aaps Summer Scientific Forum
JULY 22–25 2024
INTERCONTINENTAL KANSAS CITY AT THE PLAZA
KANSAS CITY, MO

Putting Cell Counting in Context (*of use*): Analytical Measurements Supporting Cellular Therapies

July 24, 2024

Virginia Litwin, Ph.D.

eurofins | Clinical Trial Solutions

Session Description

- When the drug product consists of living cells, distinct analytical measurements are needed. Flow cytometry, the premier technology for single cell characterization, has become an indispensable analytical tool supporting all stages cellular therapy development.
- Monitoring the post-injection distribution phase and subsequent expansion, contraction, and persistence phases, requires not only enumerating the amount of circulating cell product, but extensively characterizing the cells. For CAR T cells, the extent to which the injected cells become activated or exhausted will influence clinical outcomes, thus the expression of a large repertoire of T cell markers including differentiation, activation, exhaustion, and checkpoint molecules are routinely measured. When administering any type of immune therapy, including CAR T cells, it is informative to also evaluate the disposition of the patient's immune cells.
- Using flow cytometry, upwards of 30 measurements can now routinely be made simultaneously on thousands of single cells, relatively rapidly, and with exquisite sensitivity and specificity. This allows for extensive phenotyping of both the resident and infused T cells in the same sample, minimizing the sample volume requirements.
- While there is little controversy in the field regarding the optimal process for developing and validating complex, high-parameter immunophenotyping methods, when it comes to cell enumeration assays which generate an absolute count expressed, as the number of cells per unit volume, there is a surprising lack of understanding/
- This presentation discuss the pros and cons of various methods to generate cell counts using flow cytometry and will review the progress of the NIST Flow Cytometry Standards Consortium.

Objectives

- Participants will gain an understanding of the value of high-parameter flow cytometry in monitoring cellular therapies.
- Participants will learn the pros and cons of various methods to generate cell counts using flow cytometry.
- Participants will be updated on the progress of the NIST Flow Cytometry Standards Consortium which was launched in 2021 in order to accelerate the adoption of quantitative flow cytometry in biomanufacturing of cell and gene therapies.

Biography and Contact Information

Virginia Litwin
Scientific Affairs Director at Eurofins Clinical Trials Solutions
Virginia.Litwin@bcl.eurofins.com

- President-Elect of the International Society for the Advancement of Cytometry (ISAC)
- 2023 recipient of the International Clinical Cytometry Society (ICCS) Wallace H. Coulter award in recognition of contributions to the science, education and practice of Clinical Cytometry
- Associate Editor for Cytometry Part B: Clinical Cytometry
- Co-founder of the AAPS Flow Cytometry Community
- Clinical and Laboratory Standards Institute (CLSI) Expert Panel and document development chair for H62 and H42
- Member of the National Institute of Standards and Technology (NIST) Flow Cytometry Standards Consortium

Cellular Therapies

- One of the most exciting and promising new therapeutic modalities
- Genetically engineered cells or normal cells
- Derived from autologous or allogenic origin
- Chimeric antigen receptor (CAR) T-cells
  - Genetically engineered to recognize cancer cells
  - CD19 CAR T-cells
    - Targeting B cell malignancies
    - Impressive remission rates in patients
- Other applications being evaluated
  - Solid tumors
  - Autoimmune conditions
  - Infectious diseases

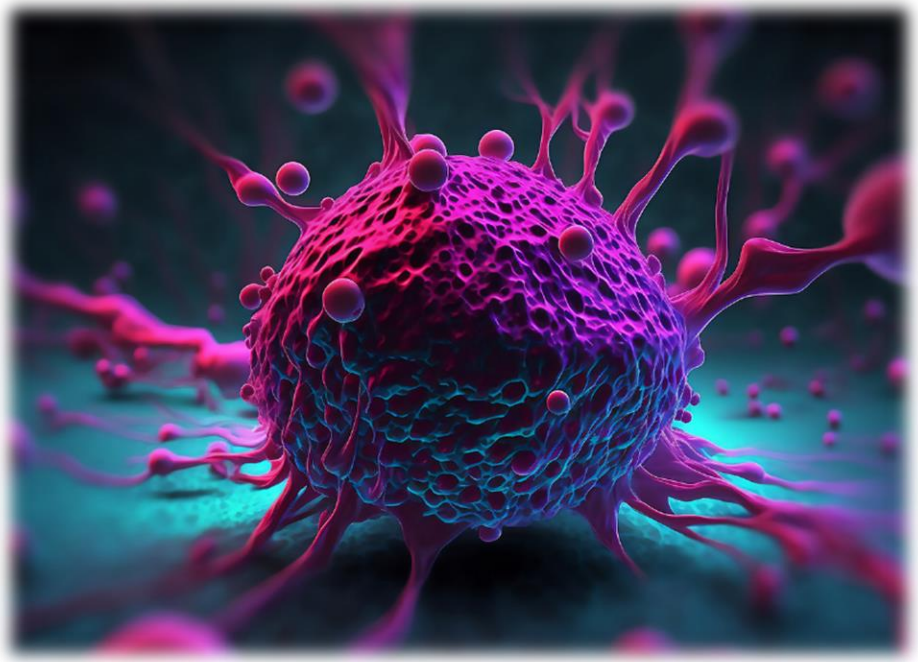


Figure from : Current State and Future State of Sample Preparation in Cell and Gene Therapy. S. Virolainen and C. Eberle https://promo.genengnews.com/curiox-next-gen-sample-prep/

Analytical Measurements Supporting Cellular Therapies

- Protein-based immunotherapies and small molecule drugs
  - Highly purified, single entities
- Cellular therapies
  - Heterogenous populations
  - Dynamic living cells
  - Require distinct analytical measurements
- **Flow cytometry**
  - **Premier technology for single cell characterization**
  - **Indispensable analytical tool supporting all stages cellular therapy development**

Clinical Trials Supporting Cellular Therapies

- Elucidate the factors which influence clinical response and CAR T-cell persistence
- **What to measure**
- **How to measure it**

What to Measure- CAR T-cells

- Cellular Kinetics (CK)
  - Absolute counts (needs to be developed and validated for rare events)
  - Distribution, expansion, contraction, and persistence phases
- Pharmacodynamics (PD)
  - Expression level of CAR (Quantitative Flow Cytometry)
    - At dosing
    - Post-dose monitoring
  - Extensive immunophenotyping
    - At dosing
    - Post-dose monitoring
    - Distribution/balance of naïve, central memory and effector memory T-cells populations
    - Activation, Exhaustion/Senescence

The duration of detectable CAR T cell may correlate with event free survival

Heterogenous and dynamic populations of living cells

What to Measure- Target Population

- Efficacy
  - Measurable Residual Disease (MRB) (needs to be developed and validated for rare events)
  - Duration of disease-free survival
- B cell hematological malignancy e.g. B-Cell Acute Lymphoblastic Leukemia (B-ALL)
  - Phenotyping
  - ✓ Baseline tumor burden
  - ✓ Expression level of CAR Antigen e.g. CD19 (Quantitative Flow Cytometry)

✓ Thought to impact efficacy

What to Measure- Endogenous Immune Cells

- T cells
  - Distribution/balance of naïve, central memory and effector memory
  - Activation, Exhaustion/Senescence
- Regulatory Cells
  - Treg
  - Myeloid derived suppressor cells (MDSC)

Simultaneous monitoring the endogenous counter parts can serve as a biological control

Endogenous immune factors are thought to impact efficacy

How to Measure- Flow Cytometry

- Multi-parametric Flow Cytometry
  - Conventional
  - Spectral
- Extensive Immunophenotyping
- Rare Events
- Quantitative Flow Cytometry
- Absolute Counts

The AAPS Journal (2021) 23: 98
DOI: 10.1208/s12248-021-00633-6

Review Article

The Evolution of Single-Cell Analysis and Utility in Drug Development

Shibani Mitra-Kaushik,[1] Anita Mehta-Damani,[2] Jennifer J. Stewart,[4] Cherie Green,[5] Virginia Litwin,[3] and Christèle Gonneau[6,7]

