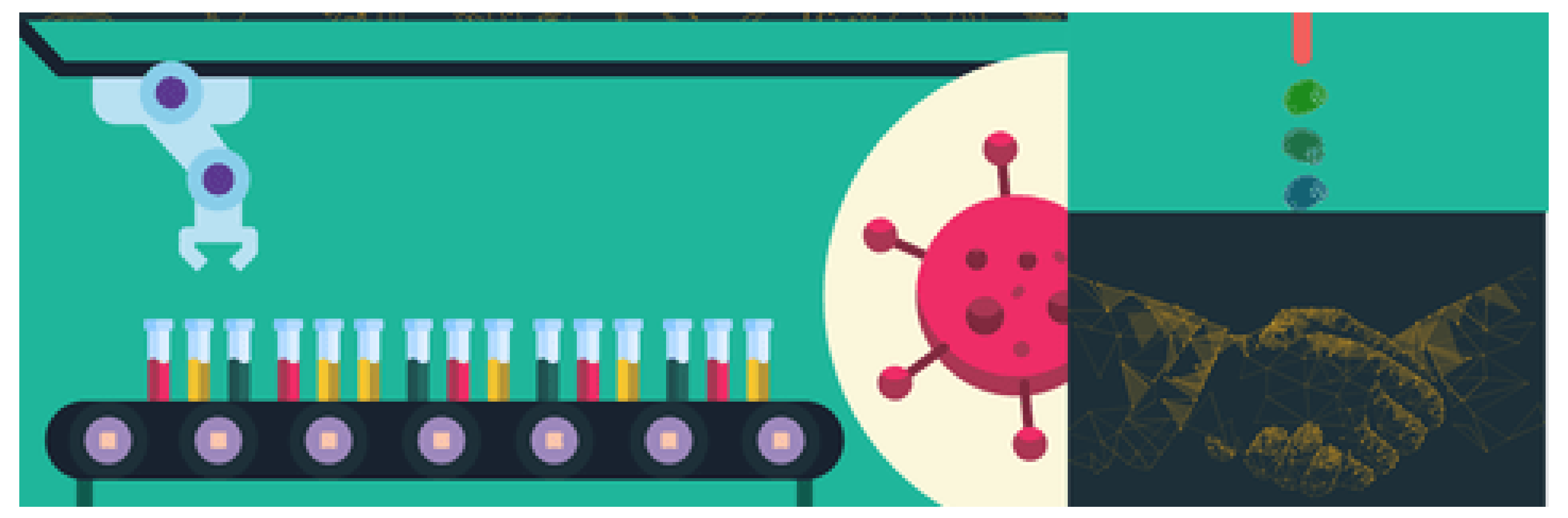


Flow Cytometry Standards Consortium Workshop Day Two

Sumona Sarkar

NIST, Biosystems and Biomaterials Division

Feb 17, 2021

Workshop Goals

- Launch and inform future directions of the Flow Cytometry Standards Consortium.
- State-of-the-art in flow cytometry applications
- Identify pressing measurement challenges and standards needs
- Provide initial feedback on the proposed directions of the Consortium.

Workshop Day-One Summary

- Overview of NIST and the NIST flow cytometry program
- FDA’s perspectives on standardization of flow cytometry
- Overview of existing standards efforts: documentary and reference materials
- Use Cases where flow cytometry was a crucial measurement technique
- Challenges, needs, gaps, and opportunities

Highlights of Needs and Challenges

- Flow cytometry is an important mode of measurement for (e.g.)
  - Cell therapy product characterization and testing (e.g. identity, purity, potency)
  - Detection and evaluation of engineered T cells before and after infusion
  - Patient screening/starting cell population characterization
  - *In vitro* diagnostics

- Challenges:
  - Instrument variability – between manufacturers, between replicate instruments
  - Gate setting ambiguity – too dependent on instrument/instrument settings
  - Sample handling and preparation
  - Representative samples for flow cytometry method development – transitioning from healthy donor samples to diseased state
  - Measurement quality for rare events – FACS and molecular methods don't always agree
  - Accurate cell counts – to support product dosing

Highlights of Gaps and Opportunities

- Reference materials for instrument-to-instrument calibration
- Reference materials that represent “clean” and “dirty” samples for validation
- Reference materials for specific cell types (e.g. MSCs)

- Guidelines for data interpretation across flow platforms
- Guidelines on sample preparation
- Guidelines for panel optimization and antibody titration
- Guidelines for instrument qualification and performance checks

Agenda – Day 2

Wednesday, February 17, 2021 | 12:00 to 4:00 pm ET

12:00 PM – 12:10 PM	Workshop Day-One Summary and Goal setting for Day-Two
12:10 PM – 1:10 PM	NIST Flow Cytometry Standards Consortium and Working Group objectives
1:10 PM – 1:20 PM	Break
1:20 PM – 2:50 PM	Technology/Capability Showcase of Companies
2:50 PM – 3:50 PM	Consortium Strategy, Including Near-term and Long-term Goals of the Consortium/Working Groups
3:50 PM – 3:55 PM	Next steps and Closing Remarks
3:55 PM	Adjourn for the day

Last Session (2:50-3:50pm): Consortium Strategy, Including Near-term and Long-term Goals of the Consortium/ Working Groups

- Presenters, please join back into the presenter's link to participate in a live discussion
- Attendees, Please share your ideas in the Q/A on:
  - Activities for the Flow Cytometry Standards Consortium
  - Wish list of tools and strategies to improve flow cytometry measurements
  - Additional challenges

NIST Flow Cytometry Standards Consortium
- Reference Materials and Reagents

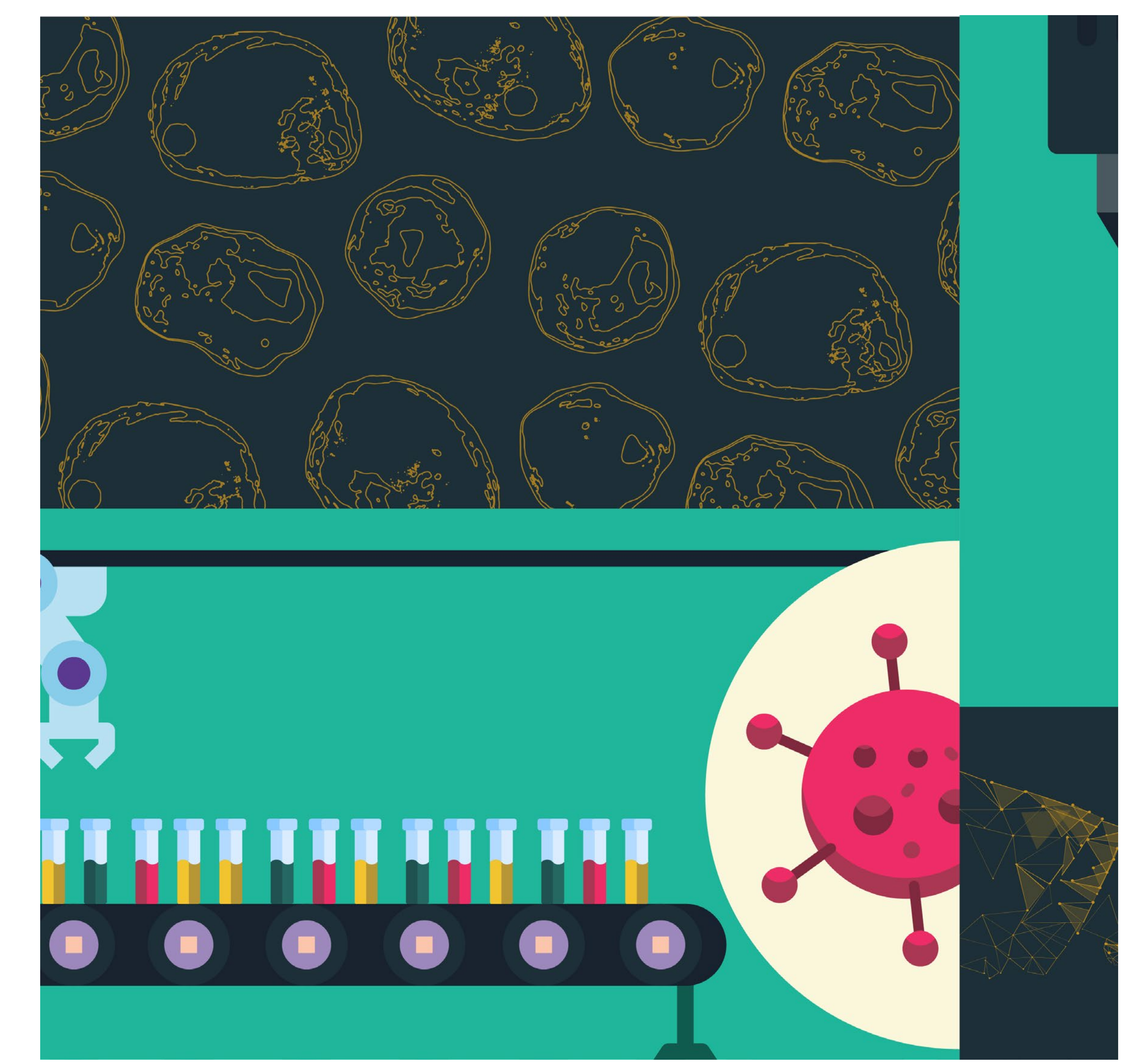
Lili Wang, Biosystems and Biomaterials Division, NIST

Flow Cytometry Standards Consortium

The consortium will develop measurement solutions and standards for flow cytometry, including improving measurement confidence by establishing traceability and assisting measurement comparability.

Measurement applications to be addressed include the use of flow cytometry for the characterizing and testing cell identity, purity, health, count, potency, biomarker expression, and associated critical reagents and starting materials for the cell and gene therapy products.

https://www.nist.gov/programs-projects/nist-flow-cytometry-standards-consortium



NIST-FDA Flow Cytometry Workshop: Building Measurement Assurance in Flow Cytometry

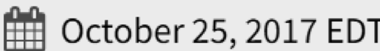
NIST Organizers: Lili Wang, John Elliott, Sheng Lin-Gibson FDA Organizers: Steven Bauer, Heba Degheidy, Judy Arcidiacono

NIST and FDA are actively collaborating on projects that address regulatory and measurement challenges for cell therapies and regenerative medicine products. These collaborations leverage NIST expertise in measurement sciences to address specific analytical challenges as well as FDA regulatory science, research and review expertise in these advanced application areas to ensure that the science and standards developed address significant regulatory challenges that recur across the field. Building on the success of the previous NIST-FDA Cell Counting Workshop, NIST and FDA plan to host a Workshop to examine measurement challenges associated with flow cytometry.

Flow cytometry is a powerful tool for cell characterization and function analysis. Despite wide use of flow cytometry, the challenges associated with robust cellular measurands, the lack of adequate biological and non-biological reference materials, and the complexity of the instrumentation have resulted in few standards to address measurement assurance. This workshop will identify application challenges in clinical and cell manufacturing settings, and potential solutions for overcoming gaps in obtaining sufficient measurement assurance for flow cytometry. The expected outcome of the workshop is a Whitepaper to be published in a peer reviewed journal as well as input into standards and best practices for quantitative, comparable flow cytometry.

Building Measurement Assurance in Flow Cytometry. Cytometry Part A, 95A, 626-630 (2019)

WORKSHOP


October 25, 2017 EDT

NIST, 100 Bureau Drive, Gaithersburg, Md 20899 (Building 101 West Square)

Registration has closed.
All attendees must be pre-registered to gain entry to the NIST campus. Photo identification must be presented at the main gate to be admitted to the conference. International attendees are required to present a passport. Attendees must wear their conference badge at all times while on the campus. There is no on-site registration for meetings held at NIST.

Result of Workshop Survey

Result of Workshop Survey, Cont.

Pre-workshop Survey Results

Survey (23/25)

2) What will be the biggest help to aid the measurement assurance in flow cytometry?
(1/2)

a. Common control material(s) — 20 %

b. Reference standard(s) — 24 %

c. Measurement procedure(s) — 4 %

d. Data reporting requirements — 4 %

e. Improved cytometer performance — 7 %

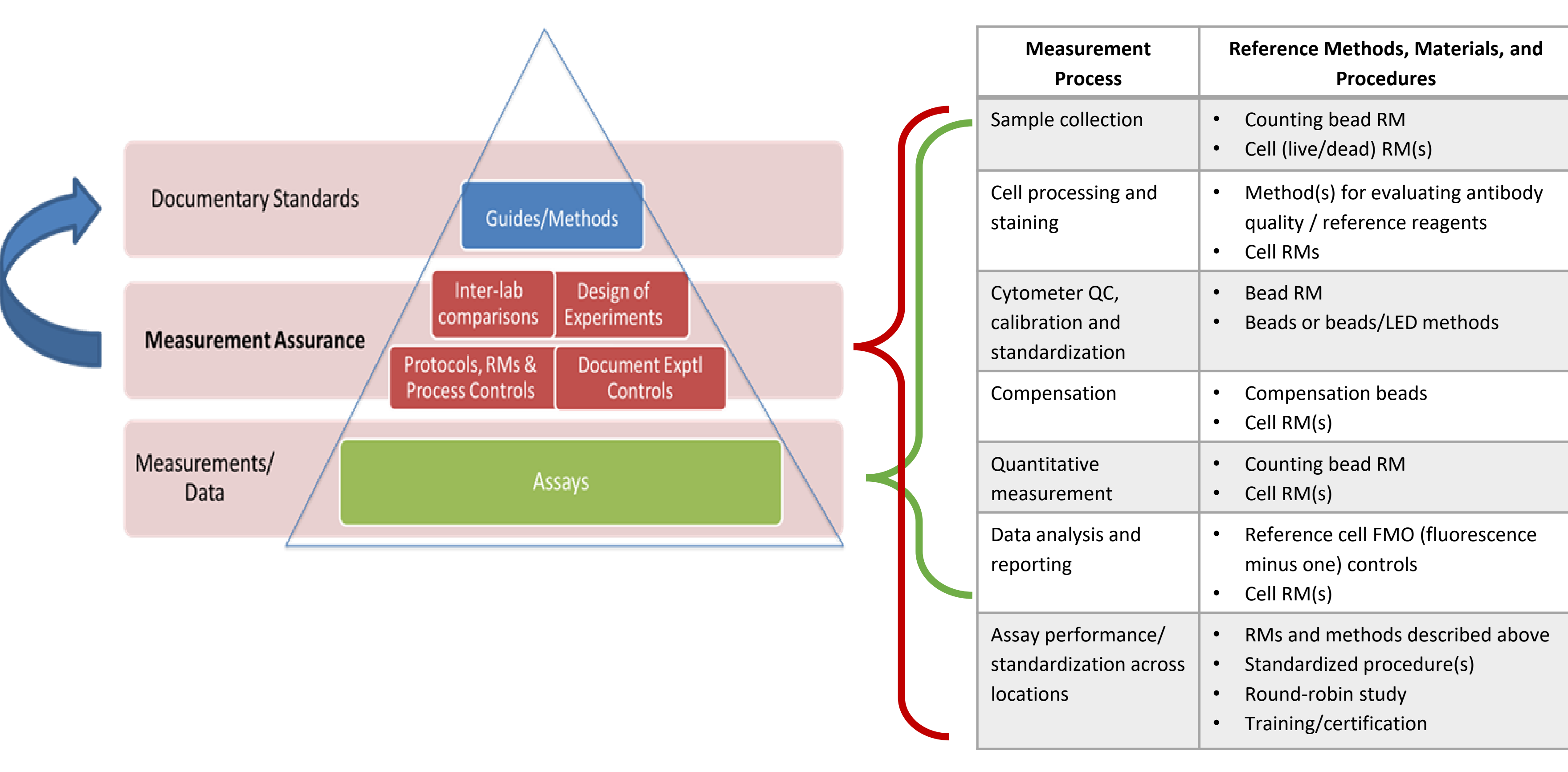
f. Combination of the above or other, please specify on next screen: — 42 %

Reference Materials, Methods, and Procedures for Quantitative Flow Cytometry

Building Measurement Assurance in Flow Cytometry. Cytometry Part A, 95A, 626-630 (2019)

Measurement Process	Sources of Variability	Reference Methods, Materials, and Procedures
Sample collection	• Fixed vs. fresh samples • Anticoagulant • Cell count and viability • Cell debris	• Counting bead reference • Cell (live/dead) reference control material(s)
Cell processing and staining	• Antibody quality: fluorophore labeling quality, binding affinity and titer • Cell debris	• Method(s) for evaluating antibody quality • Cell reference material(s)
Cytometer QC, calibration and standardization	• Linearity, sensitivity and resolution • Instrument threshold and voltage setting • Volumetric cytometers: volume calibration	• Bead reference materials • Beads or beads/LED methods
Compensation	• Linearity range • Choice of labeling fluorophores/panel design	• Compensation beads • Cell reference material(s)
Quantitative measurement	• Tube-to-tube variability of counting beads • Cell reference material(s) with known cell concentration and/or antigen expression • Assay format (single tube or separate tubes)	• Reference counting beads • Cell reference standard(s)
Data analysis and reporting	• Number of events collected • Population gating • Underlying assumptions of automated software	• Reference cell FMO (fluorescence minus one) controls • Cell reference standard(s)
Assay performance/ standardization across locations	• All issues described above • Different cytometer operators • Different assay procedures	• Reference materials and methods described above • Standardized procedure(s) • Round-robin study • Training/certification

Hierarchy for Development of Reference Materials (RM) and Documentary Standards



Measurement Process	Reference Methods, Materials, and Procedures
Sample collection	• Counting bead RM • Cell (live/dead) RM(s)
Cell processing and staining	• Method(s) for evaluating antibody quality / reference reagents • Cell RMs
Cytometer QC, calibration and standardization	• Bead RM • Beads or beads/LED methods
Compensation	• Compensation beads • Cell RM(s)
Quantitative measurement	• Counting bead RM • Cell RM(s)
Data analysis and reporting	• Reference cell FMO (fluorescence minus one) controls • Cell RM(s)
Assay performance/ standardization across locations	• RMs and methods described above • Standardized procedure(s) • Round-robin study • Training/certification

Cellular Assays on Flow Cytometry Devices Cleared by CDRH

Purpose	Measurand	'quantitative', 'qualitative' or 'semi-quantitative'	Standard used
Assessment of CMV-specific immune status and risk of CMV reactivation in immunosuppressed stem cell transplant recipients.	CMV specific CD8 MHC tetramer or dextramers	Quantitative	Beads
Immunologic assessment of patients having or suspected of having immune deficiency.	CD3+, CD3+CD4+, CD3+CD8+	Quantitative and Qualitative	Beads
monitor forms of immunodeficiency	CD3+CD4+, CD3+CD8+	Quantitative	Beads
Immunologic assessment of patients having or suspected of having immune deficiency.	CD3+CD4+, CD3+CD8+, CD3+, CD19+ and CD3-CD56+	Quantitative	Beads or on a dual platform
Immunologic assessment of patients having, or suspected of having, immune deficiency.	CD3+, CD3+CD4+, CD3+CD8+, CD3-CD19+, CD3-CD56+ and/or CD16+, CD45+ Low SS, and CD45+	Qualitative and quantitative	Beads or on a dual platform
HIV positive patients	CD4/Hgb	Quantitative	CD4 count/volume of the imaged field of views
Multiparameter immunophenotyping aid in the differential diagnosis of hematopoietic neoplasms	T1: CD2, CD56, CD7, CD5, CD45 T2: CD8, CD4, CD3, CD45 B1: Kappa, Lambda, CD19, CD5, CD45 B2: CD20, CD10, CD19, CD38, CD45 M: CD7, CD13, CD34, CD33, CD45	Qualitative	None
Multiparameter immunophenotyping aid in the differential diagnosis of hematopoietic neoplasms	T: TCRγδ, CD4, CD2, CD56, CD5, CD34, CD3, CD8, CD7, CD45 B: Kappa, Lambda, CD10, CD5, CD200, CD34, CD38, CD20, CD19, CD45 M1: CD16, CD7, CD10, CD13, CD64, CD34, CD14, HLA-DR, CD11b, CD45 M2: CD15, CD123, CD117, CD13, CD33, CD34, CD38, HLA-DR, CD19, CD45	Qualitative	None

FDA Approved Cellular & Gene Therapy Products

Various HPC, Cord Blood from blood centers , medical centers, and cord blood banks

Allogeneic Cultured Keratinocytes and Fibroblasts in Bovine Collagen

BREYANZI (Juno Therapeutics, a Bristol-Myers Squibb Company)

IMLYGIC (talimogene laherparepvec, BioVex)

KYMRIAH (tisagenlecleucel, Novartis)

LAVIV (Azficel-T, Fibrocell Technologies)

LUXTURNA (Spark Therapeutics)

MACI (Autologous Cultured Chondrocytes, Vericel Corp.)

