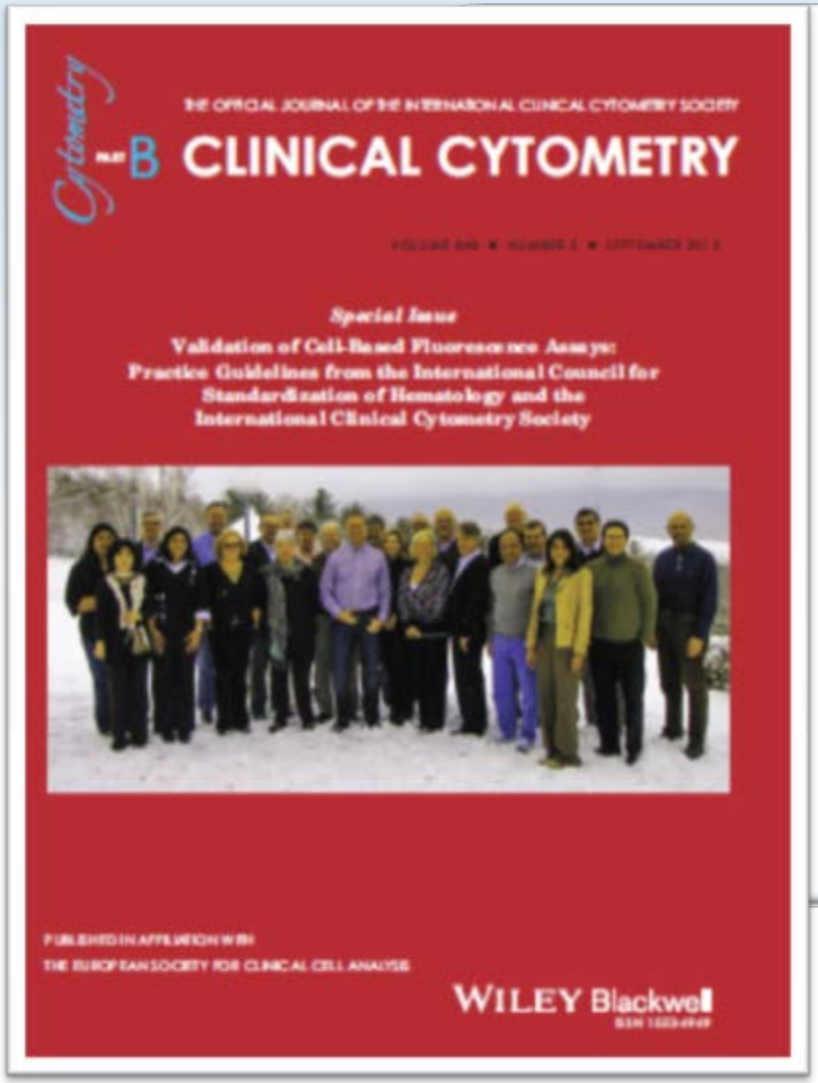
ICSH/ICCS Workgroup Deliverables



Cytometry Part B: Clinical Cytometry- Special Issue: Validation of Cell Based Fluorescence Assays: Practice Guideline

Submit to FDA for consideration as official guidance document for IVD submissions

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ICSH/ICCS Guidance Document

- Broad, expert -driven guidelines to address the uniqueness of cell based assay validations
- Pre-analytical Considerations
  - Sample storage, stability, transport
  - Cell counts, viability and use of morphology as needed
- Analytical Performance
  - Optimization/validation of instrument, sample prep, antibody/reagents, compensation and data analysis
- Performance Characteristics
  - Validation samples
  - Detailed criteria to assess required performance specifications
- Post-analytical Considerations
  - Resulting categories, data and sample storage
  - Internal and external quality assurance

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FDA Public Workshop - Clinical Flow Cytometry

Public Workshop on Clinical Flow Cytometry in Hematologic Malignancies, February 25-26, 2013.

The purpose of this public workshop is to seek input from academia, Government, laboratorians, industry, clinicians, patients and other stakeholders on the role of clinical flow cytometry in hematologic malignancies, ***in order to develop a specific regulatory policy for this class of in vitro diagnostic devices.***

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FDA Public Workshop - Clinical Flow Cytometry

- Address lack of biologic controls in hematologic malignancy testing
- Address minimal residual disease
  - Use as a surrogate marker in clinical trials
  - Discuss consensus methods

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FDA Public Workshop - Clinical Flow Cytometry

Agenda

Session 1: Analytical Challenges in Standardization and Validation of FCM

- Joint NIST ISAC Standardization Framework for Quantitative Flow Cytometry, Lili Wang, PhD
- NIAID NIH Nature Protocol for Quality Assurance for Multicolor FCM Using a Suite of Calibration Beads, Steven Perfetto, MS MT (ASCP)
- Multicolor Bead Flow Cytometry Standardization, Heba Degheidy, MD, PhD and Fatima Abbasi, MPH
- NEQAS Proficiency Testing in Acute and Chronic Leukemias, David Barnett, PhD
- College of American Pathologists (CAP) Protocol and Survey Results, Eric Hsi, MD

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FDA Public Workshop - Clinical Flow Cytometry

Agenda

Session 2: Transitioning from Analytical to Clinical QC in FCM

- The Role of Biomarkers in Clinical Trials and the Fit-for-Purpose Method Validation Approach, Virginia Litwin, PhD and Cherie Green, BS
- ICCS White Paper Validation of Fluorescent Cell Based Assays, Teri Oldaker, MLS, (ASCP), QCYM
- An Overview of CLL/BLPD and Maryalice Stetler-Stevenson, MD, PhD

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FDA Public Workshop - Clinical Flow Cytometry

Agenda

Session 3: Clinical Diagnostic Flow Cytometry

- The Bethesda Conference (US Perspective), Brent Wood, MD, PhD
- Euroflow: Standardization, Monoclonal Antibody Selection and Panel Configuration, Tomas Kalina, MD, PhD
- Different Approaches to Harmonisation and Standardisation - Experience from ERIC, EMN, Euroflow and ESCCA, Andy C. Rawstron, PhD
- A Model for Harmonizing Flow Cytometry in Clinical Trials, Phillip McCoy, PhD

Session 4: Flow Cytometry Data Analysis Software

- Perspective on Software Pitfalls and Challenges, Brent Wood, MD, PhD

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Summary

Currently a lack of guidelines for the validation of flow cytometric methods for use in:

- Drug discovery
- Drug development and clinical trial
- In vitro diagnostic
- Patient care and monitoring
- Companion Diagnostics

Different groups are pushing for guidelines

- FDA, ICCS, ICSH, AAPS

Regardless of the intended use of the data flow cytometric methods must be:

- Designed using state-of-the-art concepts
- Be validated as appropriate to the intended use of the data
- Include rigorous instrument standardization and monitoring

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