Flow Cytometry- The Basics

Fluidics

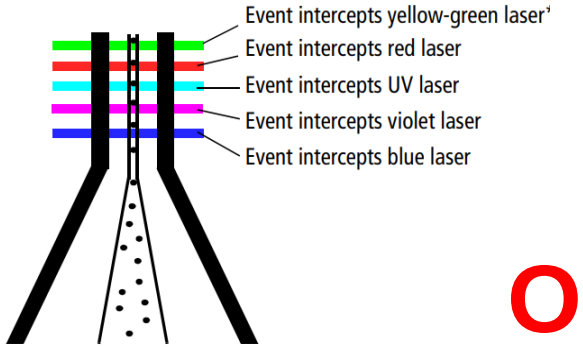
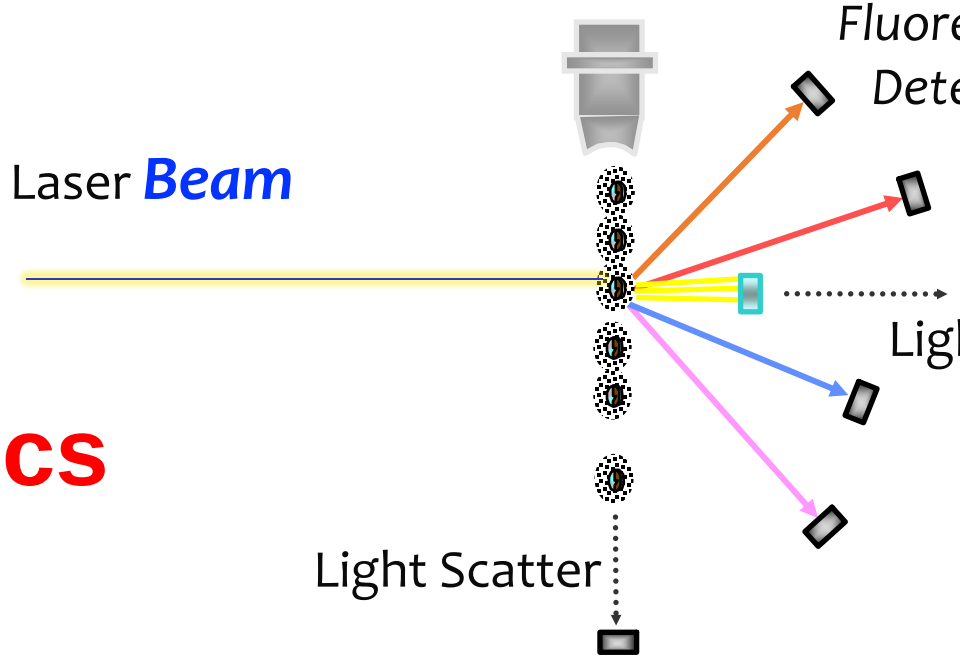


Figure from: Becton Dickinson Life Sciences

Optics

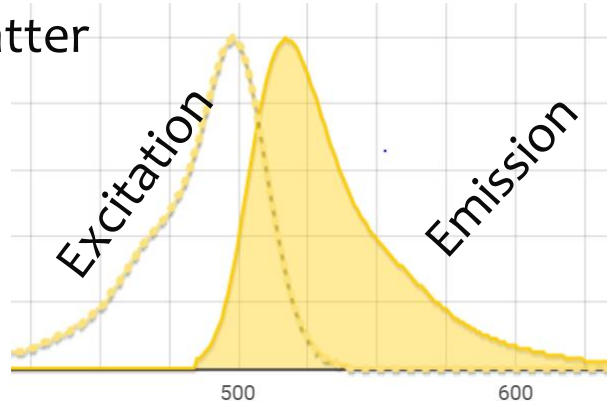
Stream

Laser ***Beam***



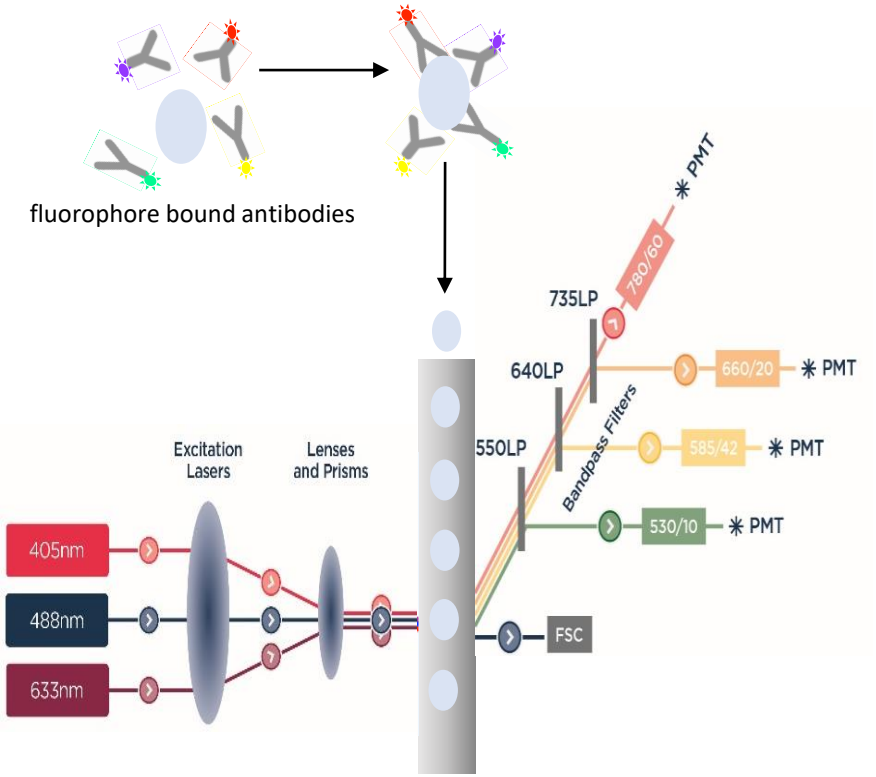
Electronics

Digitize Signal
Store



Conventional Flow Cytometry

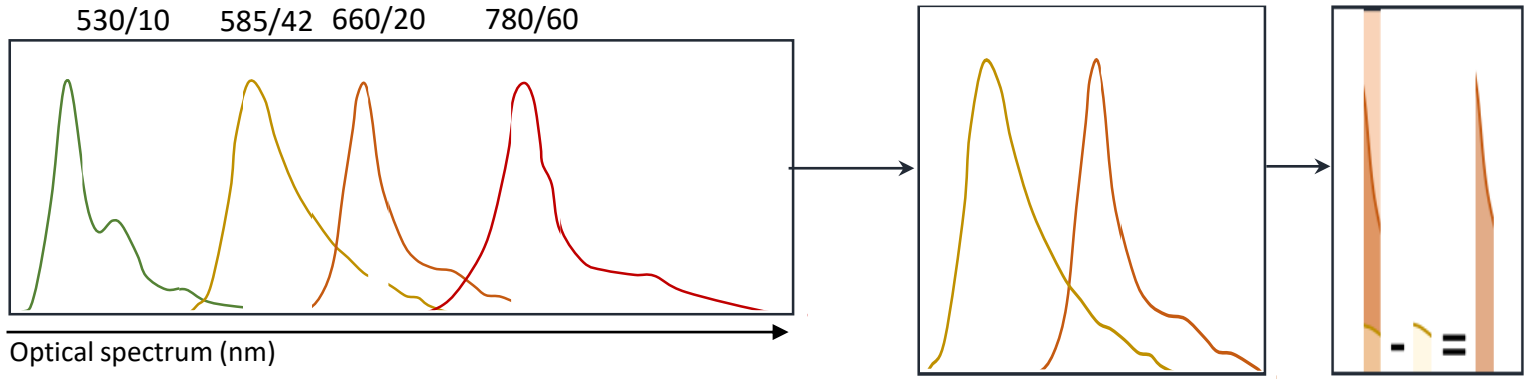
Pre-examination Phase (cell labeling)