PROVENGE (sipuleucel-T, Dendreon)

TECARTUS (brexucabtagene autoleucel, Kite Pharma)

YESCARTA (axicabtagene ciloleucel, Kite Pharma)

ZOLGENSMA (onasemnogene abeparvovec-xioi, AveXis)

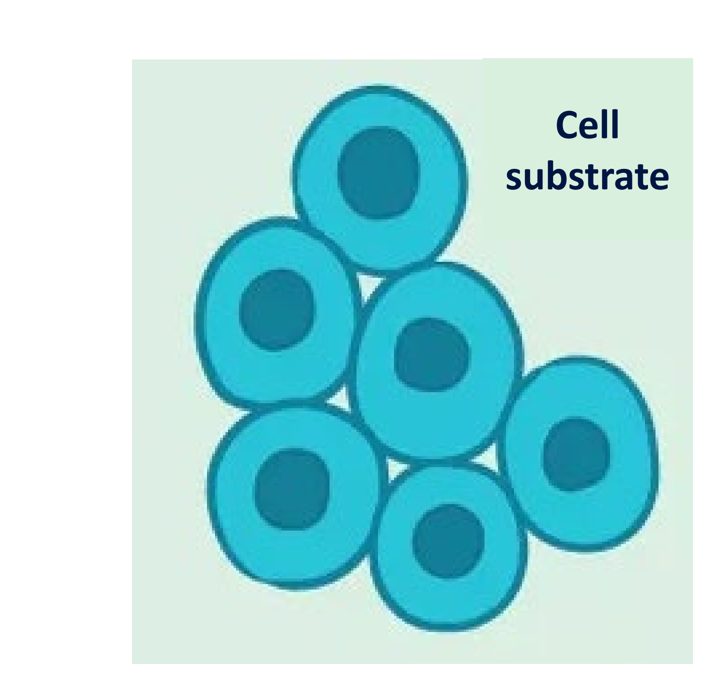
www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products

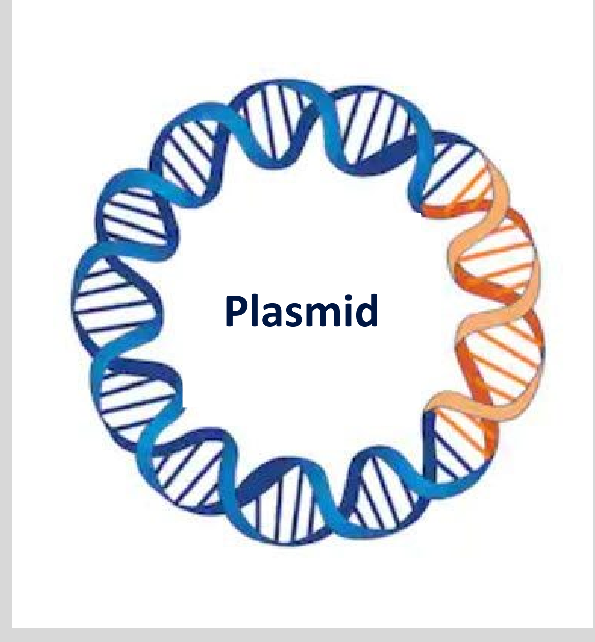
Comparability in Cell & Gene Therapies

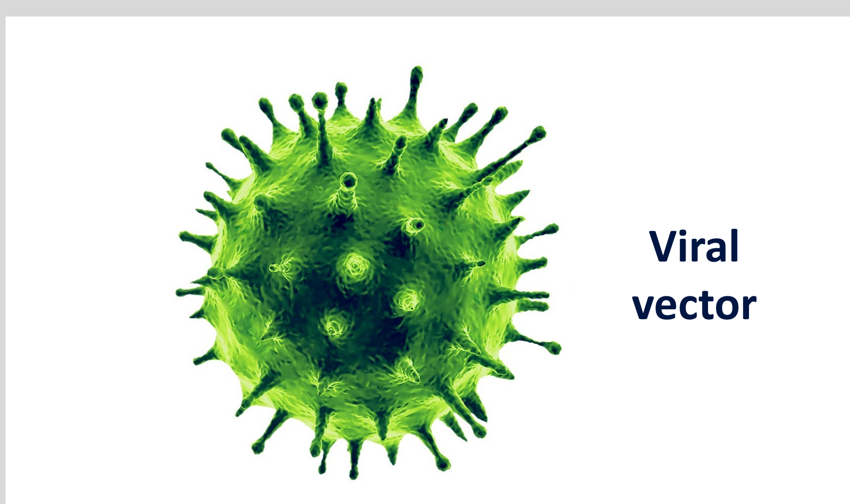
ARM-USP Workshop held on May 31, 2019

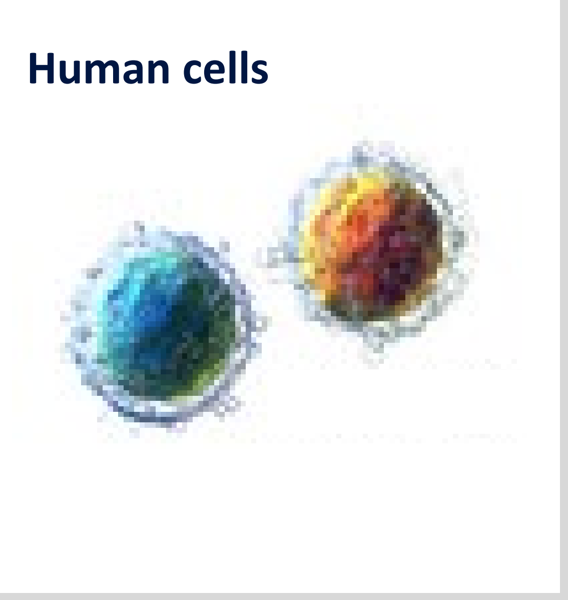
- Identify critical quality attributes (CQAs) and discuss processes to evaluate them.
- Understanding the product development and use of reference standards (RSs) and determining product comparability through analytical methods.
- Many RSs used by developers are product specific internal references.
- There remains an urgent need for new documentary and physical RSs that cover areas such as vector copy number (VCN), cell viability and cell marker standards for flow cytometry, and rapid microbial testing methods.
- Standardized analytical methods are needed to ensure product comparability.

Cell & Gene Therapies – Starting Materials









Cell & Gene Therapies – Material Characteristics

Viral Vector:

- Consistency and stability of the stock
- Infectious viral titre / total viral particles
- Infectivity
- Transgene sequence and expression
- Confirmation of transgene expression in permissive cell

Transduced cells:

- Immunophenotypic profile
- Differentiation / scenescent
- Cell number and viability
- Transduction efficiency
- Vector copy number
- Transgene sequence
- Biological characterization
- Potency
- Stability (accelerated)

Cell Product Characterization

- Identity – specific sequence, cell/cell subset phenotype, morphology, scaffold, et al.
- Purity – relevant cells, ratio of viable to non-viable, senescent
- Impurity/safety – unwanted cells, residual ancillary materials, degradation products, adventitious agents, bioactive reagents/sterility
- Potency – intended function: required for comparability, consistency, and stability

CD34+ CELL STANDARD: COMPARISON OF VOLUMETRIC AND BEAD-BASED COUNTING

USP Chapter <127> Flow Cytometric Enumeration of CD34+ Cells and CD34+ Cell Enumeration System Suitability Standard. The cell standard was used for assessing the result comparability of CD34+ cell counting using different instrument platforms.

Cytometry Part B (Clinical Cytometry) 96B:508–513 (2019)

Original Article

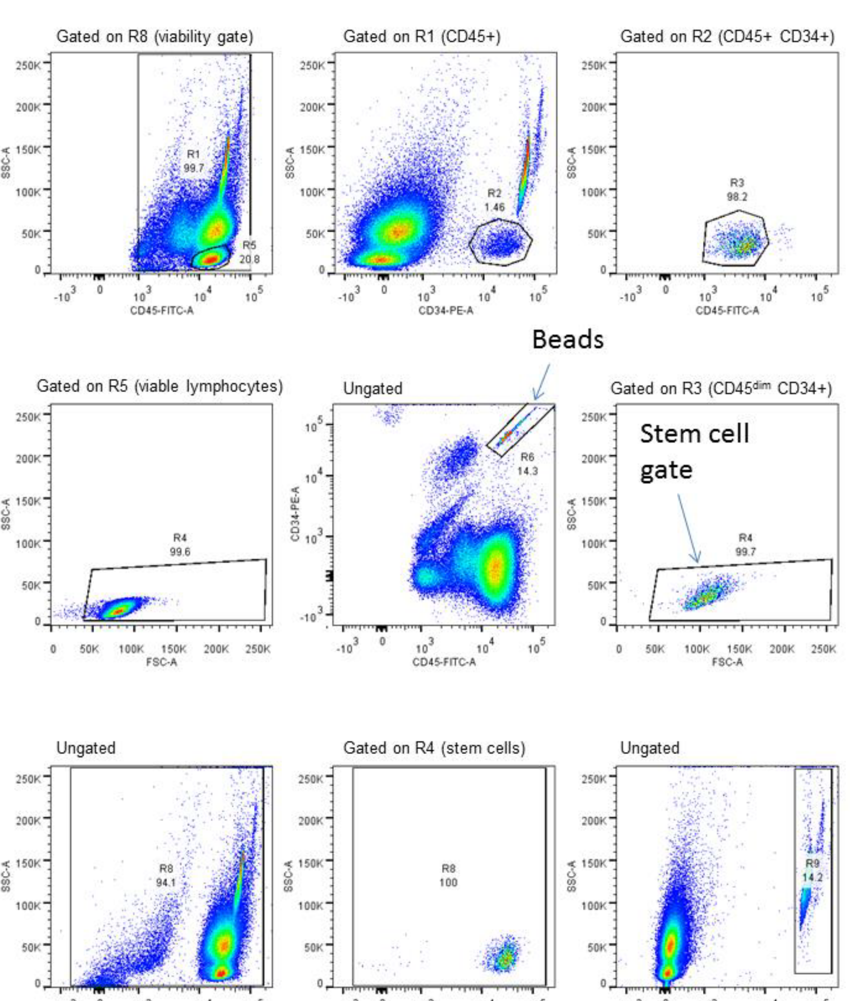
Comparison of Volumetric and Bead-Based Counting of CD34 Cells by Single-Platform Flow Cytometry

Luisa Saraiva,[1*] Lili Wang,[2] Martin Kammel,[3] Andreas Kummrow,[3] Eleanor Atkinson,[4] Ji Youn Lee,[5] Burhanettin Yalcinkaya,[6] Muslum Akgöz,[6] Jana Höckner,[7] Andreas Ruf,[7] Andrea Engel,[8] Yu-Zhong Zhang,[9] Orla O'Shea,[10] Maria Paola Sassi,[11] Carla Divieto,[11] Tamara Lekishvili,[12] Jonathan Campbell,[12] Yingying Liu,[13] Jing Wang,[13] Richard Stebbings,[1] Adolfas K. Gaigalas,[2] Peter Rigsby,[4] Jörg Neukammer,[3] and Sandrine Vessillier[1]

[1]Biotherapeutics group, National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK

[2]Biosystems and Biomaterials Division, National Institute of Standards and Technology (NIST), Gaithersburg, Maryland, 20899, United States of America

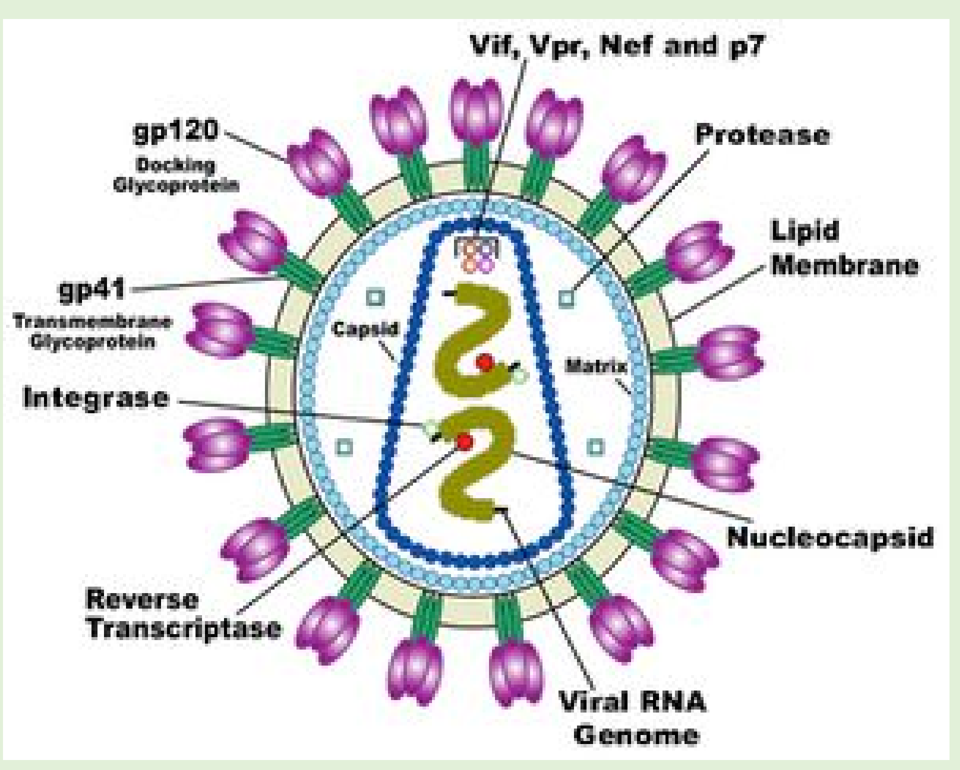
[3]Division of Medical Physics and Metrological Information Technology, Physikalisch-Technische Bundesanstalt (PTB),



Lentiviral Vector Reference Material

Vink et al Molecular Therapy Methods & Clinical Development, Vol 19. Dec 2020

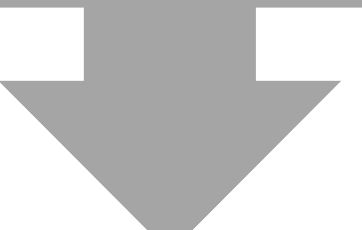
Goal: Develop standards and reference materials for more quantitative transducing unit measurements.



https://microbewiki.kenyon.edu/index.php/Lentiviral_Vectors_in_Gene_Therapy

Viral Copy Number Reference Standard Cell Lines

Jurkat cells with EF1a-GFP lentiviral vector at defined MOI – parent cell line, plus copy numbers 1, 2, 3, & 4



BBD received materials via MTA and has established master cell bank and working cell banks

VCN interlaboratory study	As potential materials for Genome Editing Consortium interlab	For cross platform study (count, flow, functional assays, etc.)	Potential product NIST VCN reference material

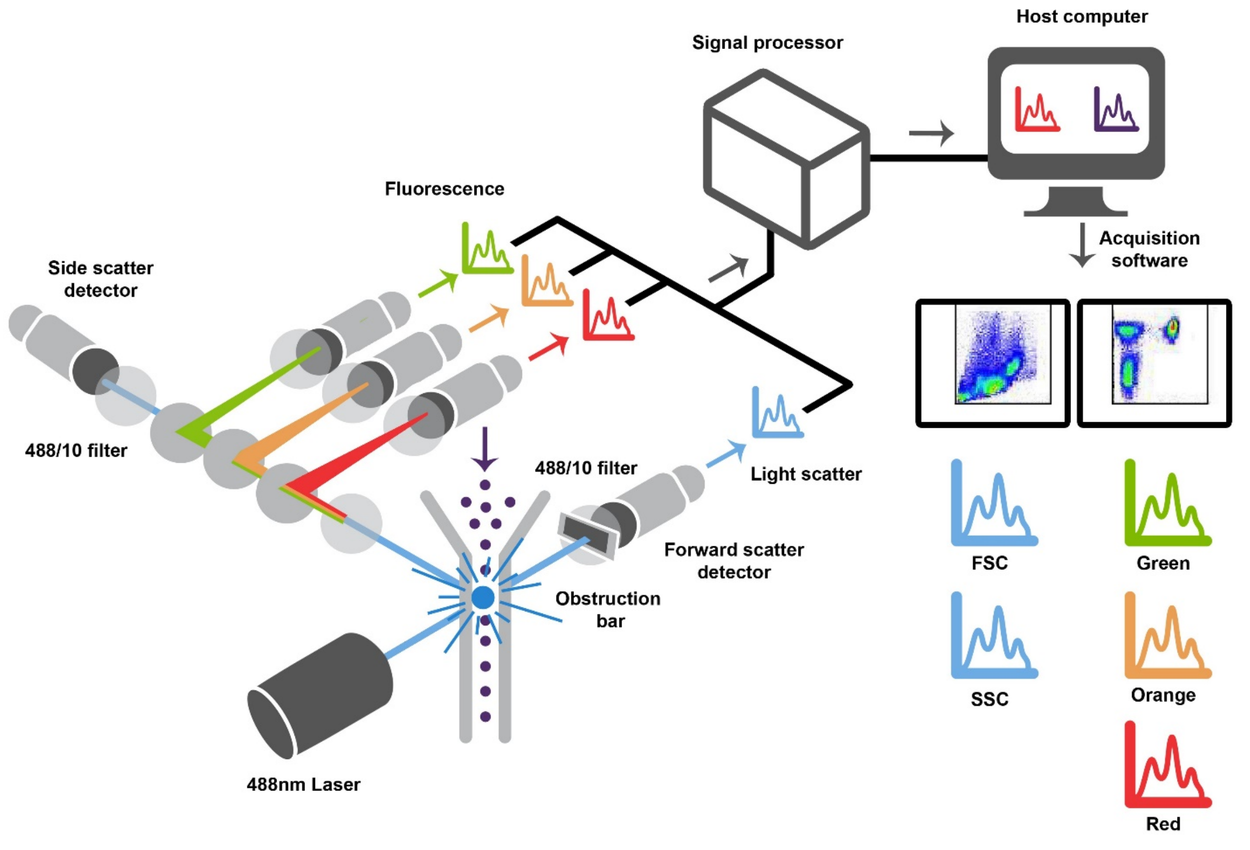
Reference standards for accurate validation and optimization of assays that determine integrated lentiviral vector copy number in transduced cells, Scientific Report, *in press*

Expanding ERF Value Assignments of Calibration Microspheres for Quantitative Flow Cytometry

- More Colors and Smaller Particles

Paul DeRose, PhD – Biosystems and Biomaterials Division
National Institute of Standards and Technology (NIST)

Fluorescence Intensity of Flow Cytometer



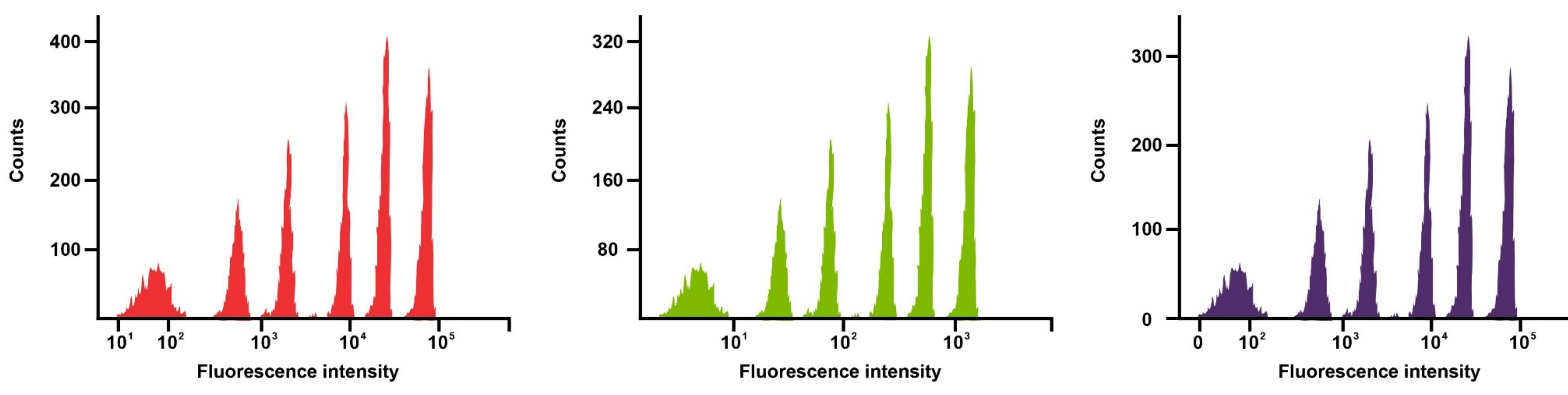
FSC ⇒ size

SSC ⇒ shape and internal complexity

Fluor ⇒ # of fluorophores attached to cell or particles (antibodies, receptors, gene expression)

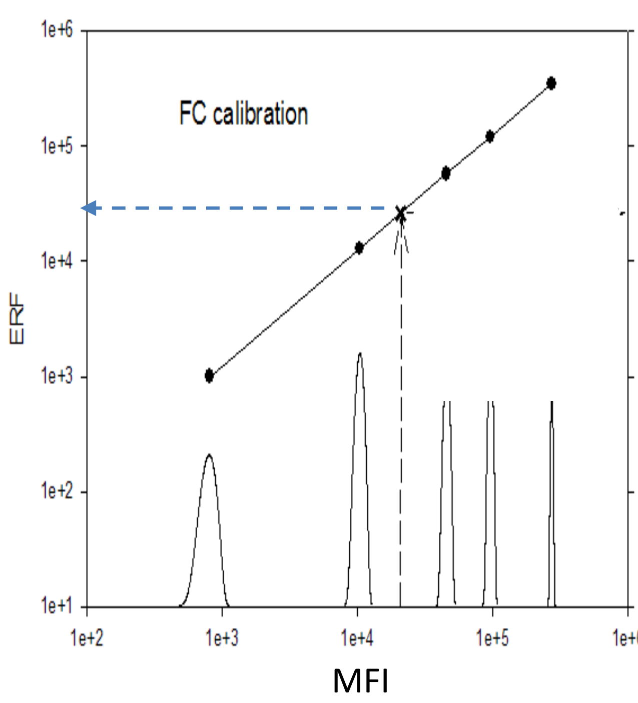
- Mean Fluorescence Intensity (MFI) $\propto$ (# of antibodies bound per cell)
- MFI values are different on every instrument

Flow Cytometer Calibration Beads



Absolute units are supplied by the bead manufacturer

- Calibration Beads – known absolute intensity



The solid line is a calibration of mean fluorescence signal (MFI) from a FC channel using calibration beads in terms of ERF.