Conventional Flow Cytometry Workflow

1 fluorophore = 1 detector

Examination Phase: Collection Optics

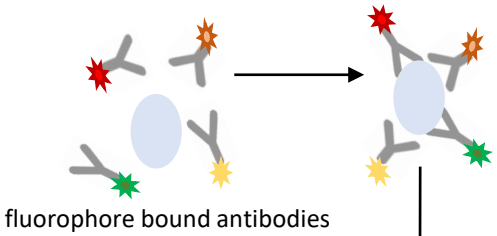


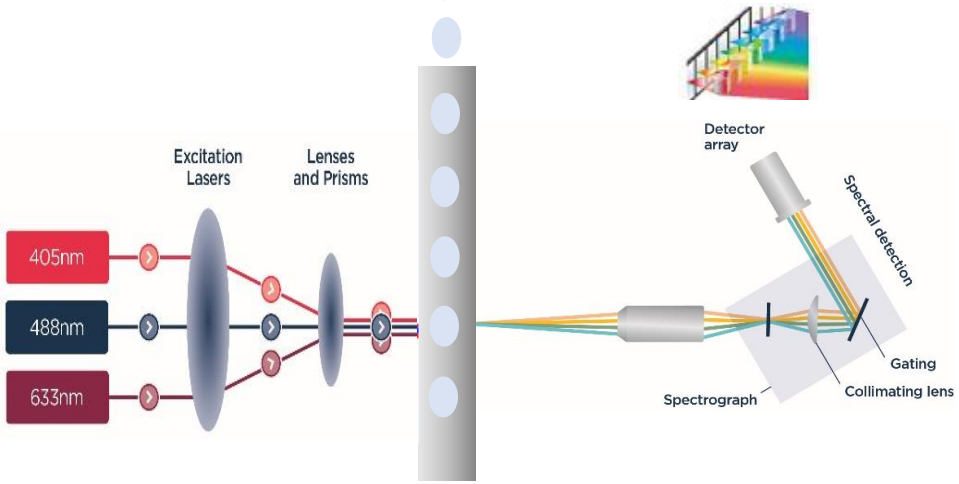
Examination Phase: Excitation/Emission Optics

Examination Phase: Spectral Overlap Compensation

Spectral Flow Cytometry

Pre-examination Phase (cell staining)





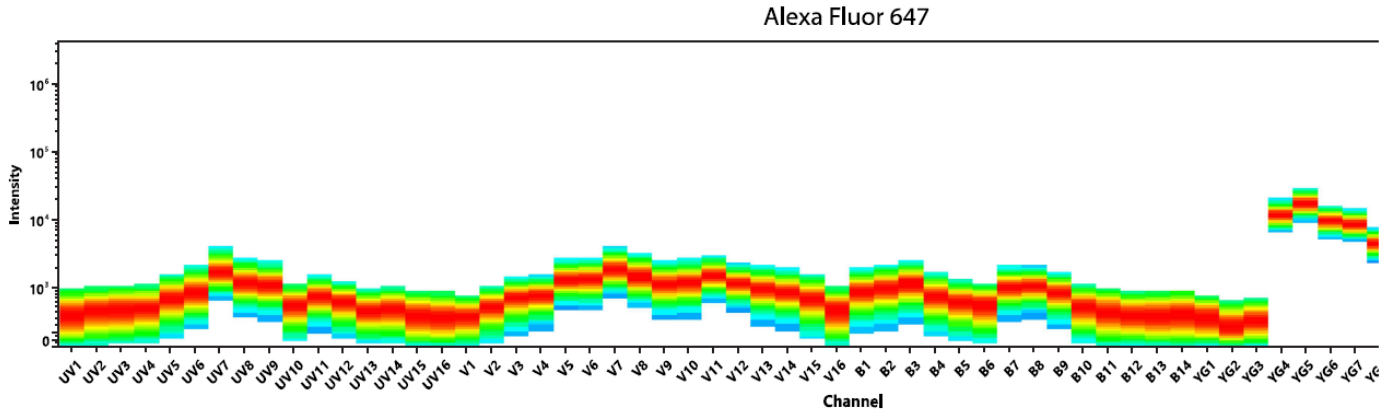
Examination Phase: Excitation/Emission Optics

Spectral Flow Cytometry Workflow

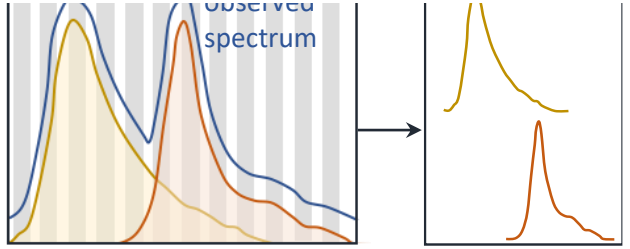
1 fluorophore = detector array

Signatures

Examination Phase: Collection Optics



Examination Phase: Spectral Unmixing



Why Spectral Flow Cytometry

- More Signal
- Less Noise

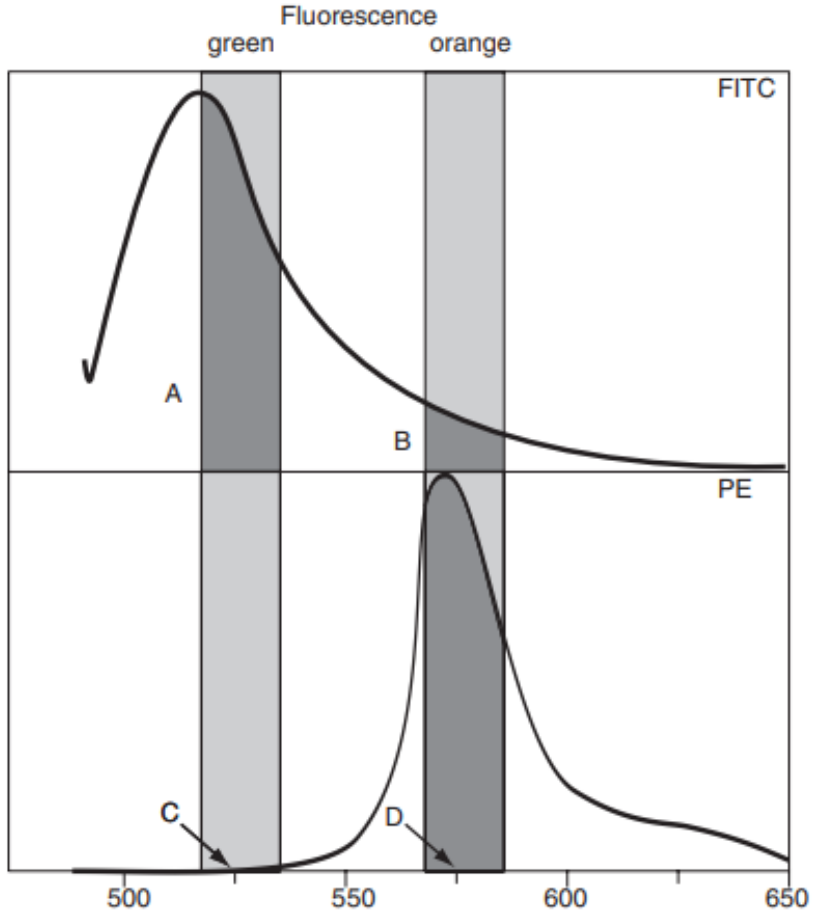
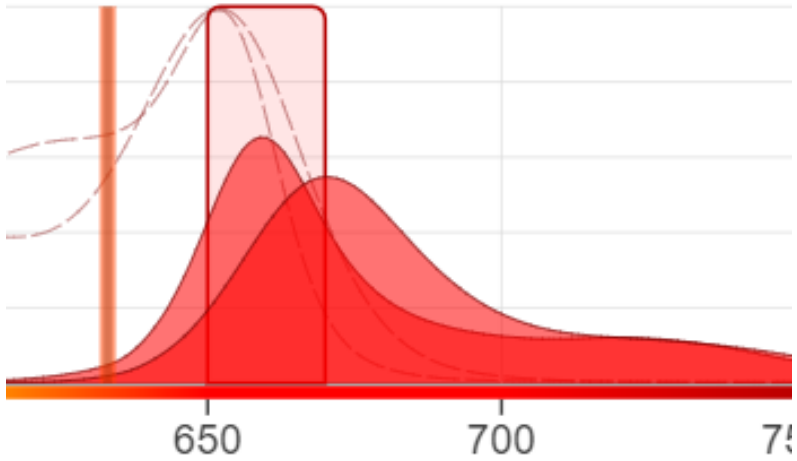
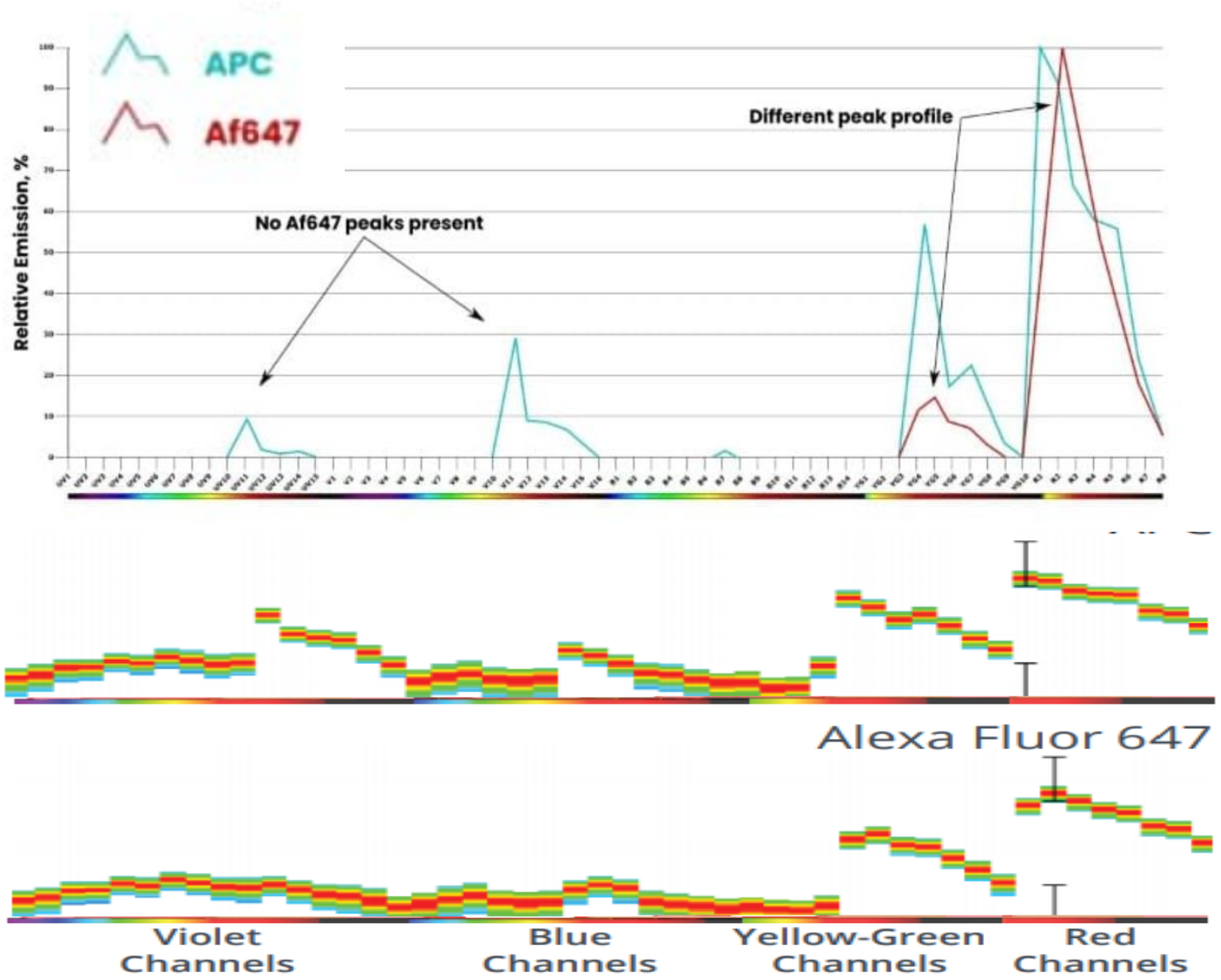


Figure from: Roederer, Current Protocols in Cytometry, 1.14.1, 2002

Why Spectral Flow Cytometry

- Enables higher parameter detection
- Allows us to use fluorophore together which cannot be used together in conventional cytometry





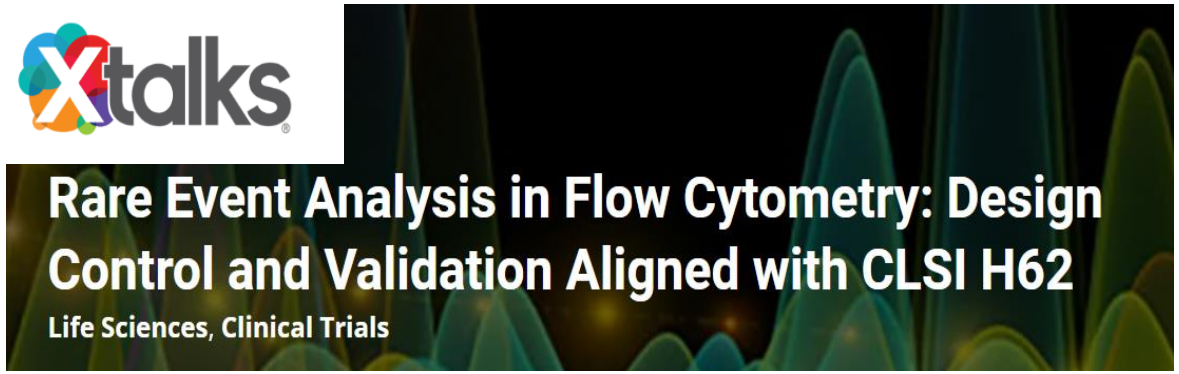
Rare Event- Flow Cytometry

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

High-sensitivity flow cytometric assays: Considerations for design control and analytical validation for identification of Rare events

Ulrike Sommer[1] | Steven Eck[2] | Laura Marszalek[3] | Jennifer J. Stewart[4] | Jolene Bradford[5] | Thomas W. McCloskey[6] | Cherie Green[7] | Alessandra Vitaliti[1] | Teri Oldaker[8] | Virginia Litwin[9]



- Highly specific
  - Positive and negative selection markers
  - Correct phenotype
  - No debris or dead cells
- Highly sensitive
  - Acquire as sufficient number of cells so that there are a minimum of 20-50 events in the gate of interest
- Validate Detection Capabilities
  - LoB, LoD, LLoQ
  - Per CLSI H62

Quantitative- Flow Cytometry

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Standardization of flow cytometric detection of antigen expression

Linhua Tian[1] | Aaron R. Nelson[2] | Tyler Lowe[2] | Linda Weaver[2] | Constance Yuan[2] | Hao-Wei Wang[2] | Paul DeRose[1] | Maryalice Stetler-Stevenson[2] | Lili Wang[1]

- Antigen quantification is vital in managing patients receiving antigen-based immunotherapy
- Antigen-based immunotherapy relies upon the level of tumor antigen expression
- Expression should not be reported as fluorescence intensity
  - Arbitrary units
- Fluorescent calibration allows measured fluorescence signals to be converted to the ABC values
- Antigen quantification requires methods for quality control and for interlaboratory and inter-cytometer platform standardization