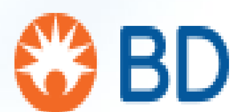
CRADA/Consortium formed in June 2016

- CRADA (NIST & Stakeholder)
  - unique needs – individual CRADAs
  - Learning Curve for both

FLOW CYTOMETRY QUANTITATION COLLABORATORS

Stakeholder representative organizations:

Calibration bead vendors:



Other stakeholders:

Clinical testing labs

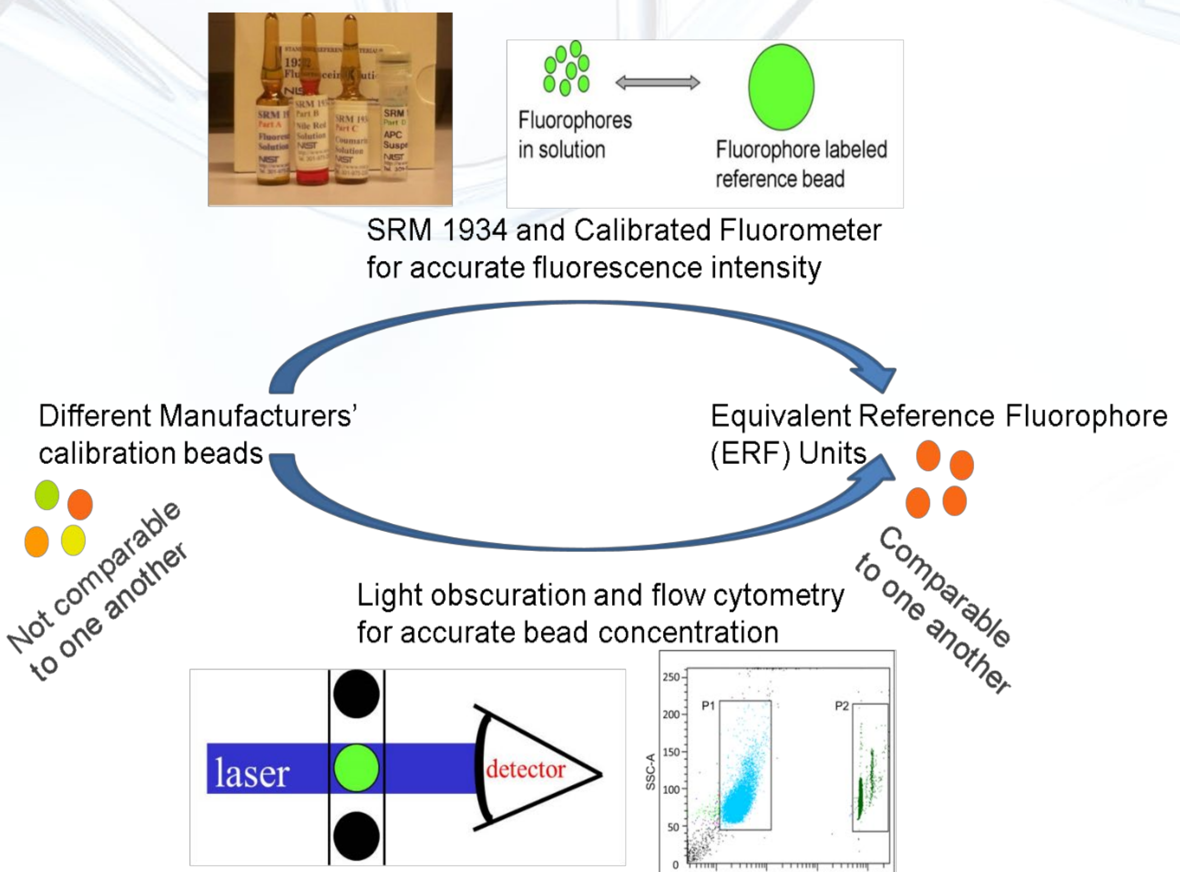
BioPharma – Water
>16 BioPharmas

Quantitative FC Applications

Documentary standards:

- **CLSI H62 Guidance Document-Validation of Assays Performed by FC**
- **USP Chapter <127> Flow Cytometric Enumeration of CD34+ Cells**

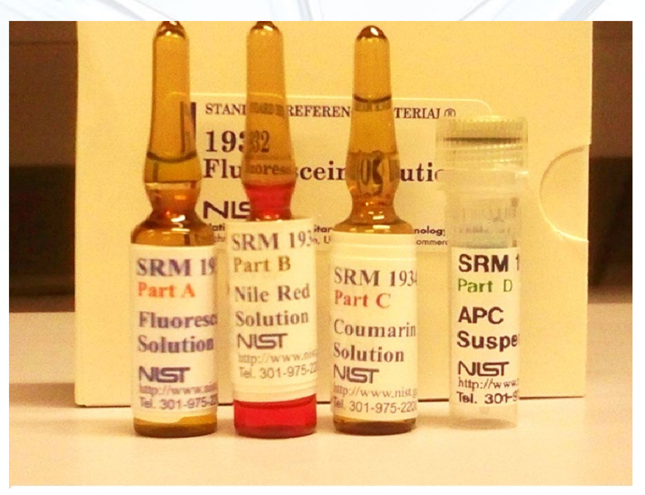
ERF Assignment of Calibration Beads

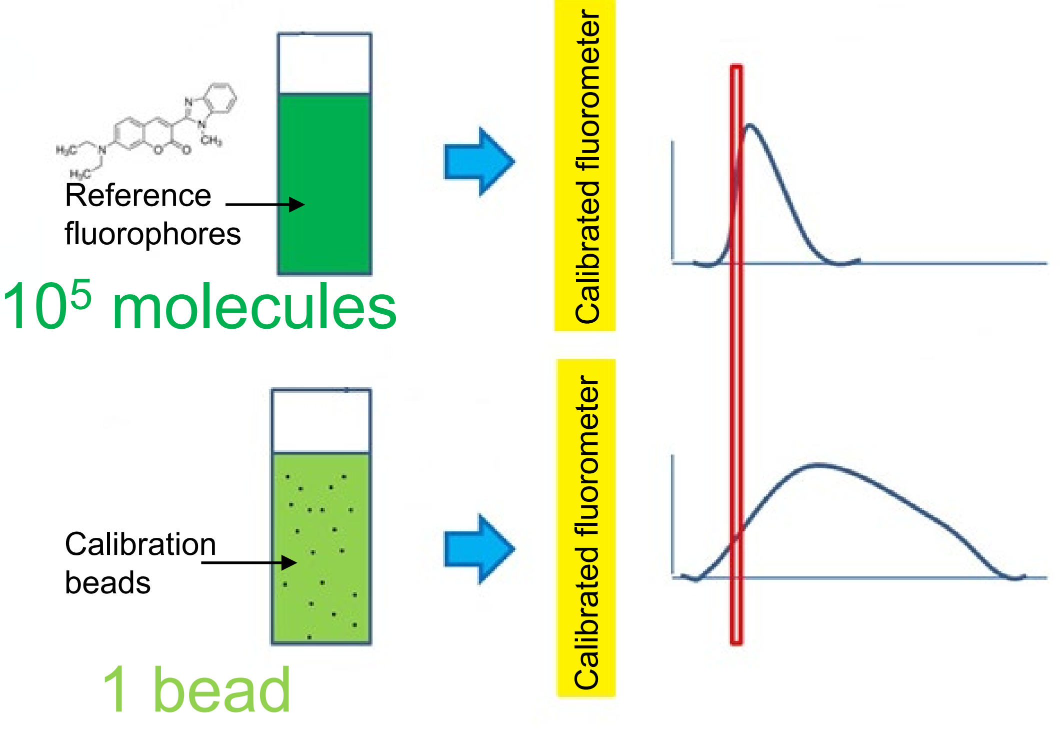


- ERF values for a particular FC Channel under a specified set of instrument conditions are comparable between instruments and over time, even when different manufacturers' calibration beads are used

- DeRose, P., Tian, L., Elsheikh, E., Urbas, A., Zhang, Y.-Z., Wang, L. "Expanding NIST Calibration of Fluorescent Microspheres for Flow Cytometry to More Fluorescence Channels and Smaller Particles," *Materials*, **13**, 4111 (2020).
- DeRose, P.C., Wang, L., "NIST Fluorescence-based Measurement Services" BioPharm Int., (Dec 2018)
- Wang, L., DeRose, P.C., Gaigalas, A.K, "Assignment of the Number of Equivalent Reference Fluorophores to Dyed Microspheres" *J.Res. NIST*, **121**, 264-281 (2016).

SRM 1934 – Four Fluorescent Dyes for Quantitative Flow Cytometry



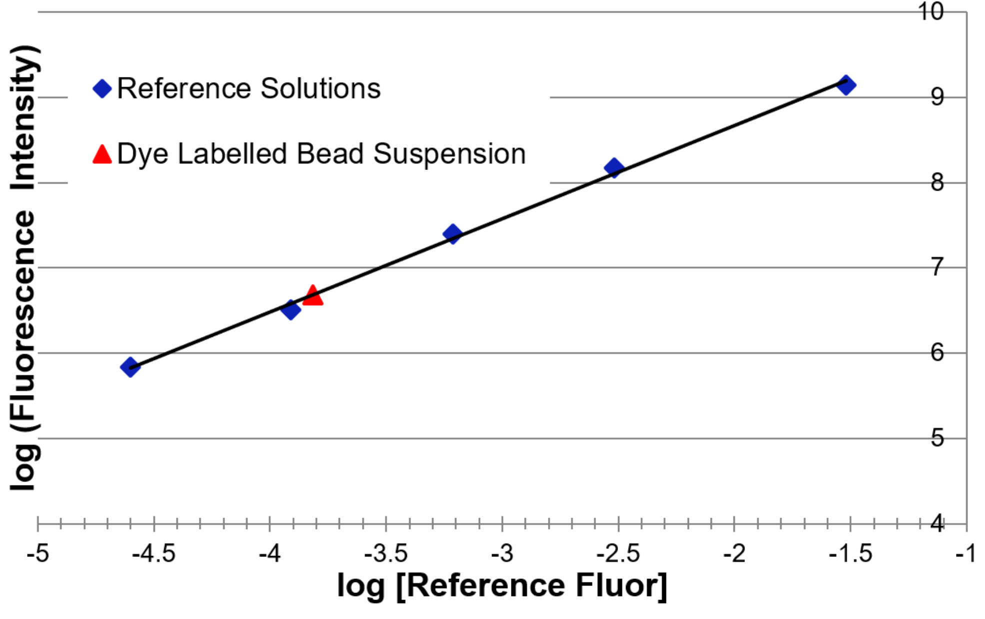


- Released by NIST May 2016
- Enables Equivalent Number of Reference Fluorophores (ERF) units to be used as a Fluorescence Intensity (FI) Scale
- Delivers More Accurate and SI-Traceable FI scale using ERF units

$\Rightarrow$ 10^5 ERF units

FC Channels and ERF Scale

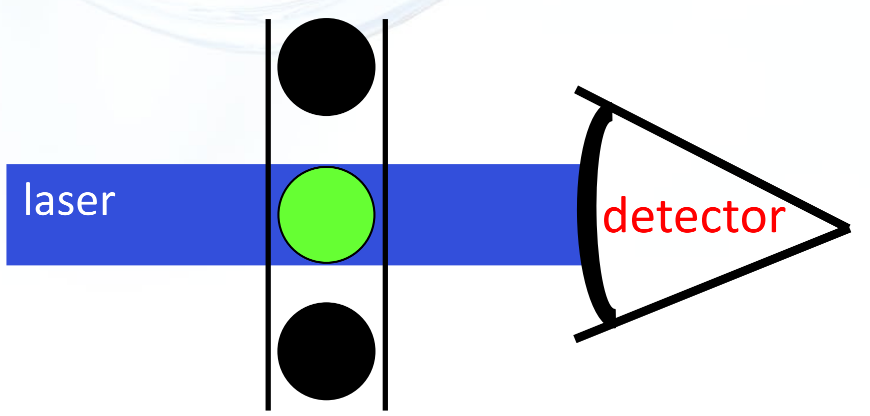
Reference Fluor	EX Laser (nm)	EM Range (nm)
Coumarin 30	375 405	390-550 420-550 (blue)
Pacific Orange	375 405	500-700 500-700 (yellow-green)
Fluorescein	488	500-580 (green)
Nile Red	488 561	570-700 580-700 (orange)
APC	633	640-740 (red)
Alexa Fluor 700	633	570-800 (red-near IR)
Alexa Fluor 750	633 808	750-850 (near IR)



- Reference fluor (SRM 1934) solutions define ERF scale
- Intensity of solutions and bead suspension measured using a fluorescence spectrometer

Bead # Concentration – Light Obscuration

SI Traceable Particle Counter



- Total Expanded Uncertainty (k=2) is about 4%for typical calibration beads

Ripple, D., DeRose, P.C., "Primary Determination of Particle Number Concentration with Light Obscuration and Dynamic Imaging Particle Counters" *J.Res. NIST*, **123** -002 (2018).

Size Limit & Uncertainties

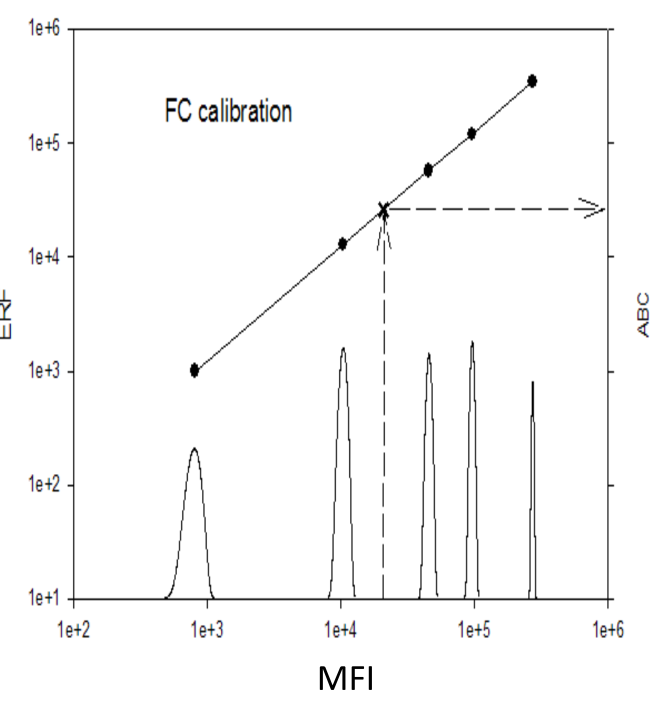
- down to 2 micron
- Particles settle in and adsorb to walls of holding container. Stirrers add particles to sample. (shake and stir strategies)
- Uncertainty in the measured volume, e.g., offset error, dead volume, timing error (volume vs. # beads detected)
- Multiple beads simultaneously detected (bead concentration vs. # beads detected)

ERF Values with Total Uncertainty Reported

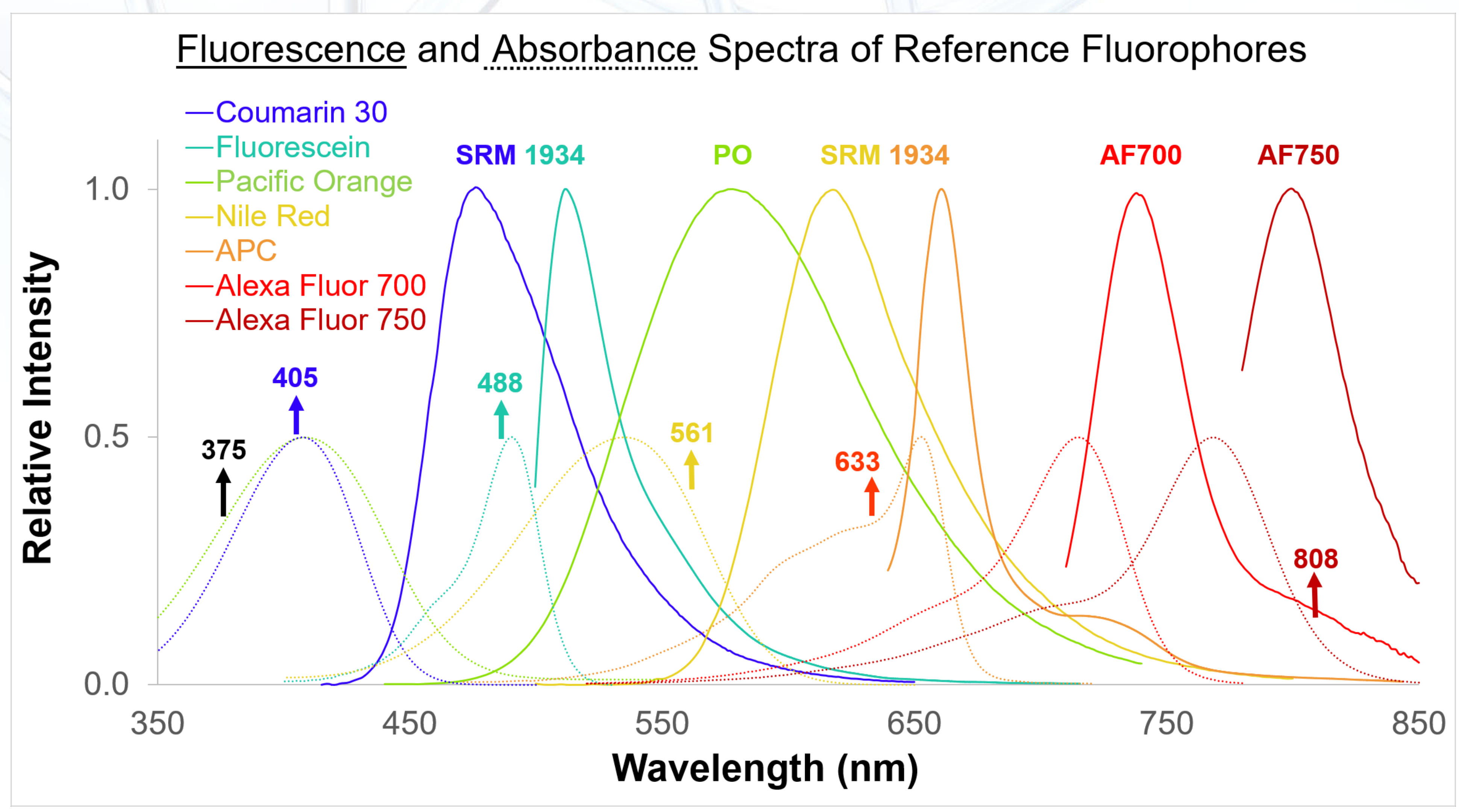
- ERF Values are SI Traceable
- Total Uncertainty in NIST ERF Assignment is 5% - 13% (k=2)

ERF & How do we get to a Biological Scale? (ABs bound per cell)

- Reference Dye
- Biological Standard



- The solid line is a calibration of mean fluorescence signal (MFI) from a FC channel using calibration beads in terms of ERF.
- The dotted lines indicate the transfer of the ERF scale to an ABC scale using standard cells with known ABC.