CAR T-cell Detection by Flow Cytometry

- Direct Staining
  - CAR specific
    - CAR antigen directly conjugated to fluor
    - Anti-idiotype mAb directly conjugated to fluor
  - Generic CAR Binding
    - Anti-Fab binding
    - Anti-Kapp Light chain binding
    - Protein L
- Indirect Staining
  - Biotin CAR Antigen/ SA-fluorophore
  - His-Tag CAR Antigen/ anti-His-fluorophore
  - Generic CAR Binding

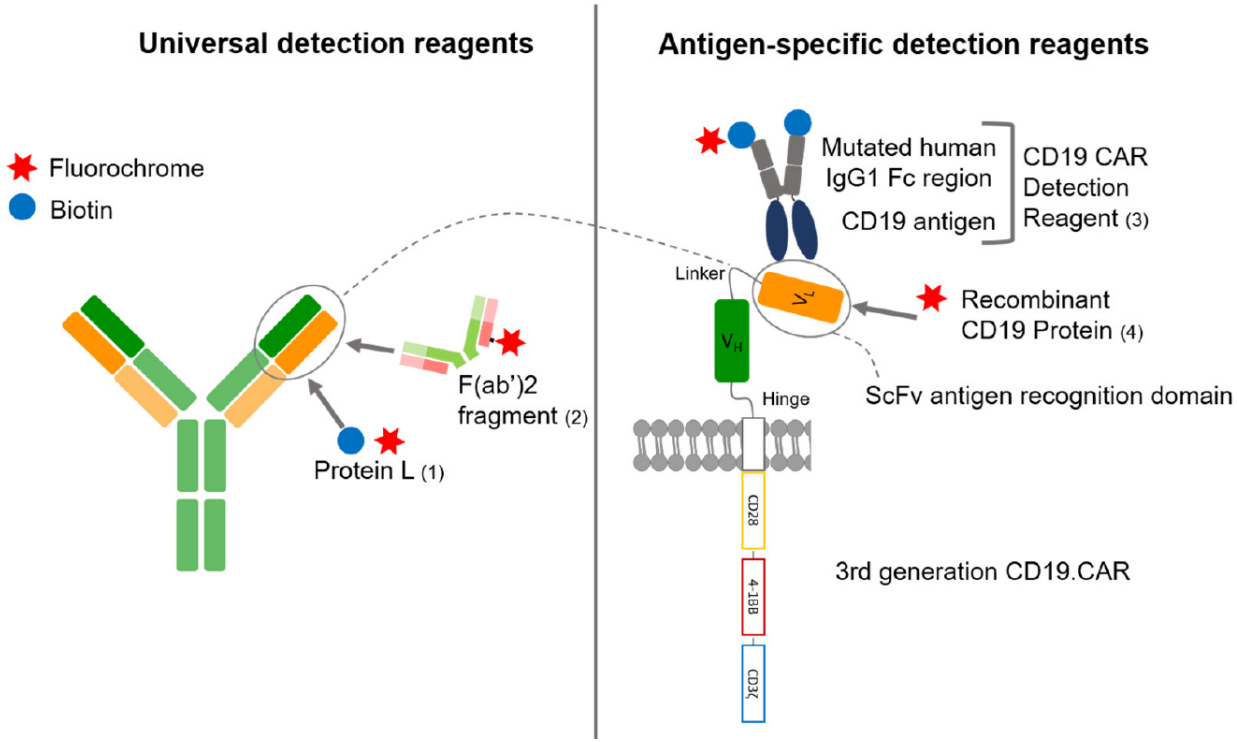


Figure from Schanda N, et al. CAR-T cell detection by flow cytometry and PCR. Cells. 2021 Nov 17;10(11):3208.

Absolute Counts by Flow Cytometry

- "Lyse/No Wash" Staining Method
- Staining is conducted in whole blood or bone marrow
  - Directly conjugated mAb
- Followed by RBC lysis step
- Directly acquired on the flow cytometer

- Quantitation is achieved when the staining is conducted in the presence of quantitation beads or when samples are acquired on an instrument which is capable of volumetric measurements

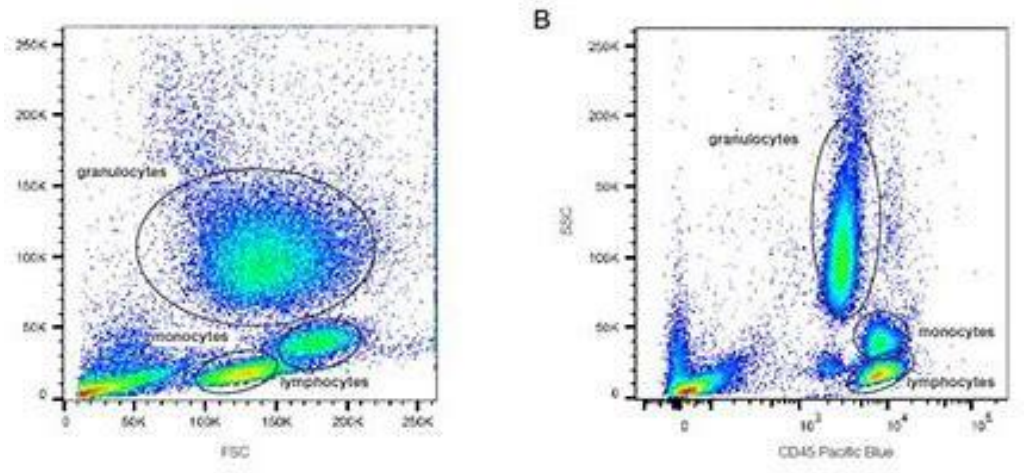
$$Cell\ abs\ count = (\#events\ in\ cell\ gate\ \div \#\ events\ in\ bead\ gate) \times (bead\ conc\ \div staining\ volume)$$

- It is not accurate to include any wash or centrifugation steps when reporting the absolute counts
- If your CAR T-cell detection assay includes a wash step, you must include a Lyse/No Wash tube for the absolute counts

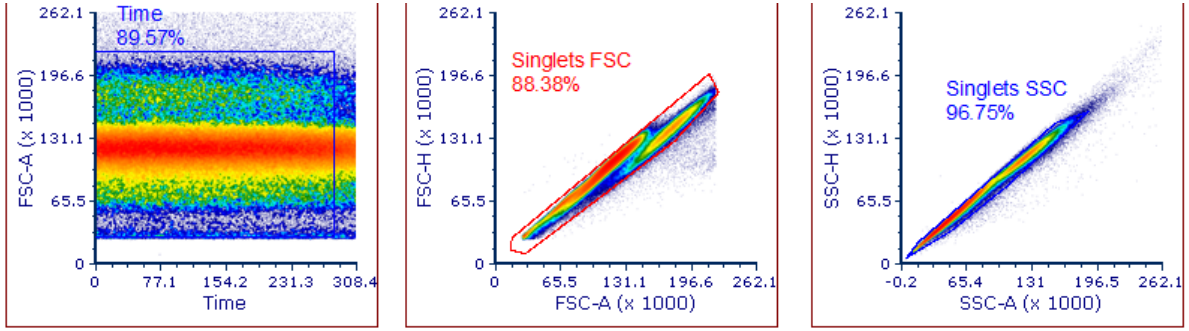
Absolute Counts by Flow Cytometry

Best Practice Recommendations (assuming a lymphocyte subset)

- Include additional markers to ensure lymphocyte gate purity
  - **CD45** lymphocytes are CD45 bright and side scatter low
  - **CD14** will exclude monocytes from the lymphocyte gate. Often in shipped samples and patient samples the monocytes degranulate and contaminate the lymphocyte gate.
  - **CD253a.** This marker will help us exclude poorly lysed RBC from the lymphocyte gate
  - **CD3** if using for CAR T-cell



- Follow best practices to ensure single cell detection
  - Time gate to exclude fluids instability
  - Forward and side scatter doublet discrimination



Absolute Counts by Flow Cytometry

Why Can't I Just Use TBNK? It's IVD/CE Marked!