Fluorescence and Absorbance Spectra of Reference Fluorophores
—Coumarin 30
—Fluorescein
—Pacific Orange
—Nile Red
—APC
—Alexa Fluor 700
—Alexa Fluor 750
SRM 1934
PO
SRM 1934
AF700
AF750
405
488
561
633
375
808
1.0
0.5
0.0
Relative Intensity
350
450
550
650
750
850
Wavelength (nm)

Summary of NIST ERF Assignments

- More than 50 fluorescence channels
- 5 lasers (6 available)
- EM from 390 nm to 850 nm
- 6 reference fluors (7 available)
- Diameters from 2 μm to 10 μm
- ERF Values are SI Traceable
- Total Uncertainty in NIST ERF Assignment is 5% - 13% (k=2)

Smaller Sizes – sub-micron particles

- Extra-cellular vesicles (EVs) – diameter = 40 nm to 1000 nm
- Mediate intercellular communications in physiology & pathology
- Many of the same surface receptors as cells
- Topic of growing interest in flow cytometry
- In principle, the same approach can be used to assign ERF values to sub-μm or nm sized particles
- Techniques that can accurately measure bead # concentration need to be established
- The few examples found in the literature are very time consuming and very expensive
- NIST collaborative efforts to develop better techniques

Sub-μm Bead # Concentration Techniques

Technique	Size Limit/Range (nm)	Sample Volume	[Sample] mL-1	Caveats
LO	2000	15 mL	10^3 to 10^4	size limit dilution error
EM	1 - 100	10 μL	10^{10} to 10^{12}	unknown volume collection time
AF4-DLS	2	10 μL	10 mg	not accurate
NTA	10-1000	12 μL	10^6 to 10^9	need standard
RPS	200	15 mL	10^4 to 10^6	need standard size limit dilution error
Next Gen RPS	60	10 μL	10^6 to 10^{10}	need standard
FCM	80	100 μL	10^5 to 10^7	need standard
Quantum FCM	30	N/D †	N/D	N/D
Virus Counter	25-300	200 μL	10^5 to 10^9	virus specific

† Not determined

Important Developments

- Flow Cytometry Standards CRADAs/Consortium is bringing stakeholders together to develop measurement services, reference materials, ref. data and reference methods (NIST/NIH co-management)
- NIST is performing ERF Assignments of Calibration Beads through CRADAs
- NIST is adding more reference dyes and laser colors to assign ERF values for all fluorescence channels for FCM
- NIST is working with stakeholders to determine # concentrations of sub-micron bioparticles and assign ERF values to sub-micron calibration beads

Acknowledgements

ERF Assignments: Lili Wang, Dolf Gaigalas, Linhua Tian, Dean Ripple, Sandra DaSilva, Nancy Lin

Modeling of Antibody Binding: Dolf Gaigalas

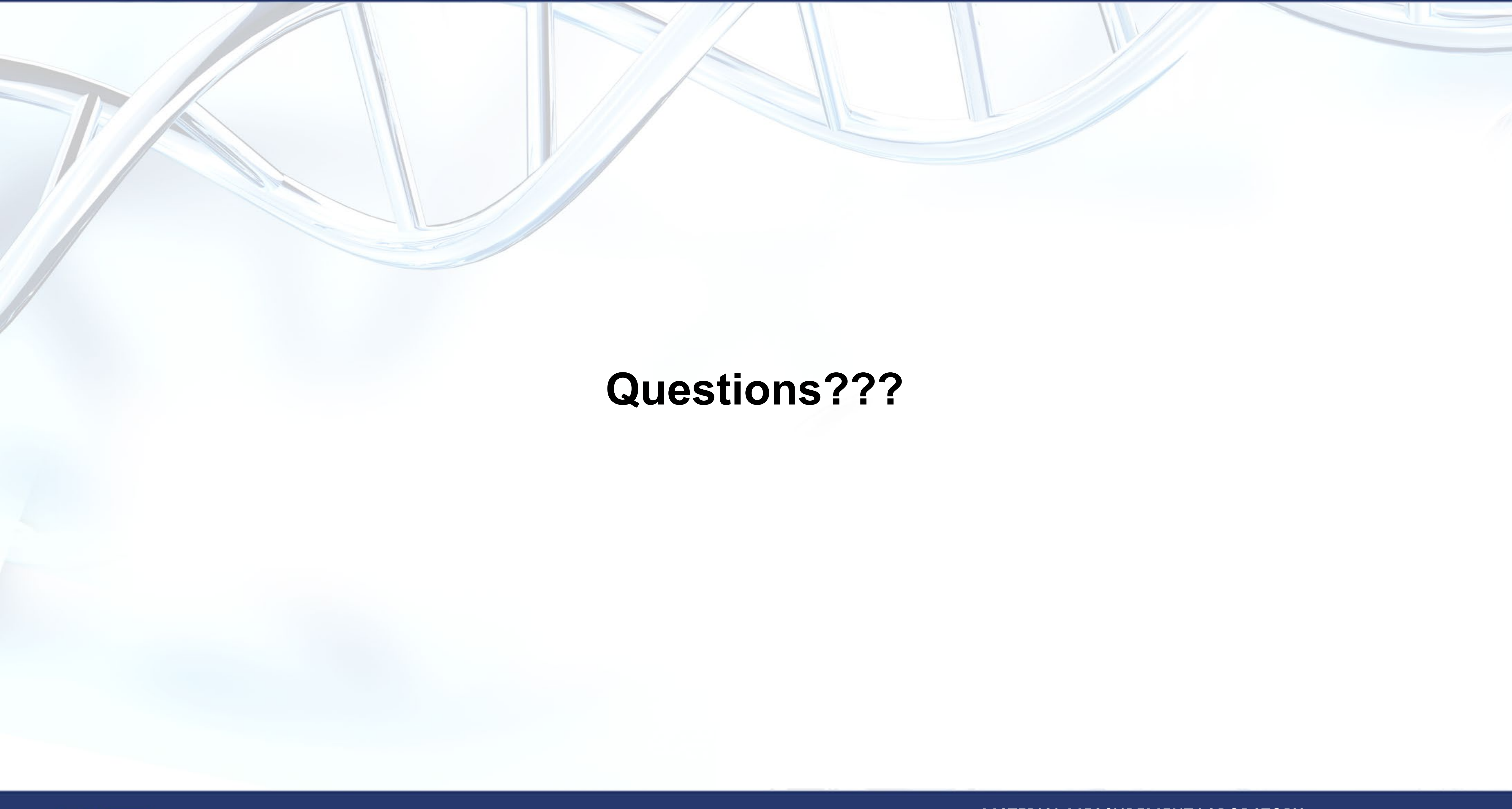
Quantitative ^{1}H NMR: Aaron Urbas, Mike Nelson

Particle Counting Discussion Group:

Kurt Benkstein, Richard Cavicchi, Sandra DaSilva, Nancy Lin, Sheng Lin-Gibson, Laura Pierce, Dean Ripple, Sumona Sarkar, Wyatt Vreeland, Lili Wang

Sub-micrometer Bead # Concentration Measurements

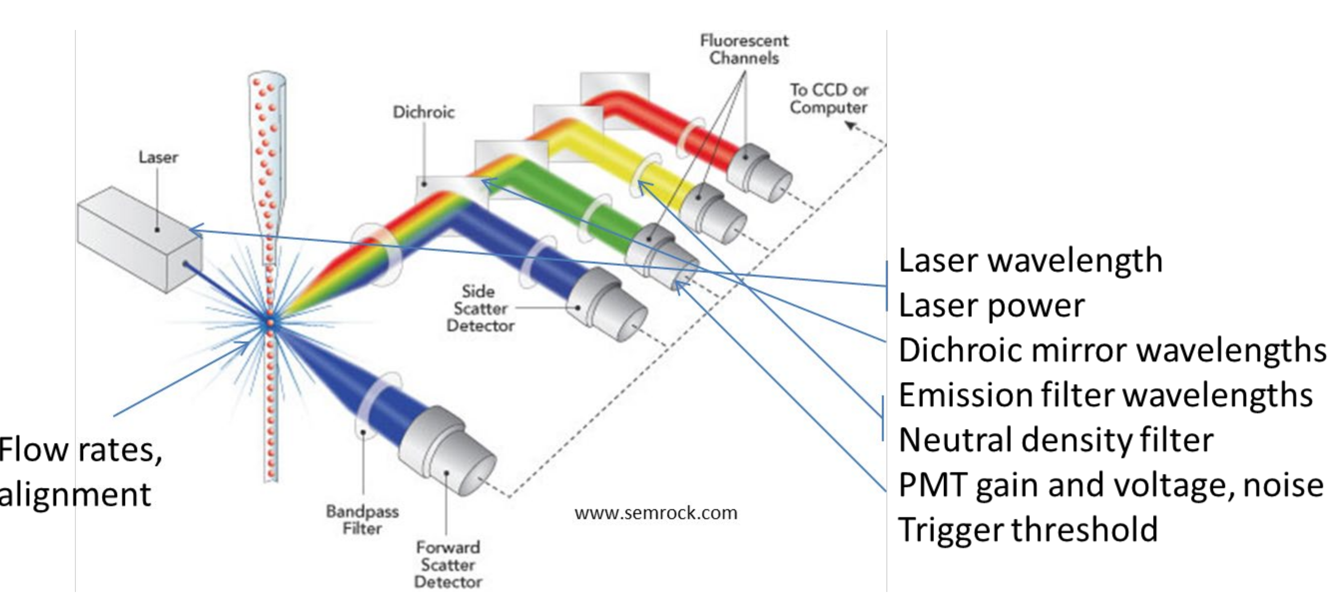
Kurt Benkstein, Elzafir Elsheikh, Sean Lehman, Bryant Nelson, Sergey Polyakov, Dean Ripple, Linhua Tian, Wyatt Vreeland, Lili Wang


Questions???

Flow Cytometry Measurement Protocols

John T. Elliott Jr., Cell Systems Science Group Leader

National Institute of Standards and Technology (NIST), Department of Commerce, Gaithersburg, MD 20899



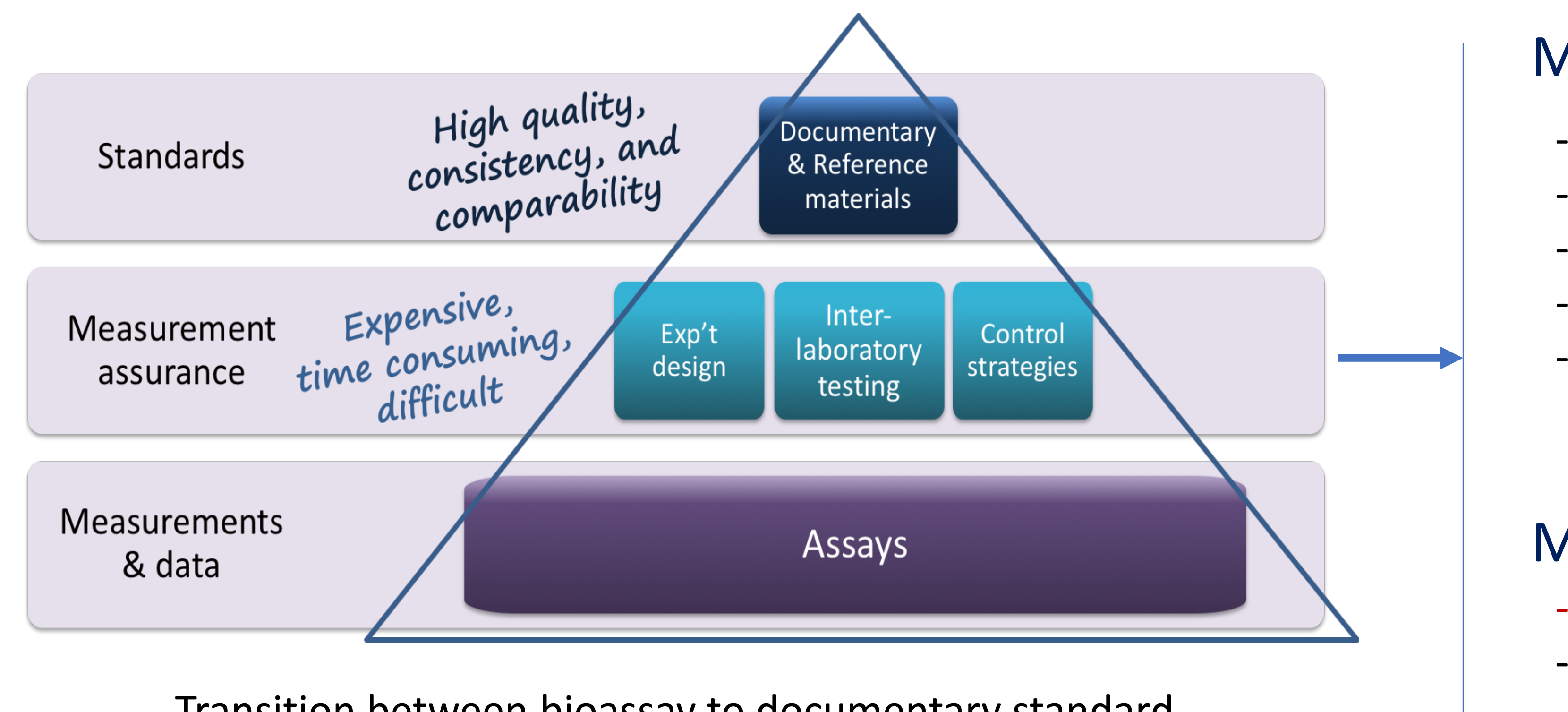
Flow rates, alignment

Laser wavelength
Laser power
Dichroic mirror wavelengths
Emission filter wavelengths
Neutral density filter
PMT gain and voltage, noise
Trigger threshold

Protocol Flow chart

- Cell Maintenance/Treatment
- Cell preparation/harvest
- Cell staining/sampling
- Flow cytometer experiment
- Generate test result

Measurement Assurance for Biological Assays



Transition between bioassay to documentary standard

Measurement Science Tools

- -Cause and effect diagrams
- -Sensitivity analysis
- -One-off controls (i.e. limit of detection)
- -In-process controls (i.e. cells OK?)
- -Interlaboratory comparisons

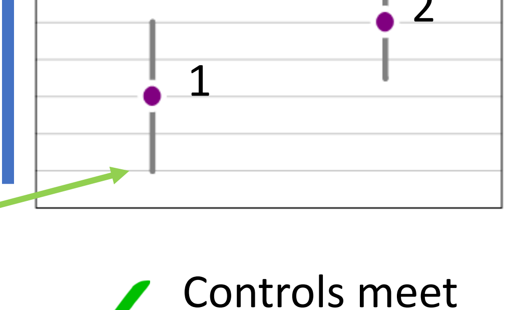
Measurement Infrastructure

- -High quality protocols
- -Control specifications
- -Reference materials

- Measurement infrastructures underpin generalized assay protocols/platforms
- Pre-competitive and consensus driven
- Can be tailored to a particular product, cell type, biomarker, assay, etc.

Developing high-quality protocols for flow cytometry: a measurement science approach

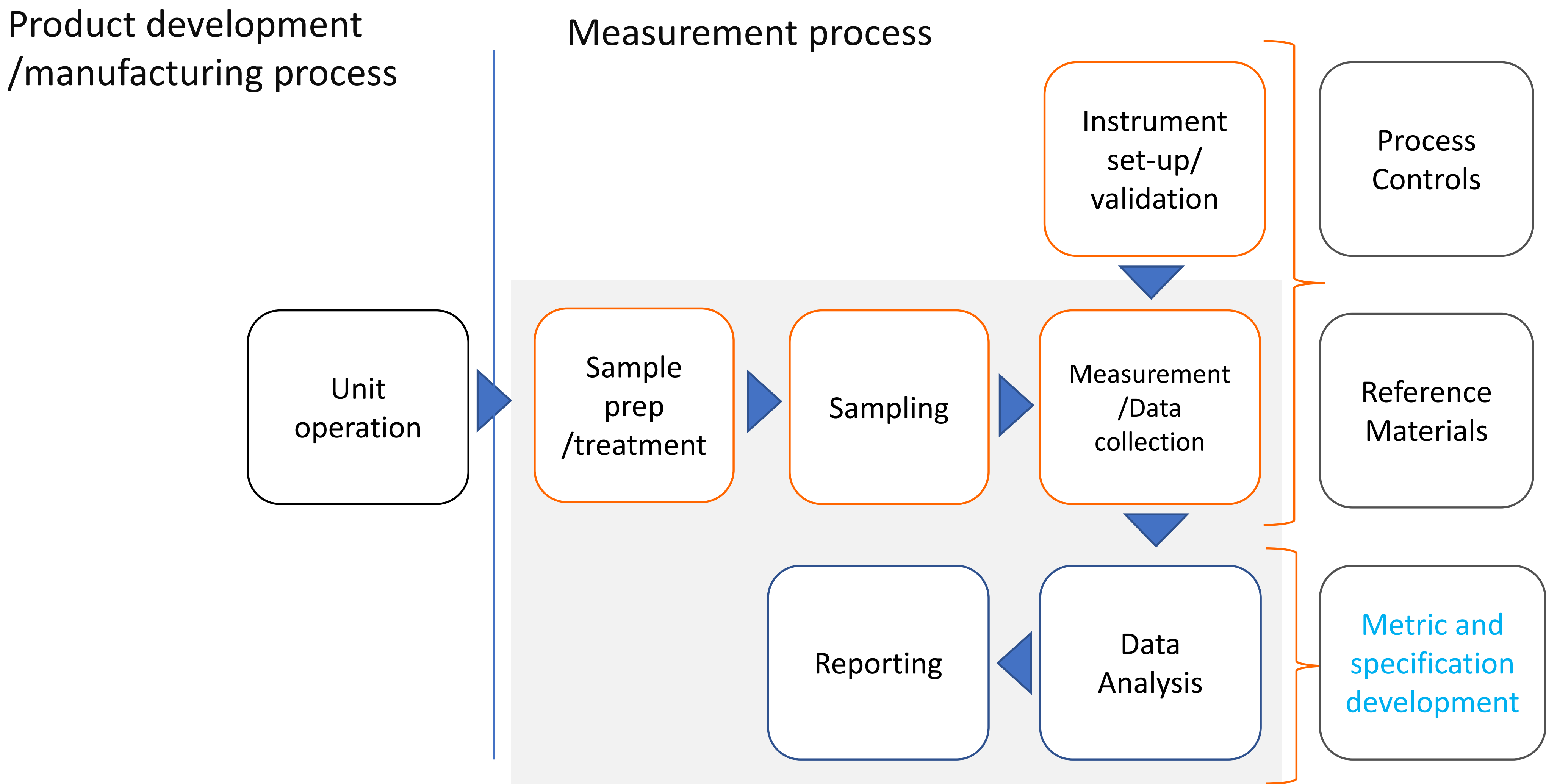
- Components of the measurement result
  - Value- it is on a scale; enables compared to other measurements
  - Uncertainty- variability in the measurement; enables statistics
  - Evidence- evaluation of the measurement system; confidence



✓ Controls meet specification

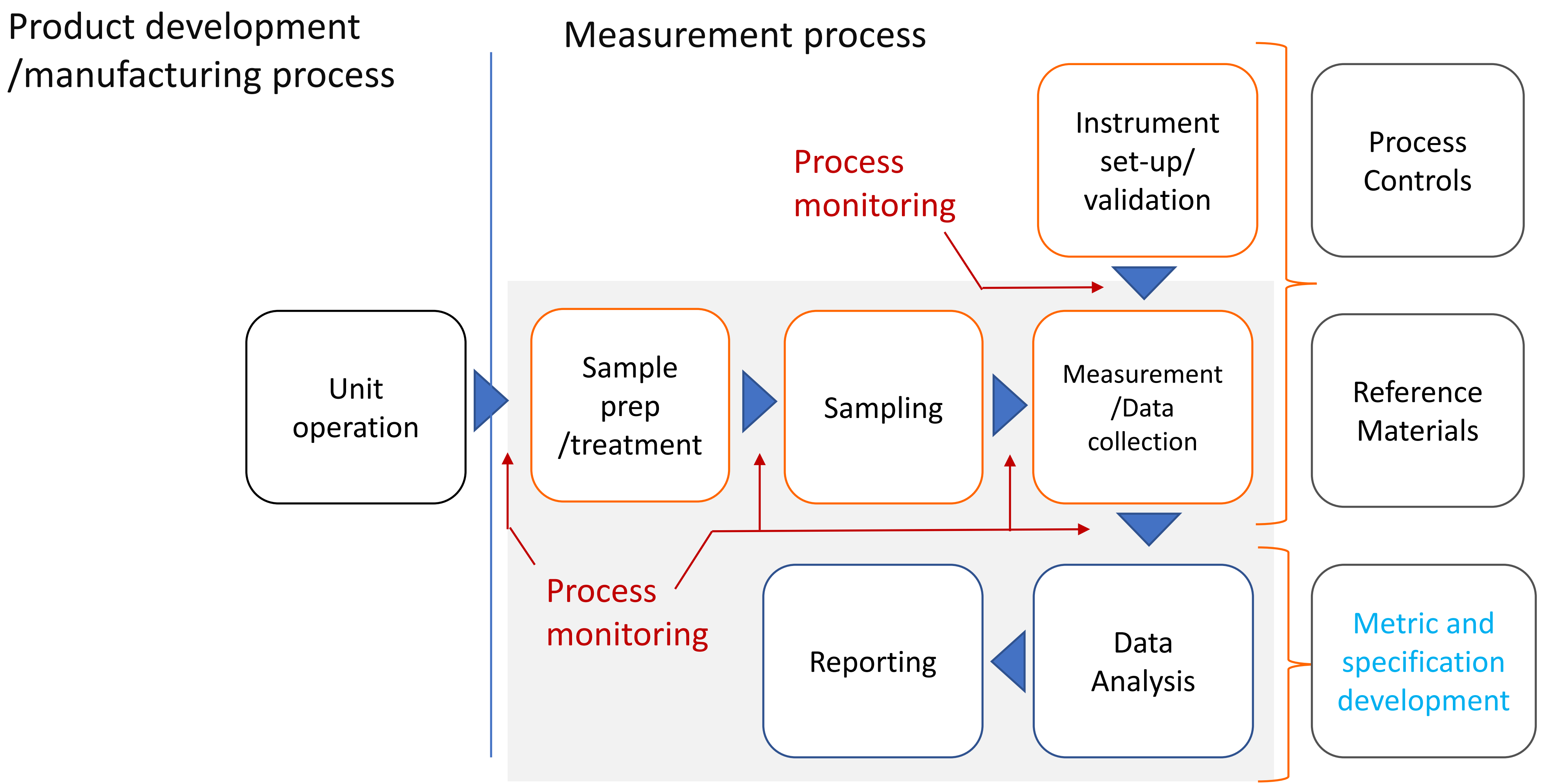
- Measurement is a process that generates a test result
  - What are the sources of variability?
  - Are the process steps working correctly?
  - Do you have specification ranges for each step?

Generalized Measurement Process for a Cell Assay



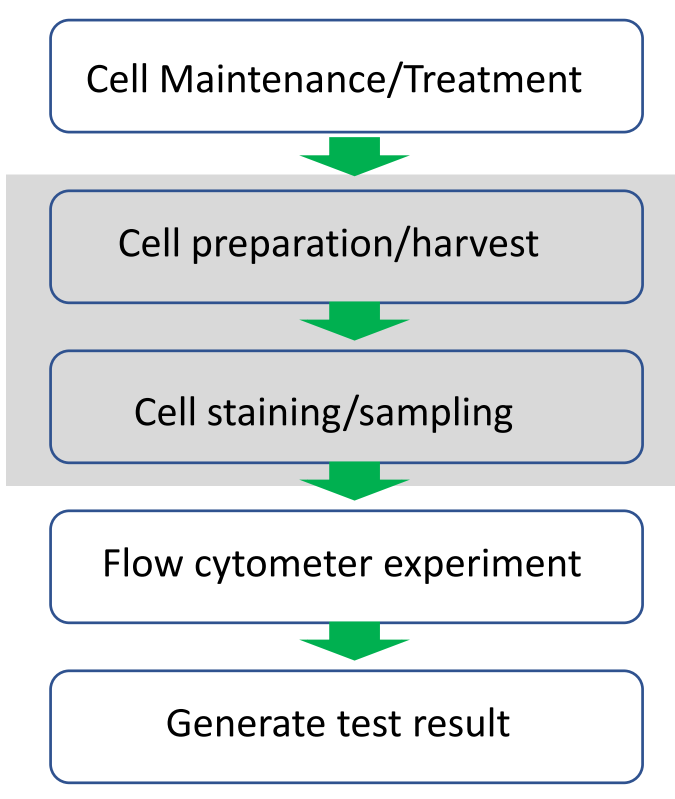
Generated from NIST Measurement Assurance Workshop 2015

Generalized Measurement Process for a Cell Assay



Generated from NIST Measurement Assurance Workshop 2015

Flow Chart for a Simple Treatment Flow Experiment



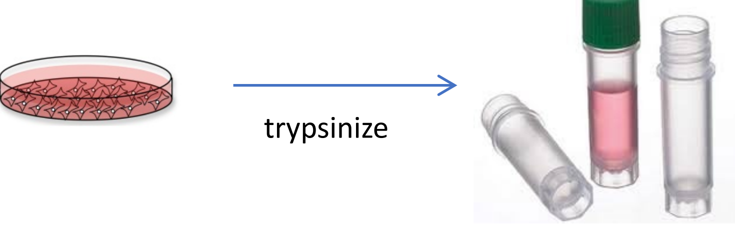
- Consider the measurement as a process.
- What are the general steps of the process?
- What kinds of problems can happen in each step?

NOTE: Each step includes many manual timed steps, pipet mixing/resuspension steps, rinsing steps, reagent additions, instrument parameter settings.

Ideal conditions for sample prep/staining

Cell Harvest:

Cells are analyzed in suspension

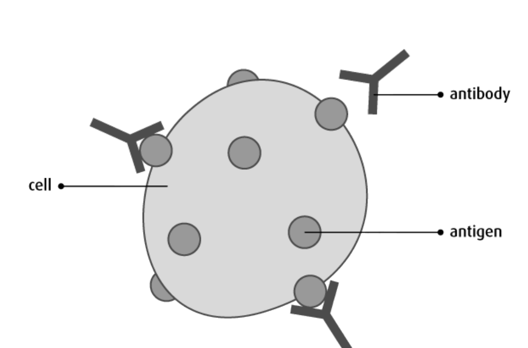


Ideal conditions:

-Confirmed single cell suspension
-little debris, few cell clumps
-adequate cell density
-Stable storage matrix
-viable (if required)
-fixed (if robust fixative)

Cell Staining:

Fluorescent antibodies
Nuclear dyes
Membrane dyes



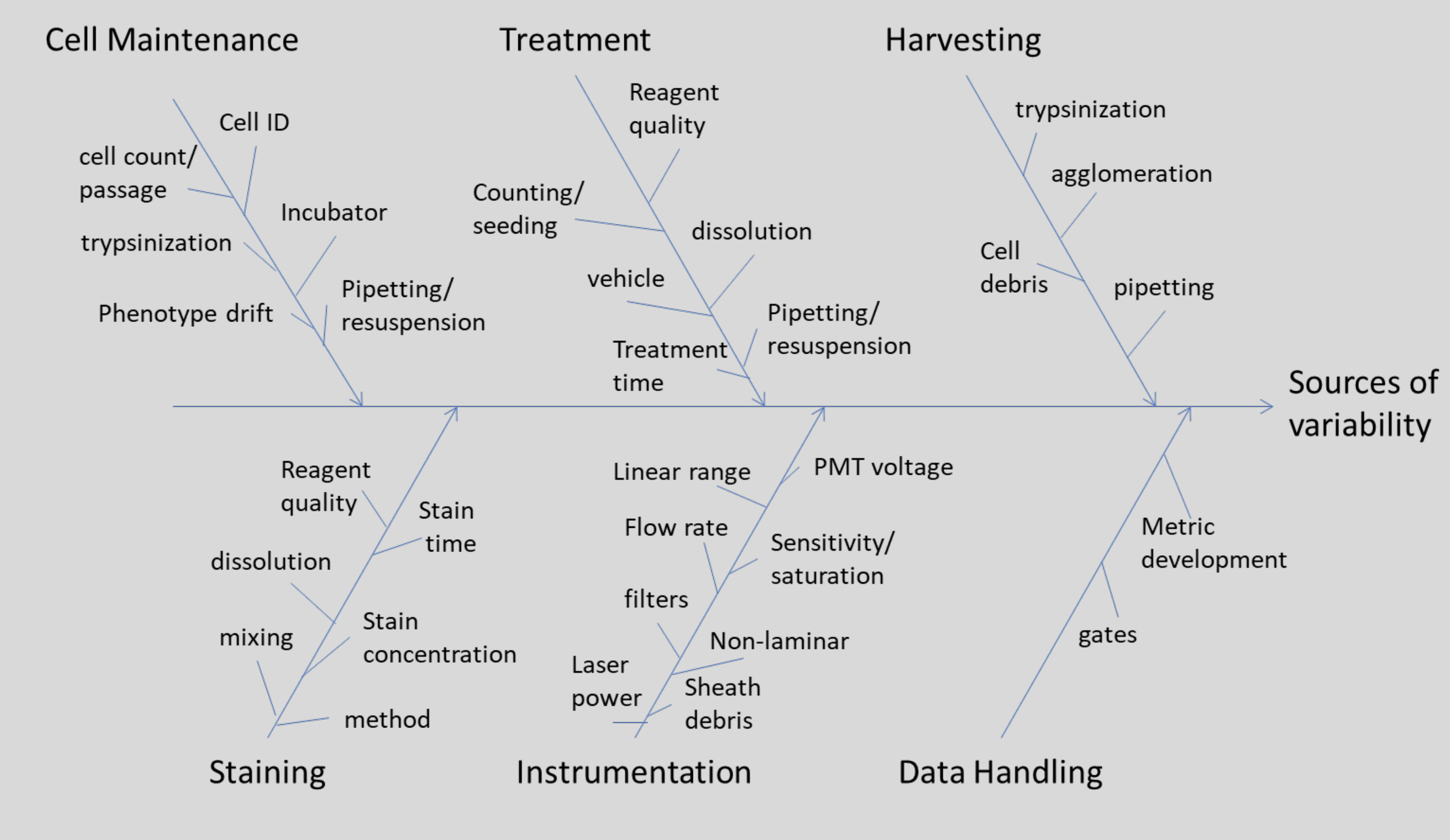
Ideal conditions:

-high-quality stains (known antigen, conjugates, ultra high affinity antibodies, low non-specific binding)
-well-defined staining protocol
-controls to verify staining procedures
-bright fluorophore, photostable, not sensitive to environment
-known measurement stability
-traceable to a fluorescence reference system

- Un-ideal conditions may reduce confidence in the measurement system

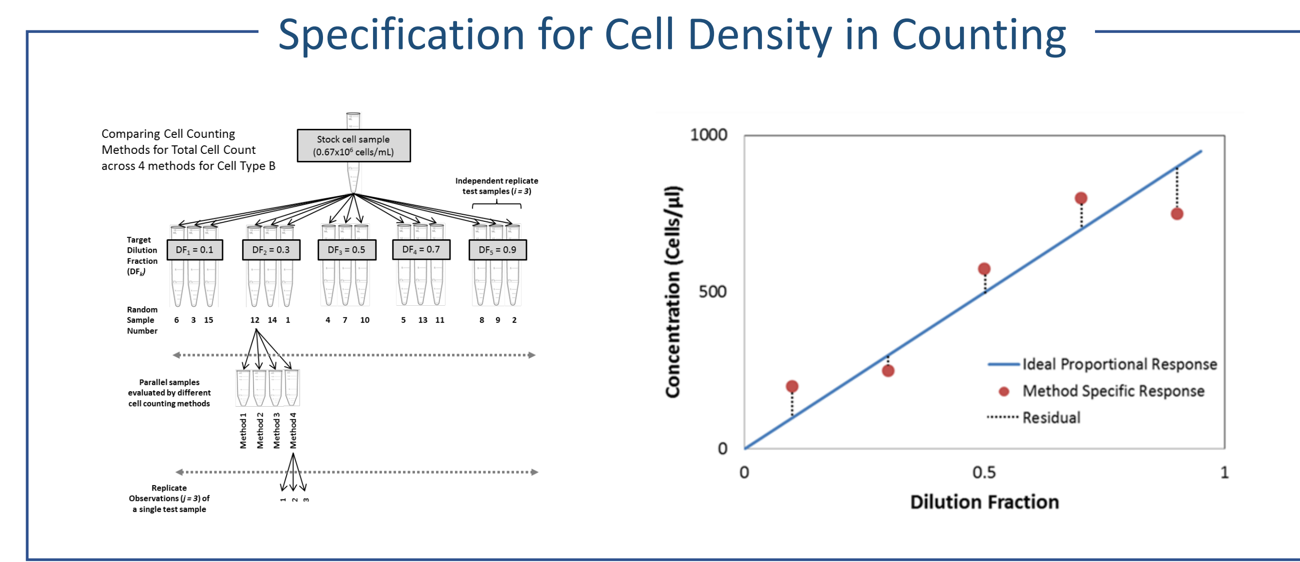
Sources of Variability in a Flow Cytometry Assay

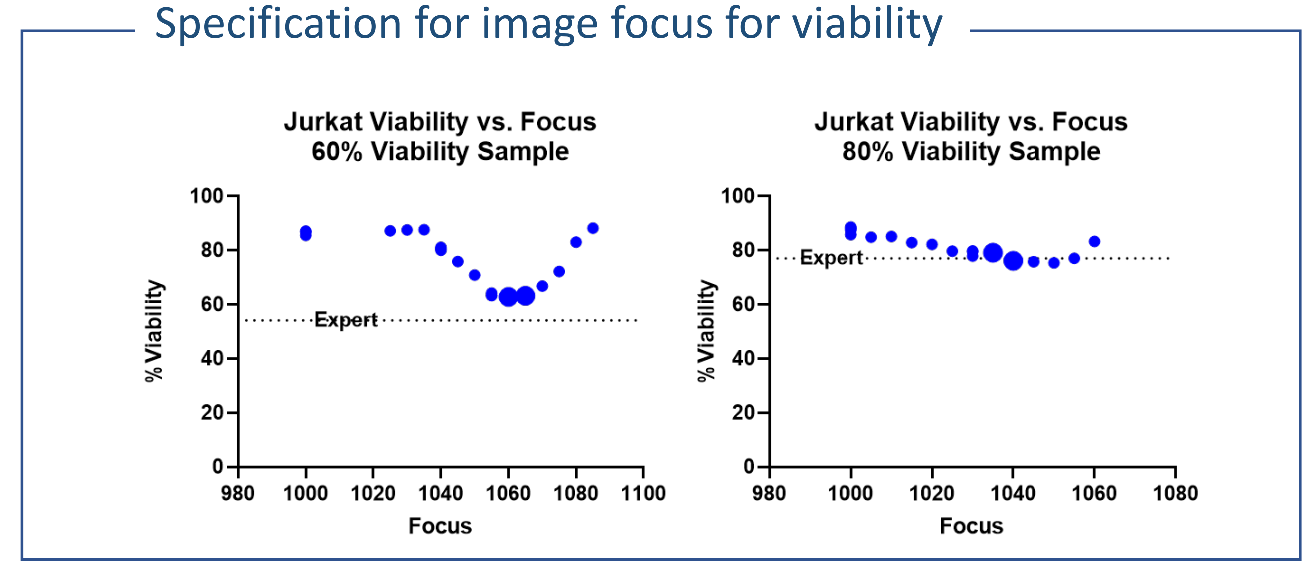
- A large number of factors can influence a test result.
- Identify the potential sources of variability
- Design control experiments to assess assay sensitivity
- Identify conditions where measurement system is stable.



One-off Control Experiments

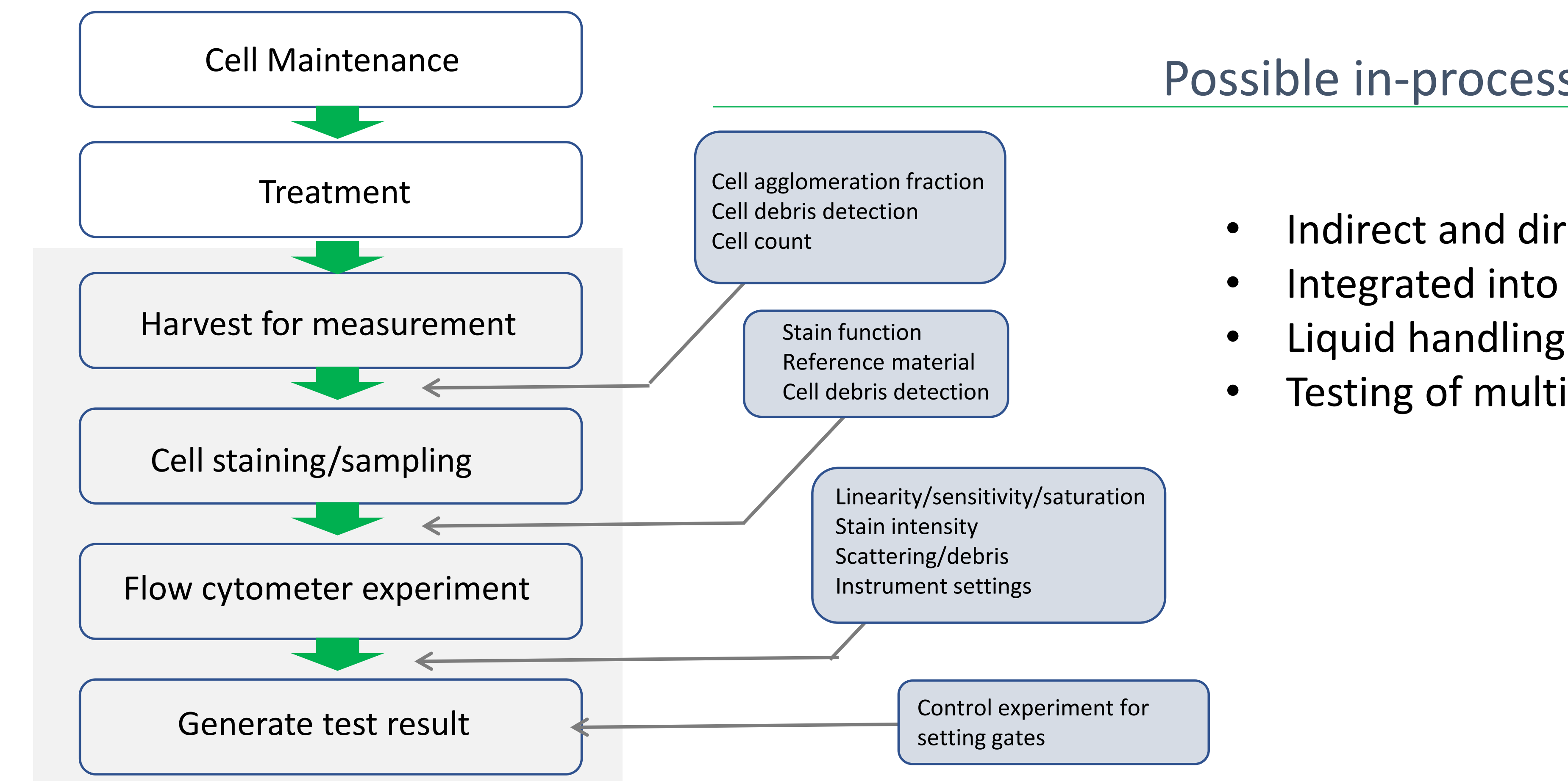
- Not necessarily performed frequently
- General platform suitability
  - readout interferences
  - Sample compatibility
  - sample quality
- Specific product suitability
  - custom test material
  - matrix
- production method
- May require actual sample





In-Process Measurement Controls

- Performed frequently (e.g. every experiment)
- Realtime evidence of measurement system status
- Must meet specifications to trigger confidence in measurement result

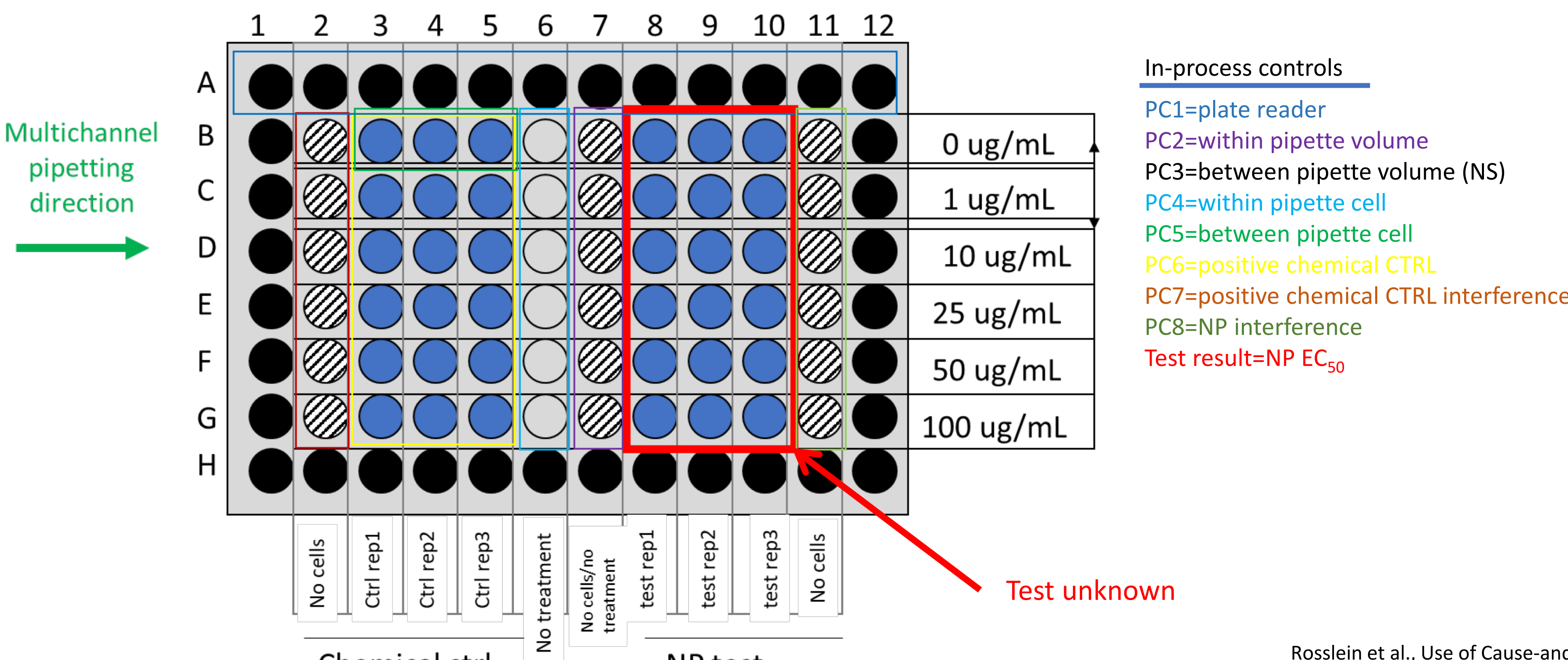


Possible in-process controls

- Indirect and direct measurements
- Integrated into protocol
- Liquid handling and sample handling
- Testing of multiple steps