- COU is not correct
  - Designed to determine if major lymphocyte subsets are with normal ranges
  - Low CD4 counts indicates immune deficiency
    - Hallmark of HIV infection
  - High CD19 counts indicates B cell malignancy
- Not designed for Rare Events
  - Error message is 2,500 lymphocyte events are not collected
  - All samples from lymphodepleted patients would give error messages
- Does not incorporated technological advances
  - No time gate
  - No doublet discrimination
- Could be more specific
  - No CD14, Not CD235a

CAR T-cell Absolute Counts by Flow Cytometry

Best Practice Recommendations (assuming a two-tube assay)

- The CAR T-cell phenotyping tube must include (Lyse/Wash)
  - All antigens used in the counting tube (Lyse/No Wash)
  - The same upstream gating used in the counting tube

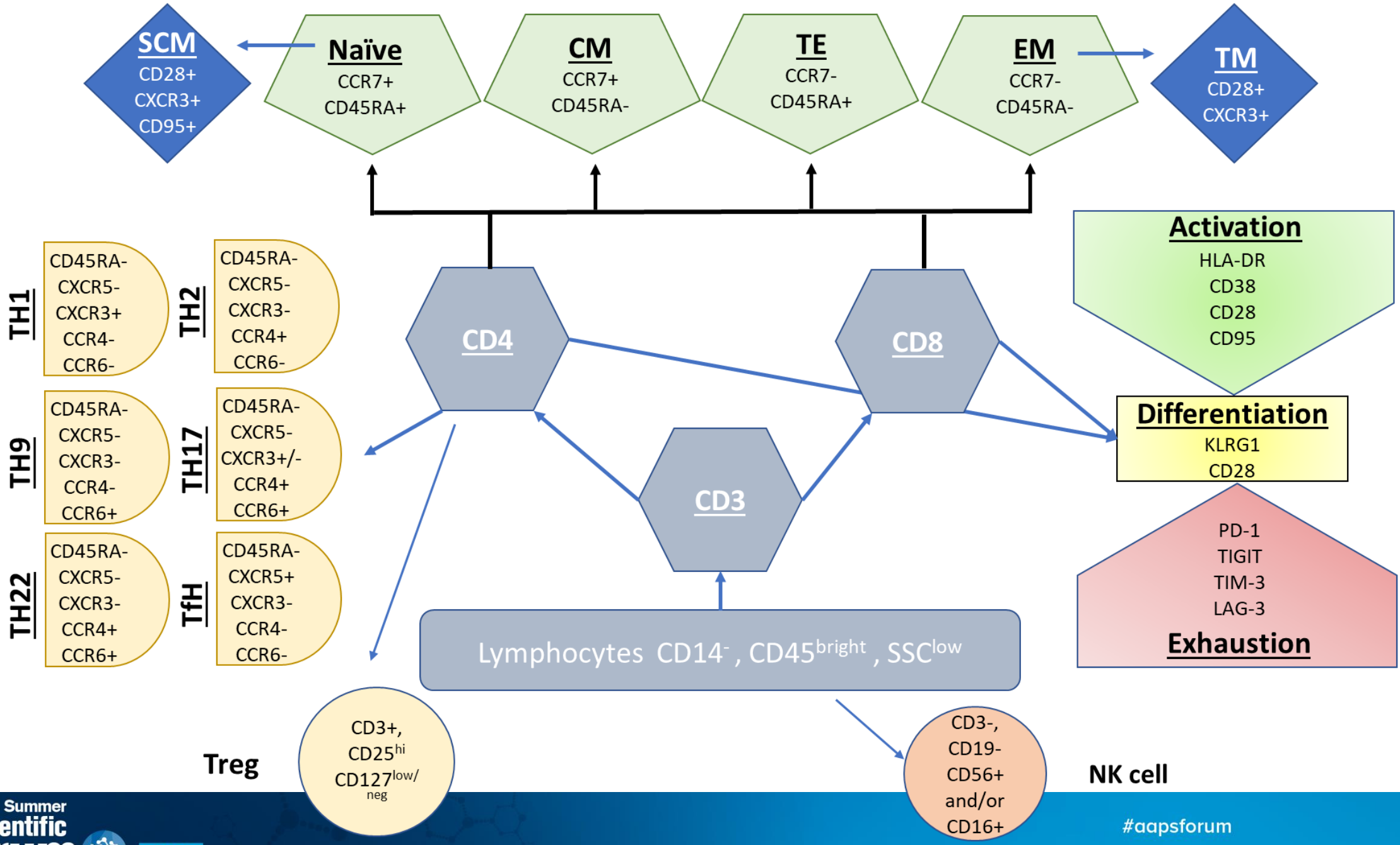
$$CAR\ T\ cell\ \ abs\ count = \%\ CD3\ Tcells\ expressing\ CAR\ Ag\ from\ Lyse\ Wash\ assay \times CD3\ abs\ count\ from\ Lyse\ No\ Wash\ Assay$$

Why Do We Need So Many Markers?

Benefit of increased dimensionality

- Provides a deeper understanding
  - of each cell's function
  - the overall biology of the immune system
- Adds to the specificity of the population identified
  - Current, accepted phenotype
  - Negative and positive selection markers
- Enables a deeper understanding of the impact of novel therapies
  - on the immune system
  - the targeted disease
- Increased the efficiency of sample usage
  - Important in clinical trials to decrease specimen collection volumes per patient visit

25 Marker T cell Panel



S.No	Antigen	Purpose
1	CD127	Treg surface staining
2	CD14	Lineage (monocyte, lymph gate purity)
3	CD16	NK cells
4	CD183 (CXCR3)	TH subsets
5	CD185 (CXCR5)	TH subsets
6	CD19	Lineage (B cell, NK gate purity)
7	CD194 (CCR4)	TH subsets
8	CD196 (CCR6)	TH subsets
9	CD197 (CCR7)	Differentiation
10	CD223 (LAG-3)	Co-inhibitory
11	CD25	Treg surface staining
12	CD279 (PD1)	T cell exhaustion
13	CD28	Differentiation
14	CD3	Lineage
15	CD366 (TIM-3)	Co-inhibitory
16	CD38	Activation marker
17	CD4	Lineage
18	CD45	Pan leucocyte
19	CD45RA	Differentiation
20	CD56	NK cells
21	CD8	Lineage
22	CD95	Differentiation
23	HLA-DR	Activation marker
24	KLRG1	Senescence
25	TIGIT	Co-inhibitory

- 25-marker T cell panel
- Add 235a
- Add B-CLL MRD
- MDSC
- Add CAR detection
- →42 markers

CAR Ag Detection	Unlysed RBC, lymph gate purity	B-ALL MRD	MDSC
???	CD235a	CD34	CD11b
		CD10	CD14
		CD123	CD15
		CD19	CD33
		CD20	CD66b
		CD22	CD84
		CD304	CXCR1
		CD38	HLA-DR
		CD45	
		CD66c	
		CD73	
		CD81	

S.No	Antigen	Purpose
1	CD127	Treg surface staining
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3	CD16	NK cells
~~4~~	~~CD183 (CXCR3)~~	~~TH subsets~~
~~5~~	~~CD185 (CXCR5)~~	~~TH subsets~~
6	CD19	Lineage (B cell, NK gate purity)
7	~~CD194 (CCR4)~~	~~TH subsets~~
8	~~CD196 (CCR6)~~	~~TH subsets~~
9	CD197 (CCR7)	Differentiation
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14	CD3	Lineage
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17	CD4	Lineage
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20	CD56	NK cells
21	CD8	Lineage
22	CD95	Differentiation
23	HLA-DR	Activation marker
24	KLRG1	Senescence
25	TIGIT	Co-inhibitory

- 25-marker T cell panel
- Remove Th subset
- Add 235a
- Add B-CLL MRD
- MDSC
- Add CAR detection
- →38 markers

CAR Ag Detection	Unlysed RBC, lymph gate purity	B-ALL MRD	MDSC
???	CD235a	CD34	CD11b
		CD10	CD14
		CD123	CD15
		CD19	CD33
		CD20	CD66b
		CD22	CD84
		CD304	CXCR1
		CD38	HLA-DR
		CD45	
		CD66c	
		CD73	
		CD81	

How High Can We Go?