Assay Plate Designs

- Leverage multiwell plate designs to include process control measurements
- Large fraction of quality “real-estate” on the assay plate
- Control data is collected during the measurement
- Plate design depends on the measurement needs



In-process controls

PC1=plate reader

PC2=within pipette volume

PC3=between pipette volume (NS)

PC4=within pipette cell

PC5=between pipette cell

PC6=positive chemical CTRL

PC7=positive chemical CTRL interference

PC8=NP interference

Test result=NP EC_{50}

Rosslein et al., Use of Cause-and-Effect Analysis to Design a High-Quality Nanocytotoxicology Assay., 2015, Chem Res Toxicol. 2015 Jan 20;28(1):21-30

Robustness Testing- Intra and interlab studies

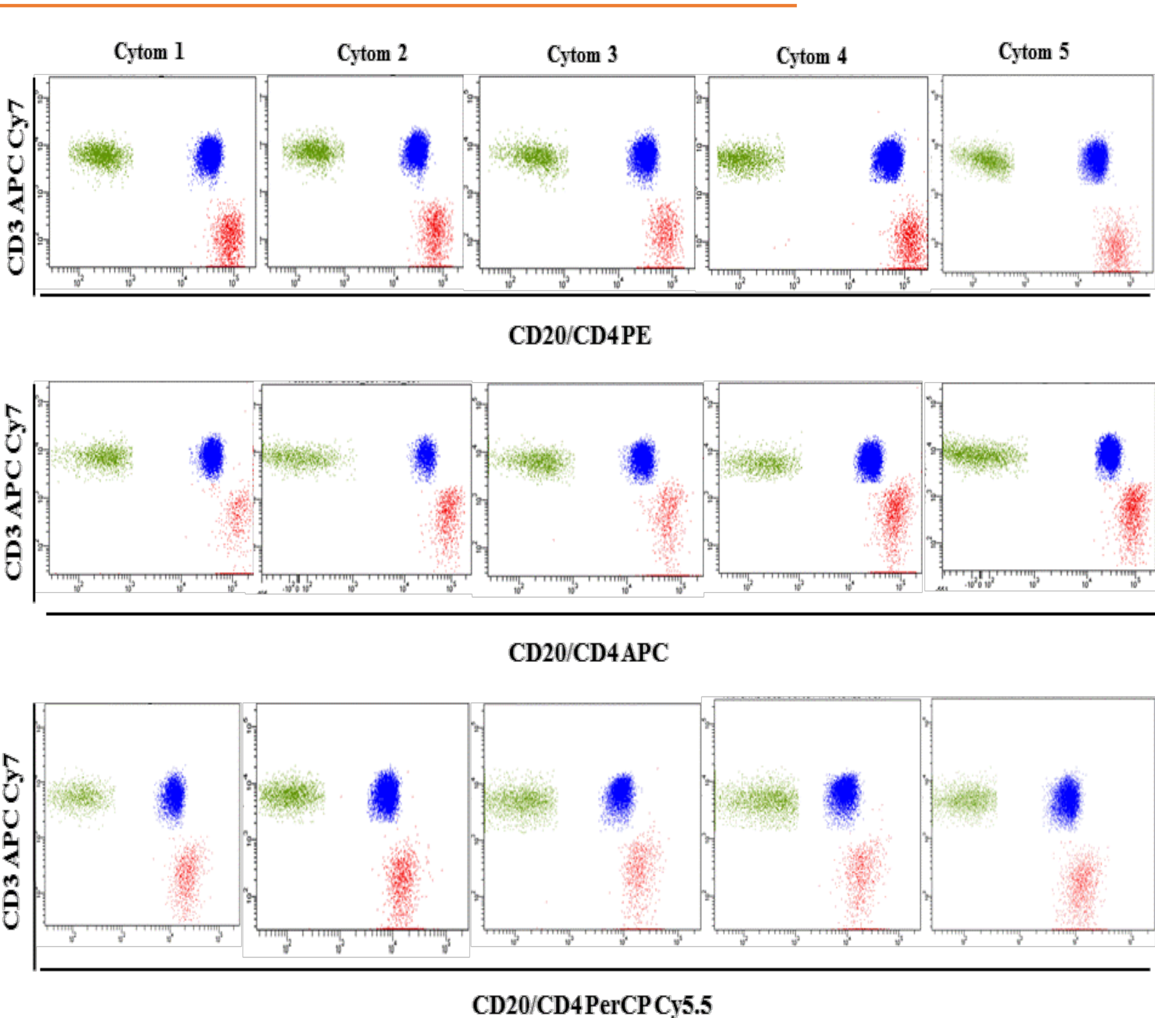
Quantifying CD20 Expression Based on CD4 Reference

Cytometer Setting

1. Identify a target cytometer with largest SD_{EN}
2. Set up PMT voltages ensuring that autofluorescence of unstained whole blood sample are within 2.5-3 times of SD_{EN} of that detector and signals from positively stained samples are within the detection range
3. (a) Run hard-dyed calibration beads and ensure linearity in the channel used for biomarker quantification; (b) record mean fluorescence intensity (MFI) of a bead population with medium intensity for all channels, and transfer the MFI to all other cytometers
4. Perform cytometer compensation with singly stained controls
5. Run test samples

Samples

Whole blood samples were stained with a cocktail of the following antibodies under saturation conditions: CD45 AmCyan, CD3 APC-Cy7, CD19 V450, either CD4 PE / CD20 PE, CD4 APC / CD20 APC, or CD4 PerCP-Cy5.5 / CD20 PerCP-Cy5.5



Cytometry Part B (Clinical Cytometry) 90B:159-167, 2016

Modified assay protocol

3. Protocols

Materials to Be Supplied by the User

- 96-well plates suitable for tissue culture
- repeating pipettes, digital pipettes or multichannel pipettes
- 96-well plate reader

3.A. General Protocol

1. Thaw the CellTiter 96® AQ_{ueous} One Solution Reagent. It should take approximately 90 minutes at temperature, or 10 minutes in a water bath at 37°C, to completely thaw the 20ml size.
2. Pipet 20µl of CellTiter 96® AQ_{ueous} One Solution Reagent into each well of the 96-well assay plat samples in 100µl of culture medium.

   Note: We recommend repeating pipettes, digital pipettes or multichannel pipettes for conve uniform volumes of CellTiter 96® AQ_{ueous} One Solution Reagent to the 96-well plate.

   Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO_2 atmosphere.

   Note: To measure the amount of soluble formazan produced by cellular reduction Step 4. Alternatively, to measure the absorbance later, add 25µl of 10% SDS SDS-treated plates protected from light in a humidified chamber at ro Step 4.

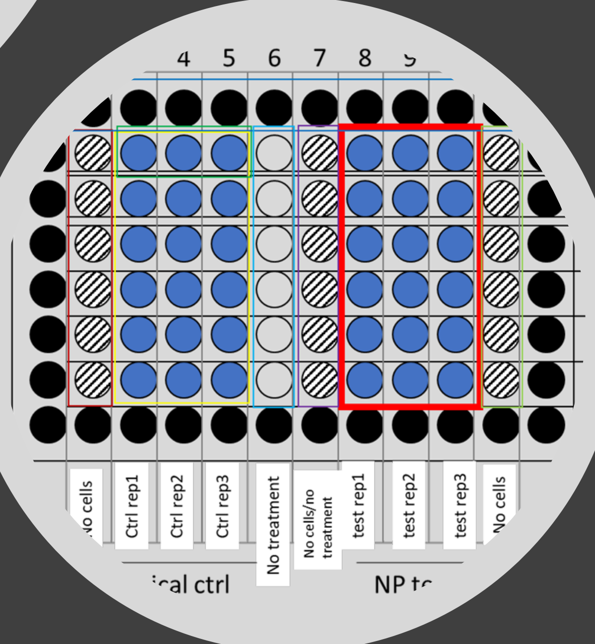
   ...ce at 490nm using a 96-well plate reader

...ications for Control Experi...

...nsus Test Result: NP EC_{50} no serum=23±3 µg/mL; NP EC_{50} serum-

Control	Serum free			Serum		
	target value	range	variability	target value	range	vari...
Control 1 (within) B6 – G6	1.8 OD	1.5-2.0 OD	<10%	2.0 OD	1.8-2.3	<7%
Control 2 (between) B3-B6 B8-B10	1.5 OD	1.3-1.8 OD	<12%	2.2 OD	1.8-2.8	<7%
Control 3A Background B7-G7	0.06 OD	0.05-0.09 OD	< 6%	see serum free		
Control 3B [1] Background Chemical Control B2-G2	0.06	0.05-0.09	<6%	see serum free		
Control 3C [2] Background NP B11-G11						
Control 4 [3] Chemical reaction control	49.9	47.5-51.5		77.2	54.3-99.4	

...he process control measurements provide evidence the ...easurement system is functioning correctly



Evidence-Based Protocols

- Modified assay protocol includes:
  - In-Process controls/Assay plate layouts
  - Specific pipetting instructions
  - One-off process controls (when required)
  - Specification targets

Evidence-based Protocols for Flow Cytometry

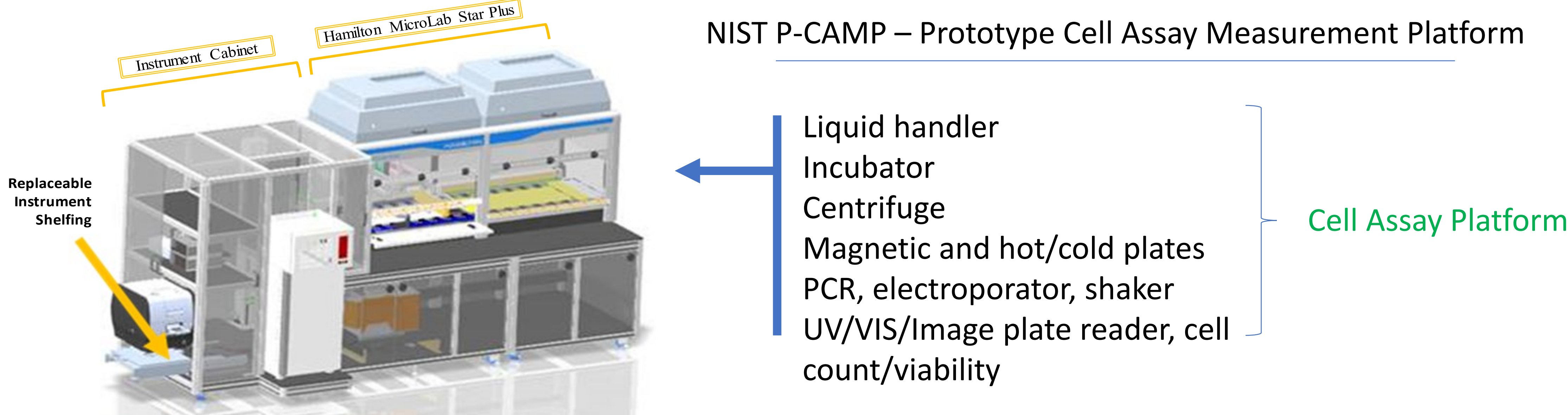
- Measurement science tools can guide a measurement assurance strategy
- Level of control depends on the fit-for-purpose application
- Precompetative, but facilitates customization for specific product, cell type, etc

Possible Protocol Needs for Consortium?

- Cell type specific biomarker detection
- Fluorescence reference systems
- Process controls for sample preparation and staining
- One-off process controls for customized system suitability
- Reference materials for instrument-independent gaiting

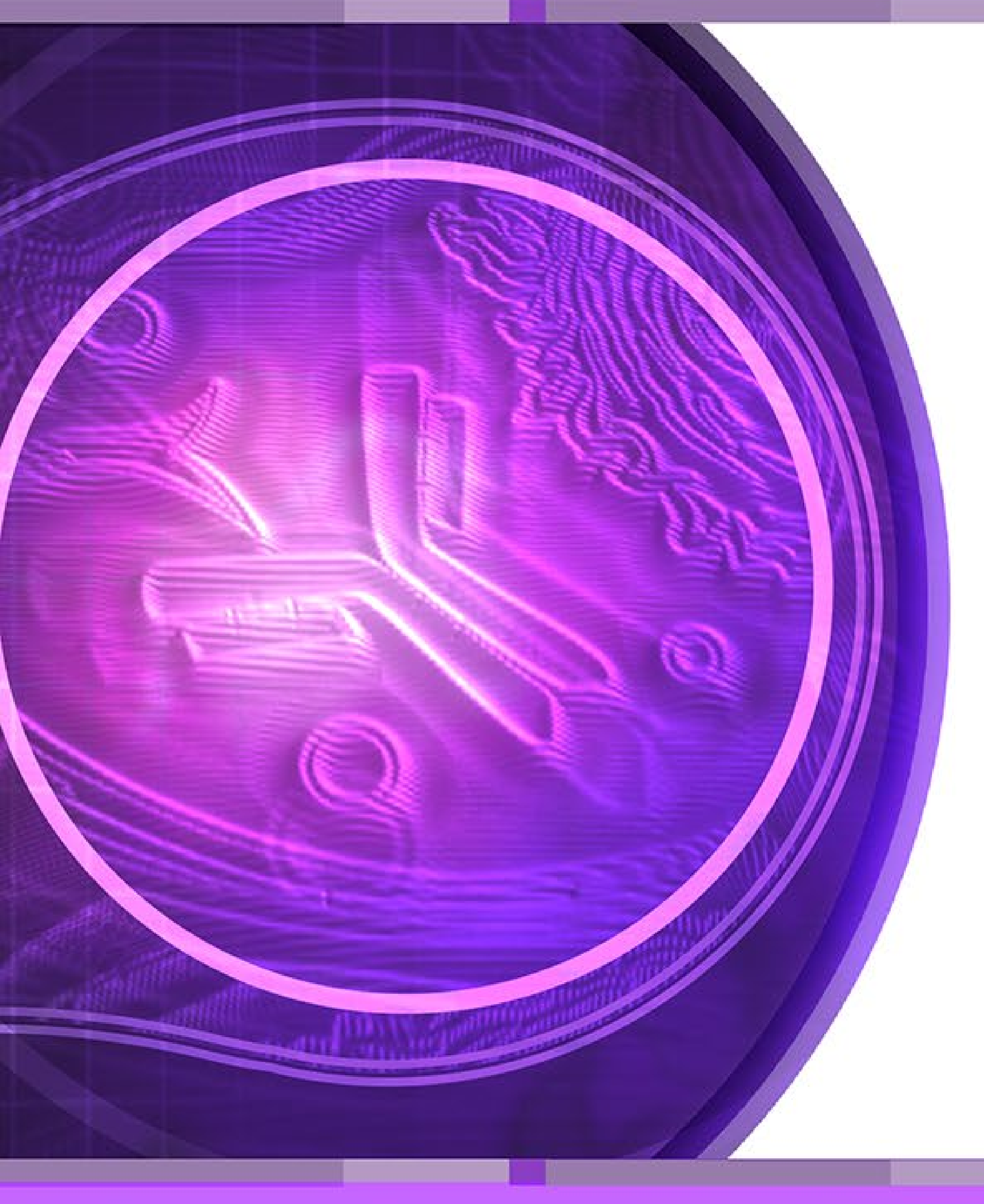
Leveraging automation

- Integrates to plate design concept
- Facilitates performing a large number of experiments
- Intentionally introduce variability
- Serve as an intra-laboratory test platform



Summary

- **Measurement science tools for protocol development**
  - Sources of uncertainty
  - One-off control experiments
  - In-process control experiments
  - Specifications on measurement system controls
  - Robustness and interlaboratory testing- possible automation assist
- **Assay Plate Design**
  - In-process controls for realtime readout of measurement system performance
  - Designed for protocol performance monitoring and not test number
- **Evidence-based Protocol**
  - Protocol with assay plate design including control experiments and specifications
  - Suitable to develop an SOP
  - Can be tailored to a specific item (i.e. cell line, biomarker, assay readout)
  - Possible consortium output and starting point for a standard.



Solutions for Consistent Cell Therapy Manufacturing

Anagha Divekar

Our Mission

Enabling Legendary Discovery from Research to Cure

Our Way….

- Privately owned company, incorporated in 2002
- Headquartered in San Diego, California
  - New five building campus opened mid-2019
- Over 27,000 Catalog products, 100+ new products per month
- Several disruptive technology platforms enabling rapidly growing applications across proteogenomics and COVID-19 research
- Certifications
  - ISO 13485:2016 for all operations since 2018
  - MDSAP certified for designated suite since 2019

Custom & Off-the-Shelf Products Tailored to Your Specific Needs

Conjugated Antibodies

Cocktails, Liquid & Dried Reagents

Assay Development Across Multiple Platforms

Recombinant Protein & Antibody Development

Soluble MHC Products: Flex-T™

Biomarker Multiplexing: LEGENDplex™

Control Cells: Veri-Cells™

Proteo-genomics: TotalSeq™

Bioprocessing & GMP Solutions

From Ideas To Solutions

Cell and gene therapy manufacturing needs

Batch to Batch Consistency

- Quality products – ISO 13485:2016, GMP
- Documented processes: SOPs, Batch Records, Product Specifications

Analysis of Potency

- Consistent standardized assays
  - Biomarker multiplexing, ELISA, functional bioassays

QC of Final Product

- Cell health – apoptosis, live/dead discrimination, mitochondrial function
- Phenotype analysis
- Assay controls (Veri-Cells™)

Batch Size

- Bulk size of validated reagents available in mg or gm size at desired specifications
- Lot to lot consistency data

Cell and gene therapy manufacturing needs

Customization

- Formats (cocktails, dried or lyophilized)
- Custom proteins or antibodies, recombinant or hybridoma
- GMP Manufacturing

Therapy effectiveness

- Single cell analysis by flow cytometry or proteogenomics
- Consistent standardized assays
  - Biomarker multiplexing, ELISA, bioassays
- Phenotyping

Veri-Cells™

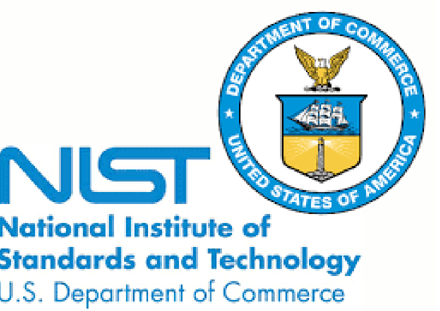
Lyophilized human cells

- Long shelf life (up to 4 years)
- Large batches available (1000’s of vials)
- Wide range of marker detection (150+)
- Customized versions to desired specifications

Lyophilized/dry down reagents

- Large batch sizes, lot to lot bridging
- Long term stability (up to 2 years)
- Customized versions to desired specifications including cocktailing service with non-BioLegend vendor reagents
- Custom packaging options
- Stimulation reagents

For more information contact us at: info@biolegend.com



Flow Cytometry Standards Consortium Workshop

Is digital flow cytometry the future ?

Single photon with SI Unit

J. Paul Robinson
Professor & CEO

Feb.17, 2021

J. Paul Robinson/M.Yamamoto

Masanobu Yamamoto
Physicist & Inventor
CTO

A Purdue Research Park Company

~2000 – Flow cytometry moved from analog to digital…

Or did it?

Really all we did was move from measuring analog signals and plotting them, to measuring analog signals and converting to digital…and then plotting them…

Yes, it helped in many ways but its had not given us a path to true quantitation. It's a little more than a band-aid, but its only a bandage…

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And the question is..

What do we do next?

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Flow signal origin is photons as an energy packet (joule)

If we can detect photon directly……

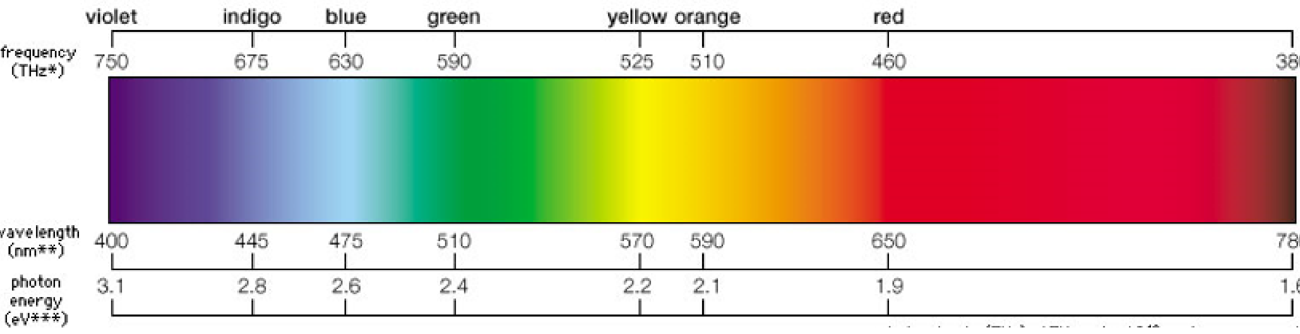
-Photon energy is determined by frequency(wavelength)
-Photon is digital in nature and " bit " equivalent
-Photon is defined by SI unit
-Photon is quantum with statistics

Challenge: sub-ns photon detection/signal processing required

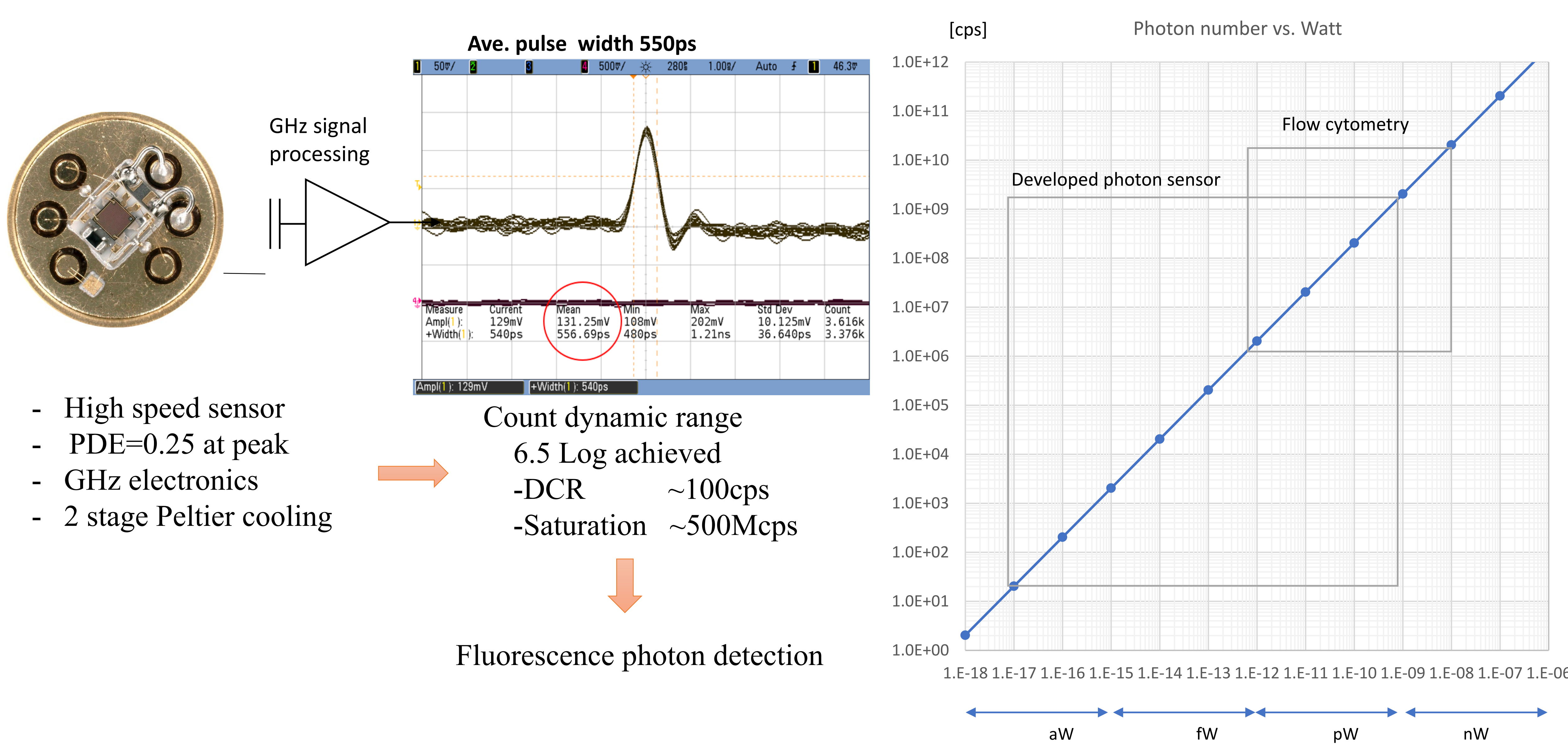
<Flow Signal>
-Rayleigh/Raman scatter
-Fluorescence
-Autofluorescence/Stray

Fluorescent Channels
To CCD or Computer
Dichroic
Laser
Side Scatter Detector
Bandpass Filter
Forward Scatter Detector

Photon Energy : **E= hν** (h: Plank constant 6.602E-34 ν= c/λ)
E= 1,240/ λ [nm] [eV] eV= 1.60E-19 [J]
400nm Photon = 3.1eV = 0.5aJ = 2 Mega photon/pW
800nm Photon = 1.55eV=0.25aJ= 4 Mega photon/pW



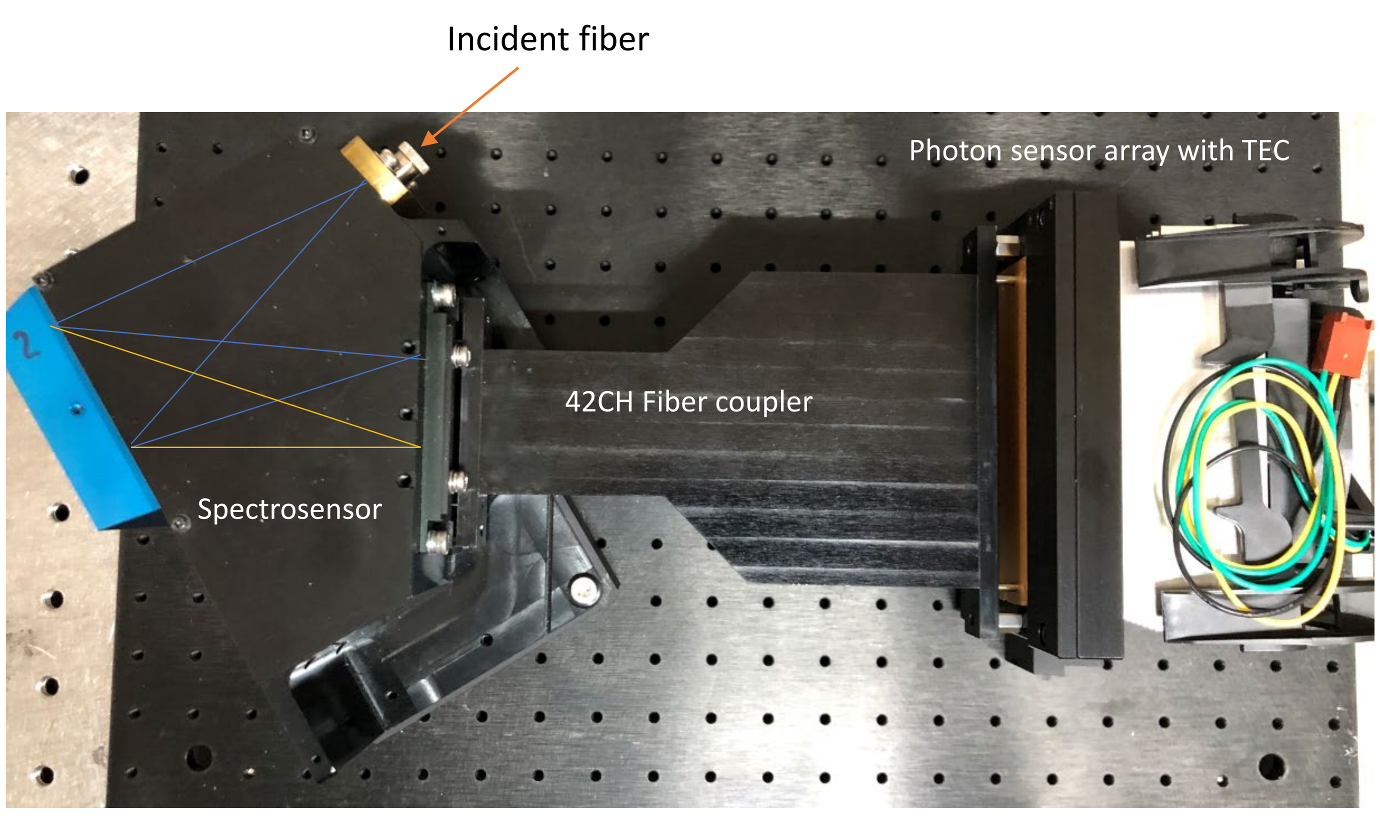
Developed sub-ns photon signal detection



Single channel to spectral photon sensor array(42CH 350-800nm)

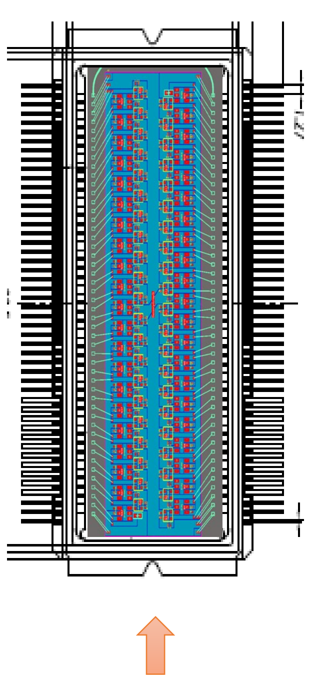
8CH was developed by NSF Award

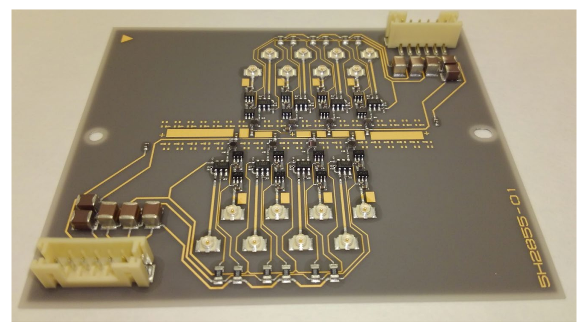
Incident fiber



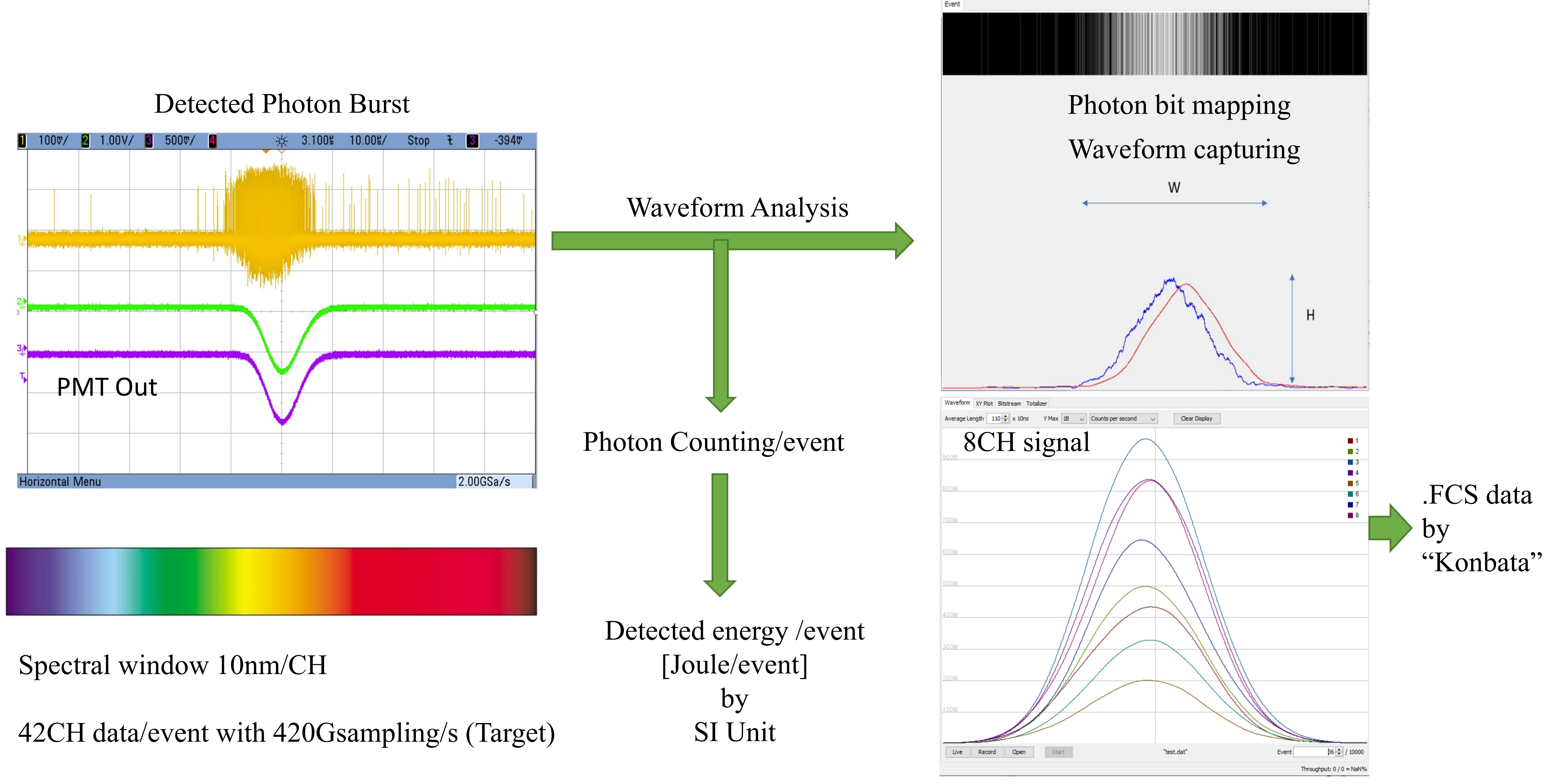
Spectral photon sensor array unit with cooling

42CH sensor array





Developed
8CH sensor array


Photon Digitizer for Photon Bit with 10Gs (100ps) time addressing
8CH 2.5Gs is operating
Detected Photon Burst
PMT Out
Horizontal Menu
2.00GSa/s
Waveform Analysis
Photon Counting/event
Detected energy /event
[Joule/event]
by
SI Unit
Photon bit mapping
Waveform capturing
W
H
8CH signal
.FCS data
by
“Konbata”
Spectral window 10nm/CH
42CH data/event with 420Gsampling/s (Target)

Discussion:
What and how photon detection contribute Flow Signal Standards ?

< Flow Cytometry Contribution?>

-Quantization

-Reproduce-ability

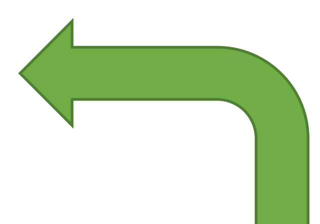
-Data Compatibility

-Relative to Absolute

-Calibration

-Data Description

- Next Flow Standards based on SI ??



Photon physical nature:

- energy packet E= hν [joule]
- digital in nature = bit
- Energy conservation principle
- SI unit
- time domain information
- Photon Statistics (Poisson base)
- Quantum characteristics

Biological Potential:

- listen “Voice from molecules”

  (photon counting is just “Loudness”)
- Sensitivity – nanoparticle detection
- Energy packet – Calibration
- Time-correlated measurement
- Lifetime /Imaging
- etc.

SI Units

NIST web:

The SI rests on a foundation of seven (7) defining constants: the cesium hyperfine splitting frequency, the speed of light in vacuum, the Planck constant, the elementary charge (i.e. the charge on a proton), the Boltzmann constant, the Avogadro constant, and the luminous efficacy of a specified monochromatic source. Definitions of all seven (7) SI base units are expressed using an explicit-constant formulation and experimentally realized using a specific *mises en pratique* (practical technique).

The seven SI base units, which are comprised of:

- Length - meter (m)
- Time - second (s)
- Amount of substance - mole (mole)
- Electric current - ampere (A)
- Temperature - kelvin (K)
- Luminous intensity - candela (cd)
- Mass - kilogram (kg)

Digital Flow = Flow paradigm by Planck constant & time keeping compatibility with conventional method

The International System of Units (SI), commonly known as the metric system, is the international standard for measurement. The International Treaty of the Meter was signed in Paris on May 20, 1875 by seventeen countries, including the United States and is now celebrated around the globe as World Metrology Day . NIST provides official U.S. representation in the various international bodies established by the Meter Convention: CGPM - General Conference on Weights and Measures; CIPM - International Committee for Weights and Measures; and BIPM - The International Bureau of Weights and Measures.

The SI is made up of 7 base units that define the 22 derived units with special names and symbols. The SI plays an essential role in international commerce and is commonly used in scientific and technological research and development. Learn more about the SI in NIST SP 330 and SP 811.

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Precision genetic device engineering with TASBE Flow Analytics

Jacob Beal

NIST Flow Cytometry Standards Consortium Workshop

February, 2021

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Three Spheres of Synthetic Biology

[Basu et al, '05]

[Danino et al., '10]

Informational

biological circuits

Structural

tissue engineering

DNA nanotech

Chemical

chemical synthesis

biofuels

synthetic vaccines

microbial fuel cells

DNA memory

organoids

biosensors

CAR-T therapy

gene drives

biomaterials

crop editing

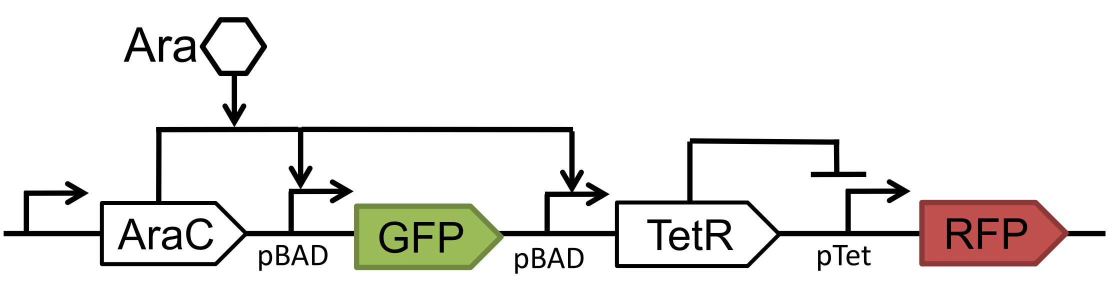
[Quadrato et al., '17]

[Ro et al., '06]

Most complex applications will require all three

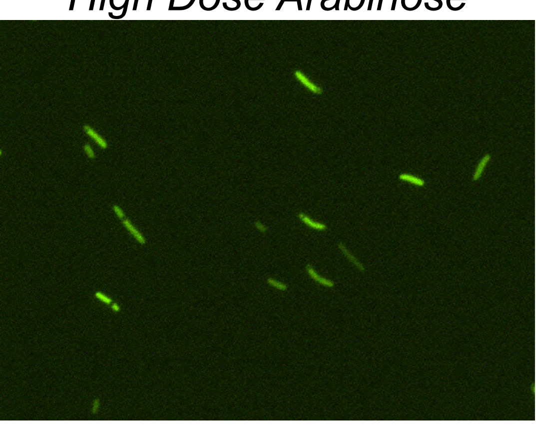
Focus: Information Processing

Biomolecular circuits regulate cell behavior:



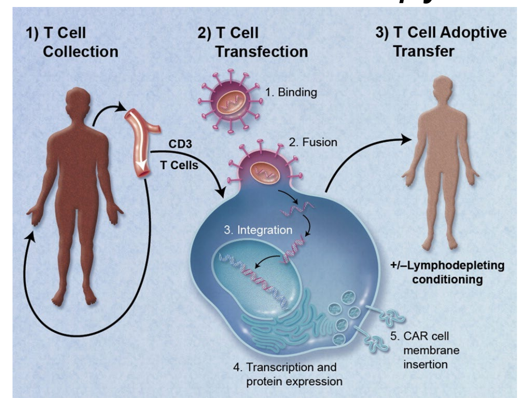
No Arabinose

High Dose Arabinose



Many potential applications:

CAR T-cell therapy



Fermentation control

Engineering: From Parts to Systems

Healthy? → NOT → Produce Drug

Systems — ***Predictable Design***

Boolean NOT

Devices — **Calibrated flow, plate → IO Range, SNR, … ???**

Parts — **BioBricks → GG, MoClo, BASIC, …**

`ttgatggctagctcagtcctaggtacaatgctagctaga...`

DNA

[Beal et al., TIBTECH '18, Endy, Nature '05]

Flow cytometry is useful for engineering

Example: RNA Replicon Cotransfection

Quantization

Resource Variation

Dose Variation

Dose Efficiency

$\log_{10}$ Blue MEFL

$\log_{10}$ Red MEFL

[Beal et al., '15]

Example: *E. coli* GFP interlab (244 teams)

iGEM

$\log_{10}$ MEFL

Microsphere Dilution

Flow Cytometer

J23101 J23106 J23117 J23100 J23104 J23116

Device

[Beal et al., '20]