Cytometry PART A
Journal of Quantitative Cell Science
ISAC

OMIP | Open Access

OMIP-102: 50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells

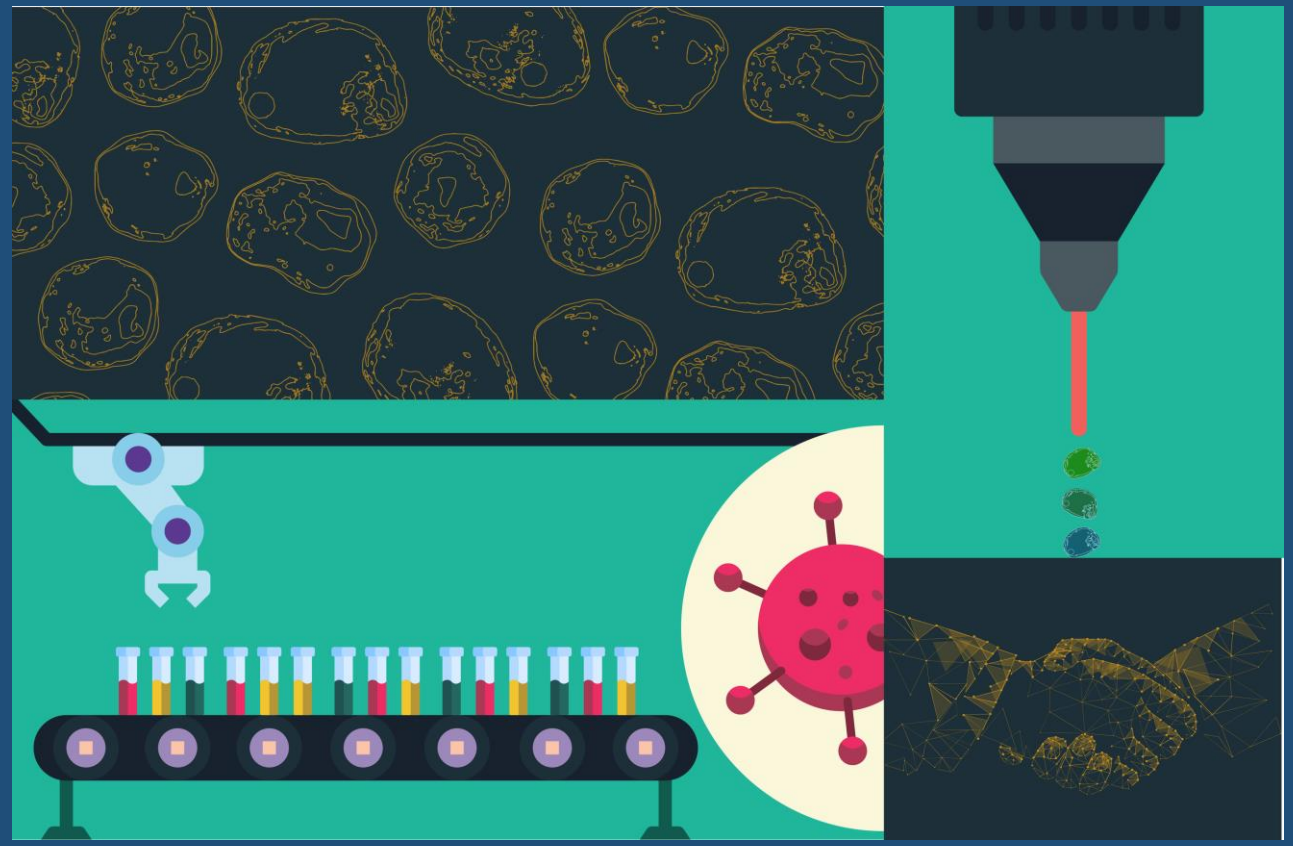
Andrew J. Konecny, Peter L. Mage, Aaron J. Tyznik, Martin Prlic, Florian Mair

First published: 18 April 2024 | https://doi.org/10.1002/cyto.a.24841

NIST Flow Cytometry Standards Consortium

What is NIST?

What is the constortium?



The National Institute of Standards and Technology is a physical sciences laboratory and a non-regulatory agency of the United States Department of Commerce

Its mission is to promote innovation and industrial competitiveness

NIST Flow Cytometry Standards Consortium

- Born out of H.R. 34, the **21st Century Cures Act** in the United States
  - In 2016, became Public Law No. 114-255
  - Aims to accelerate the discovery, development, and delivery of new medicines and medical technologies
- Title III of the Act
  - Addresses the standards for regenerative medicine and regenerative advanced therapies (RMAT)
  - Focuses on drug and device development
  - **Directs HHS (FDA), in consultation with NIST and stakeholders**
    - to facilitate efforts around development of standards for regenerative medicine therapies and advanced therapies

Directive

NIST and FDA jointly hosted three workshops to identify measurement gaps/needs and possible solutions

- cell counting nist-fda-cell-counting-workshop
- flow cytometry nist-fda-flow-cytometry-workshop-building-measurement-assurance-flow
- genome editing nist-fda-genome-editing-workshop

Outcomes

- ISO standard document on cell counting, ISO 20391-1:2018 Biotechnology -- Cell counting -- Part 1
- General guidance on cell counting methods https://www.iso.org/standard/68879.html
- Reference Standards Initiative
- **Flow Cytometry Standards Consortium**

A public-private partnership through cost sharing to address the measurements and standards needed to increase confidence and comparability of flow cytometry data in research and commercial products

NIST Flow Cytometry Standards Consortium

MISSION

Convene stakeholders in the pre-competitive space to accelerate the adoption of quantitative flow cytometry in biomanufacturing of cell and gene therapies

Why?

- Why a Consortium?
  - The challenge requires a **coordinated response** with significant input from the stakeholder community
  - **Lessens risks** being placed on any single entity
  - Helps **develop consensus**
  - **Leverages subject matter expertise** from the stakeholders

- Why NIST?
  - **Non-regulatory agency** of the U.S. Department of Commerce
  - **Neutral convener** for industry consortia, standards development organizations, federal laboratories, universities, public workshops, and interlaboratory comparability testing
  - **Cross-disciplinary expertise** in engineering and the physical, information, chemical, and biological sciences

Consortium Outcomes

- Develop standards and measurement assurance tools to enhance flow cytometry measurement confidence.
- Generate data from interlaboratory studies to support development of best practices and standard methods.
- Improve flow cytometry measurement capabilities

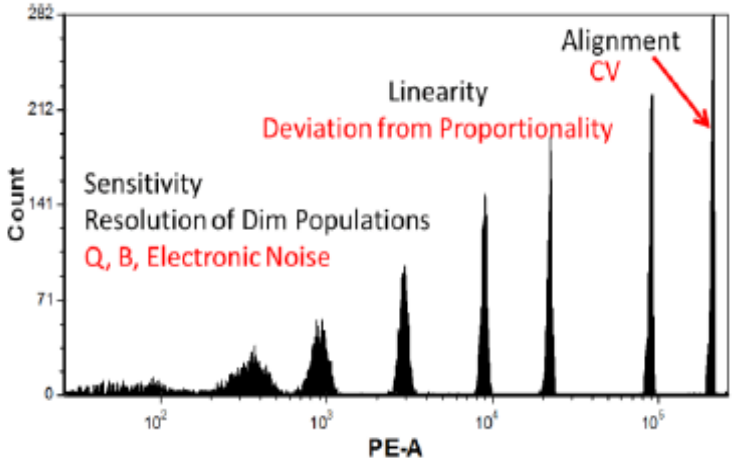
Progress

- Approved- October 2020
- Kick-off - Open Workshop- February 2021
- https://www.nist.gov/news-events/events/2021/02/flow-cytometry-standards-consortium-workshop
- Formed working groups
  - WG1- Paul de Rose
  - WG2- Lili Wang
  - WG3- John Elliot

WG1

Paul de Rose

Develop reference standards including reference materials, reference data, reference methods, and measurement service for assigning the Equivalent Number of Reference Fluorophores (ERF) to calibration microspheres and assessing the associated uncertainties and utilities, and drive cytometer standardization



WG1 Interlaboratory Study

Title

Equivalent Reference Fluorophore (ERF) Beads Variability and Assignment of Unknowns

Purpose

To evaluate the variability of the ERF calibration scale between bead sets, instruments and laboratories

Scope

Measure the mean fluorescence intensities for four different ERF-assigned bead sets from four different vendors and for a set of fluorescently-labeled PBMCs under the same gain settings for each fluorescence channel

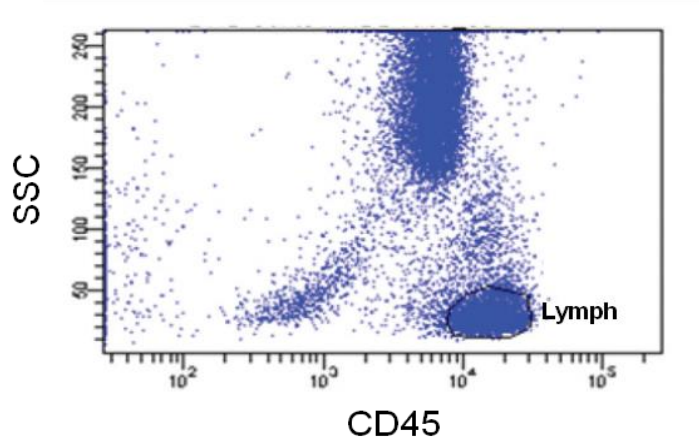
WG2

Lili Wang

Design and conduct interlaboratory studies for the development of reference standards, control materials, and best practice protocols to achieve assay standardization and reference data generating

ILS-1 TBMNK Assay
19 Affiliations
24 Locations
50 Instruments

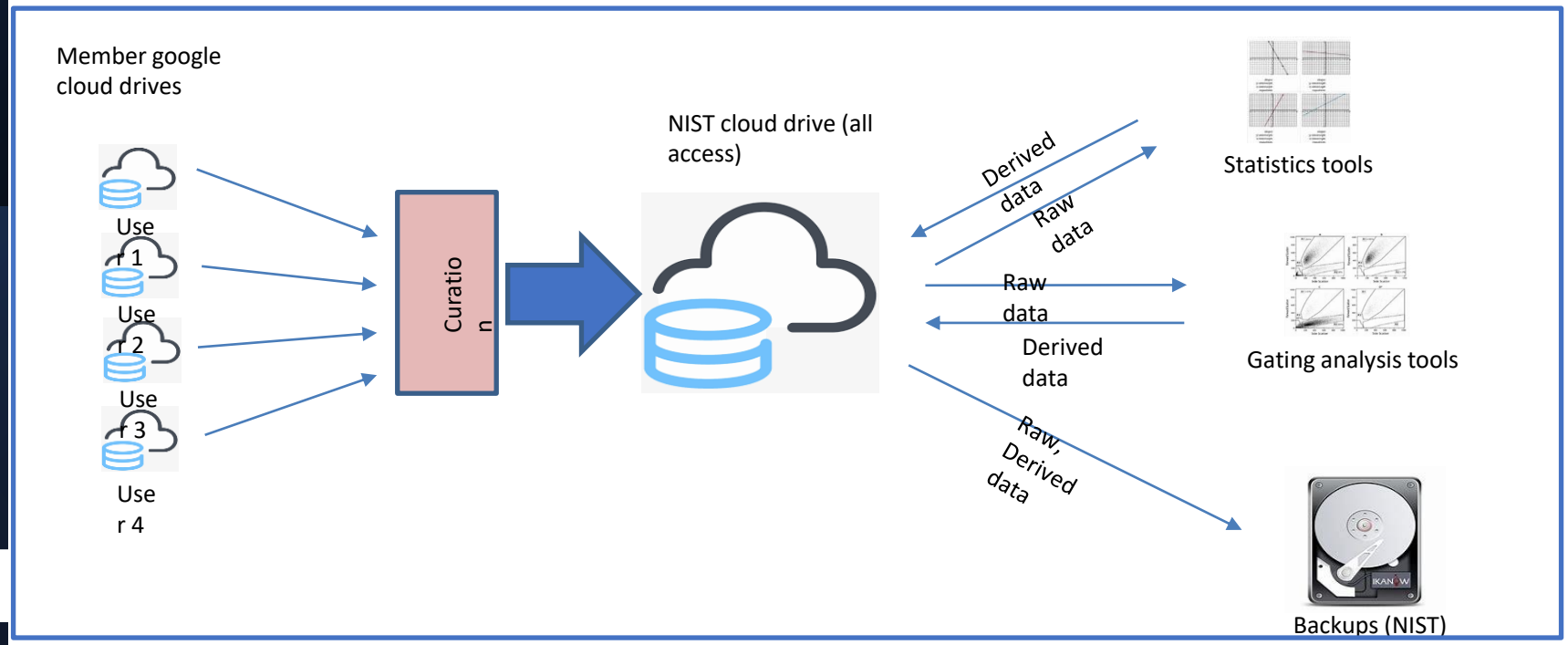
ILS-2 CAR T-cell Assays



WG3

John Elliot

Build infrastructure and capability for flow cytometry data repository and multi-modal simultaneous data analysis



WG3 Data Repository and Analysis Pipeline

- Leverage experience of members
- Ensure compatibility with analysis and statistics pipelines
- Consensus on filename scheme, directory structure, etc.
- Ensure linkages between metadata and raw flow cytometry data

How to Join this Consortium

- Visit the NIST Website
- Contact Lili Wang

Save the Date!!!!

CYTO 2025

Denver, CO

May 31- June 4

ISAC

INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

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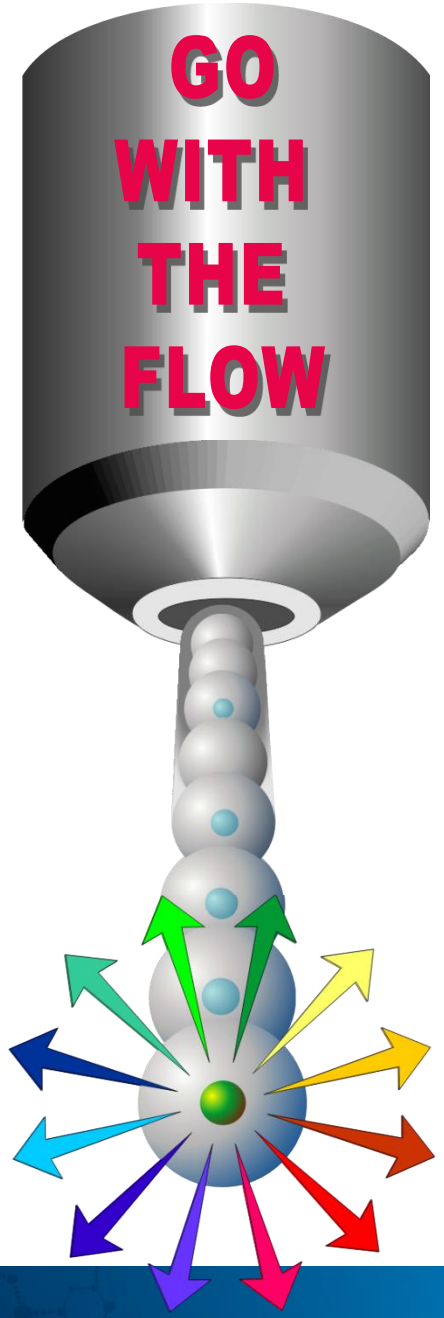

Figure courtesy of Ira Schieren, Columbia University

Questions