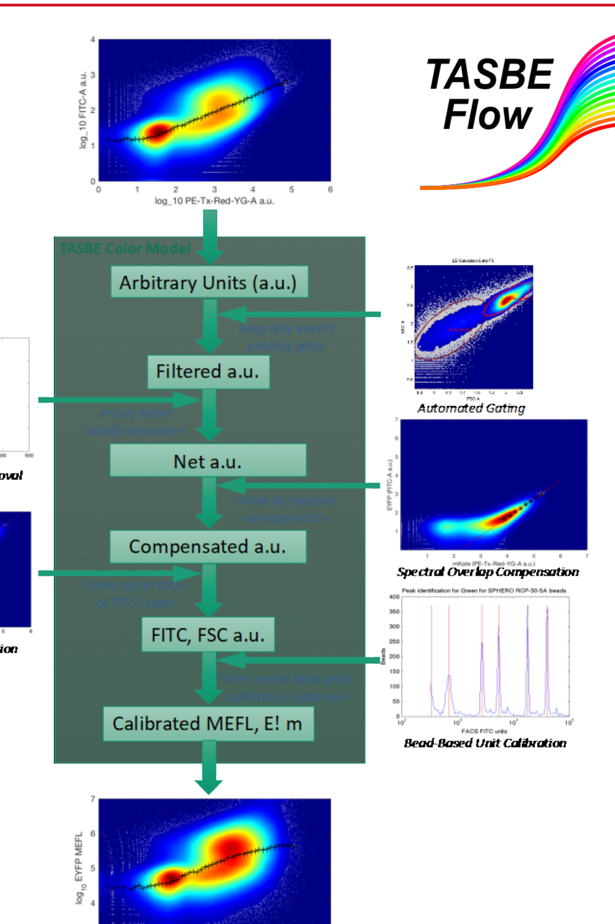
- Components of variation expose biological mechanisms & model parameters
- Calibrated flow cytometry data is readily replicated, compared, fused, and applied

TASBE Flow Analytics

- **Free & open package for Matlab, Octave, Python**
- Calibration with standard materials:
  - MEFL units from NIST-certified rainbow beads
  - Background subtraction w. WT/NT
  - Spectral compensation w. single positives
  - Color comparison w. multi-color controls
- Supports high-throughput analysis pipelines
- Optional “bench-friendly” Excel UI

- Related tools: CytoFlow (MIT), FlowCal (Rice)

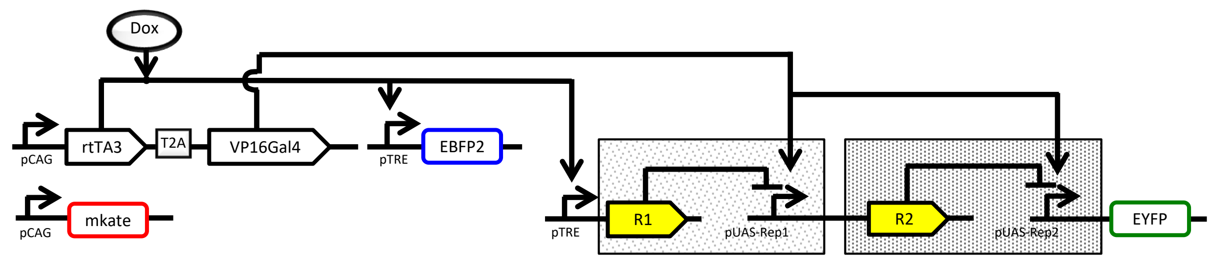
https://github.com/TASBE/TASBEFlowAnalytics



[Roederer, ‘02; Wang et al., ‘08; NIST/ISAC, ‘12; Beal et al., ‘12; Castillo-Hair et al., ’16; Beal et al.,’19]

Precision Predictions: Circuit Modeling

Example: high-precision prediction of cascades and feed-forward networks using approximate ODEs and calibrated fluorescence measurement



Prediction of Repressor Cascade

TAL14 → TAL21

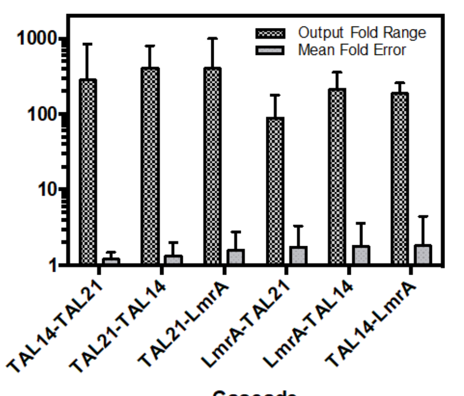
+: experimental
o: predicted

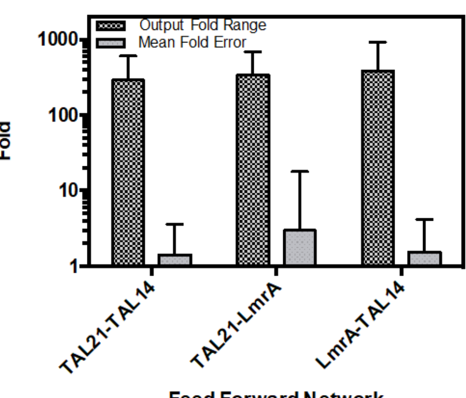
OFP MEFL
IFP MEFL

Each line is a dose/response curve for a different relative number of circuit copies.

Subpopulation identified by color on inset mKate histogram

Range vs. Error for 6 Cascades, 3 Feed-Forward Networks





[Davidsohn et al., 2014]

Precision Engineering: Replicon Expression

Example: Per-cell measurement of dose-response gives model for precision control of expression in mixtures of Sindbis RNA replicons

Example Prediction of 3-RNA Replicon Mix:

Prediction / Experimental — Fraction of Cells (0 to 0.12) vs. Mean MEFL (10^4 to 10^9)

Range vs. Error for 6 Mixtures

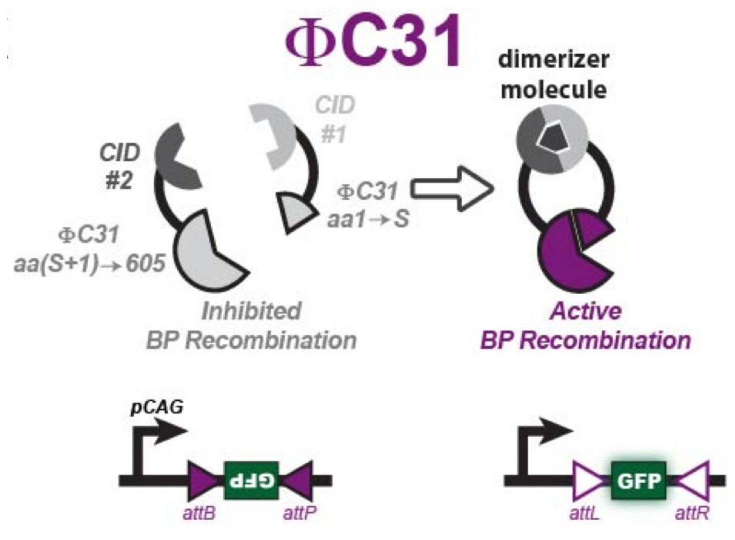
Mean Prediction Error — Fold (10^0 to 10^4) vs. Mix Number (1–6); Range / Error

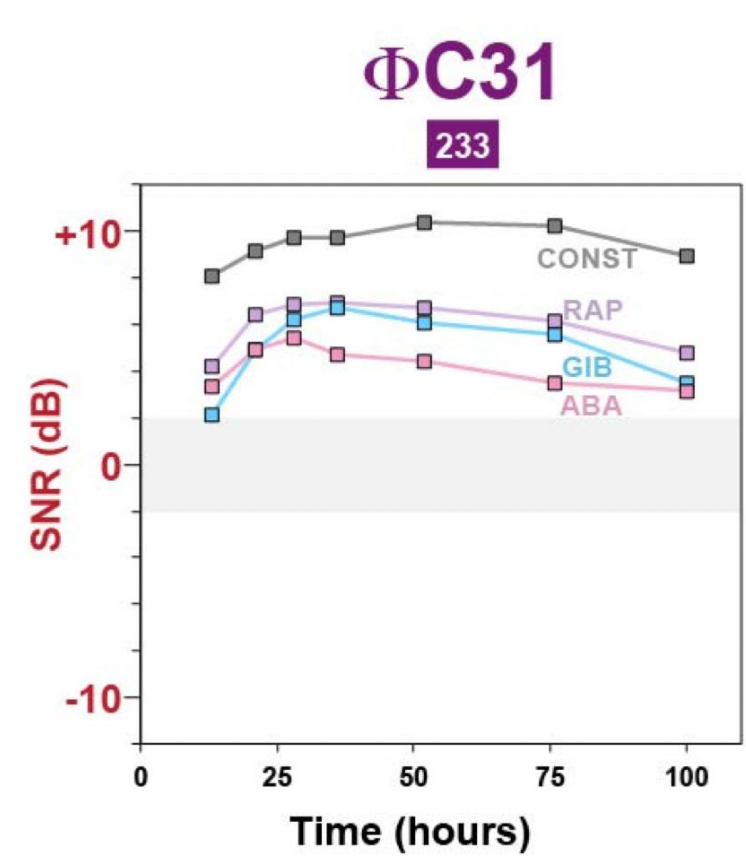
nsP1-4 → SGP → mVenus
nsP1-4 → SGP → mKate
nsP1-4 → SGP → EBFP2

Mix 1: 0.1Y, 0.1R, 0.1B
Mix 2: 0.3Y, 0.3R, 0.3B
Mix 3: 0.1Y, 0.5R, 0.4B
Mix 4: 0.2Y, 0.2R, 0.6B
Mix 5: 0.01Y, 0.1R, 0.5B
Mix 6: 0.4Y, 0.02R, 0.02B

[Beal et al., 2014]

Better Devices: High-SNR Sensors

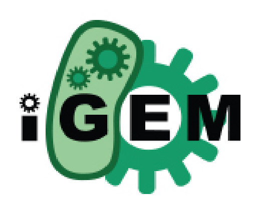




- Split recombinase amplifies dimerizing molecule sensor
- With tuning, sustained 2-6 dB differentiated expression

[Weinberg et al, Nat.Comm., '19]

Consortium Interests & Challenges

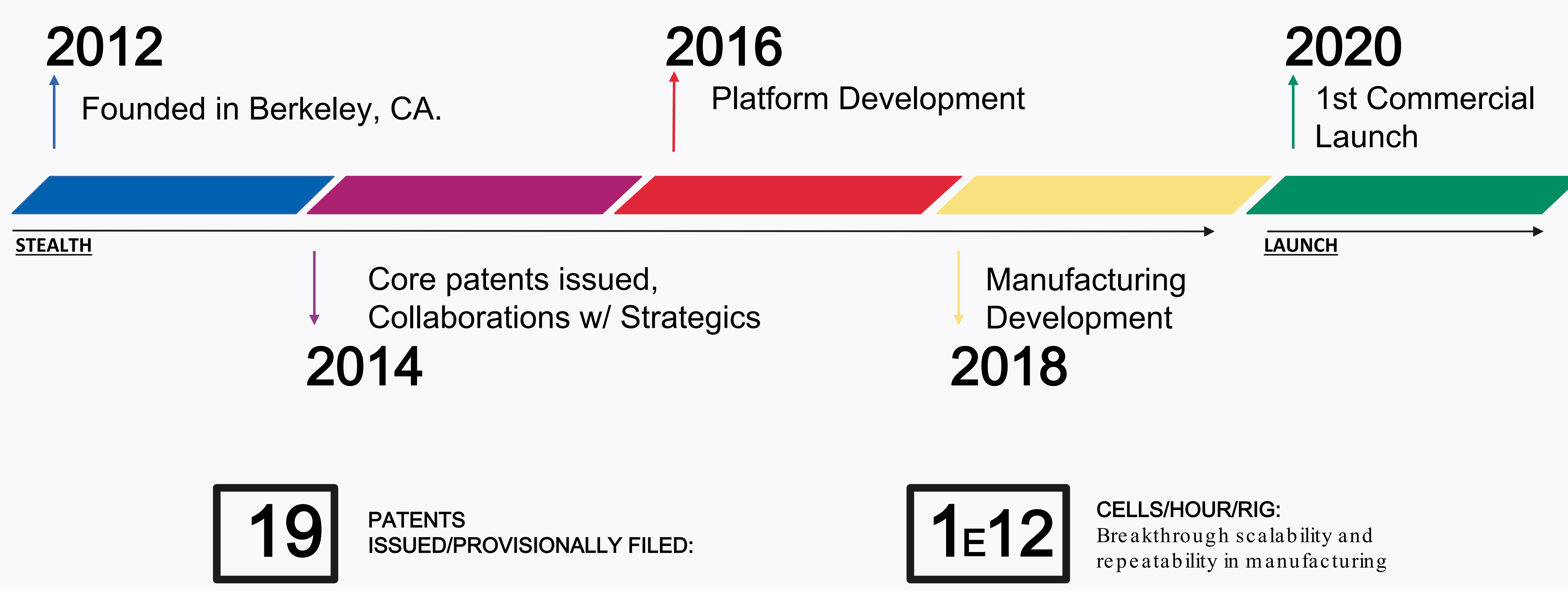


- TASBE Flow Analytics is free & open software: use it, copy it, remix it!
- iGEM Engineering Committee: collaboration opportunity
- Community challenges:
  - Calibrated units are rarely used in the scientific community
  - Machine-friendly registration of bead lot calibration values
  - Standardization of laser/filter/dye/protein combinations
- Technical challenges:
  - Cross-channel comparison
  - Fusion with other instruments: plate reader, microscopy, omics
  - FSC calibration models
  - Calibration of SSC channel, -W and -H channels
  - MEx → Molecules, POPS

SLiNGSHOT

Prepared and presented by Dr. Jeffrey Kim

Our Company History - Stealth to Launch



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Overview

Product Highlight

Applications

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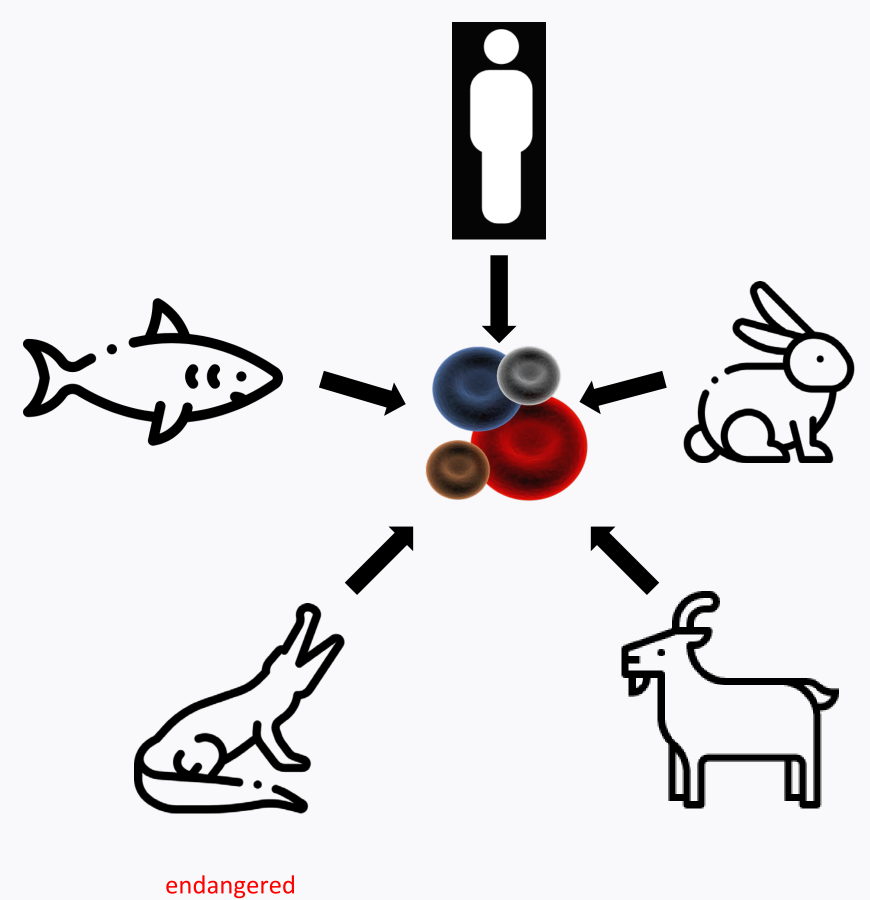
Flow Controls are Comprised of Complex, Unstable Components



Comprised of donated human blood, supplemented with components from diverse animals.

- complex supply chain*
- high variability (batch-to-batch)
- high cost ($xxx-xxxx)
- unstable (weeks->months)
- environmentally and ethically unsound.

Standard Control Market

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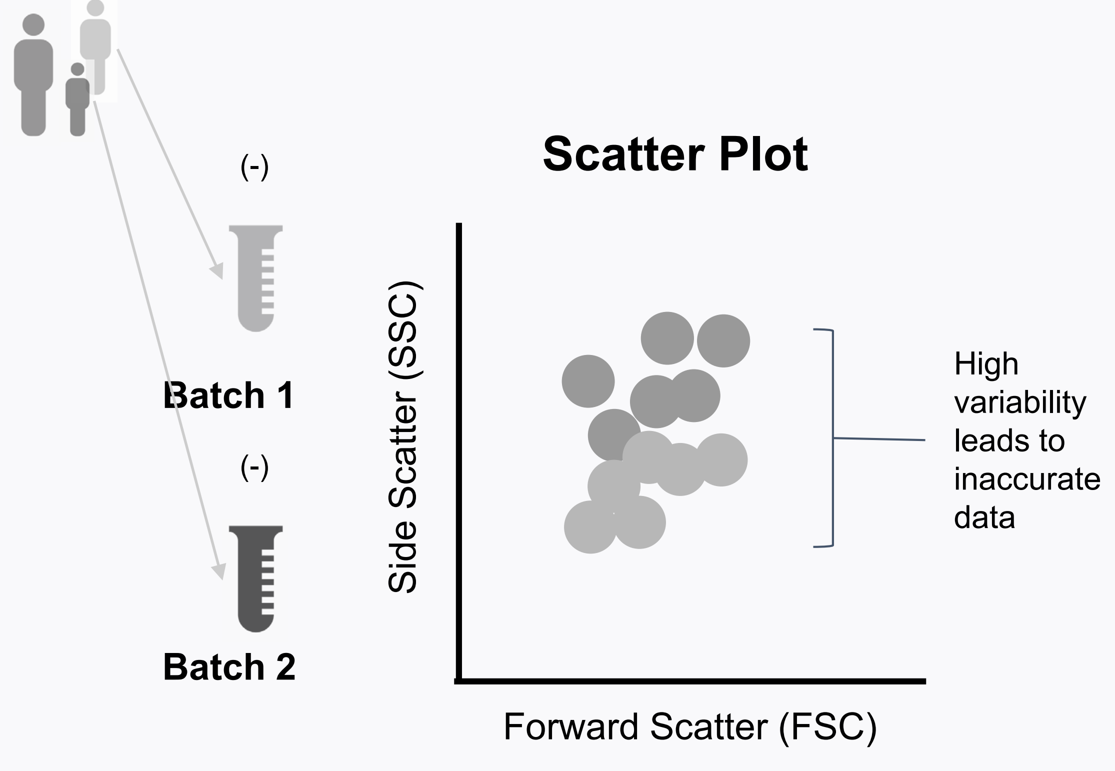
Flow Controls are Comprised of Complex, Unstable Components



Custom controls are comprised of donated human blood or stimulated cohorts from biobanks.

- complex supply chain
- high variability (batch-to-batch)
- high cost ($xxx-xxxx)
- unstable (weeks->months)
- requires $$$ crossover studies

Specialized Control Market

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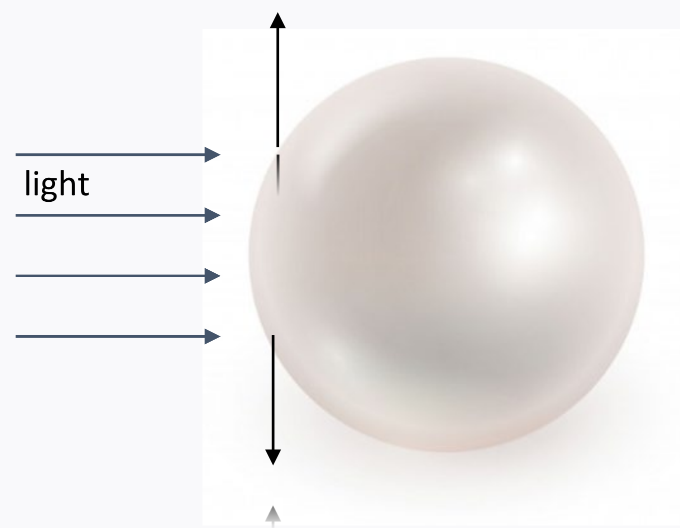
Flow Reagents are Comprised of Polystyrene/Latex

Look nothing like cells, requiring secondary, manual set up processes using cellular controls

"Beads are not cells and do not necessarily scatter light as cells do "[1]
National Institute of Standards and Technology



- Artificial Autofluorescence (Tandem Dye incompatibility)
- Opaque - no internal features
- Dense (clogging/settling)
- Fixed surface area
- Low protein binding capacity

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Cornerstones of an Ideal Flow Control Product

CELL-LIKE PROPERTIES

Can we service the >200 blood diseases that lack off-of-the-shelf reference controls to avoid biobanks or stimulated cohorts?

STABLE OVER TIME

Are the products stable enough to trust from one day\month\year to the next? Will they look the same between different sites?

REPRODUCIBLE BATCHES

Can you make batches that look and act the same to avoid crossover studies?

LOW-COST \ ACCESSIBLE

Can the product be deployed globally to help make diagnosis more consistent across centers/populations?

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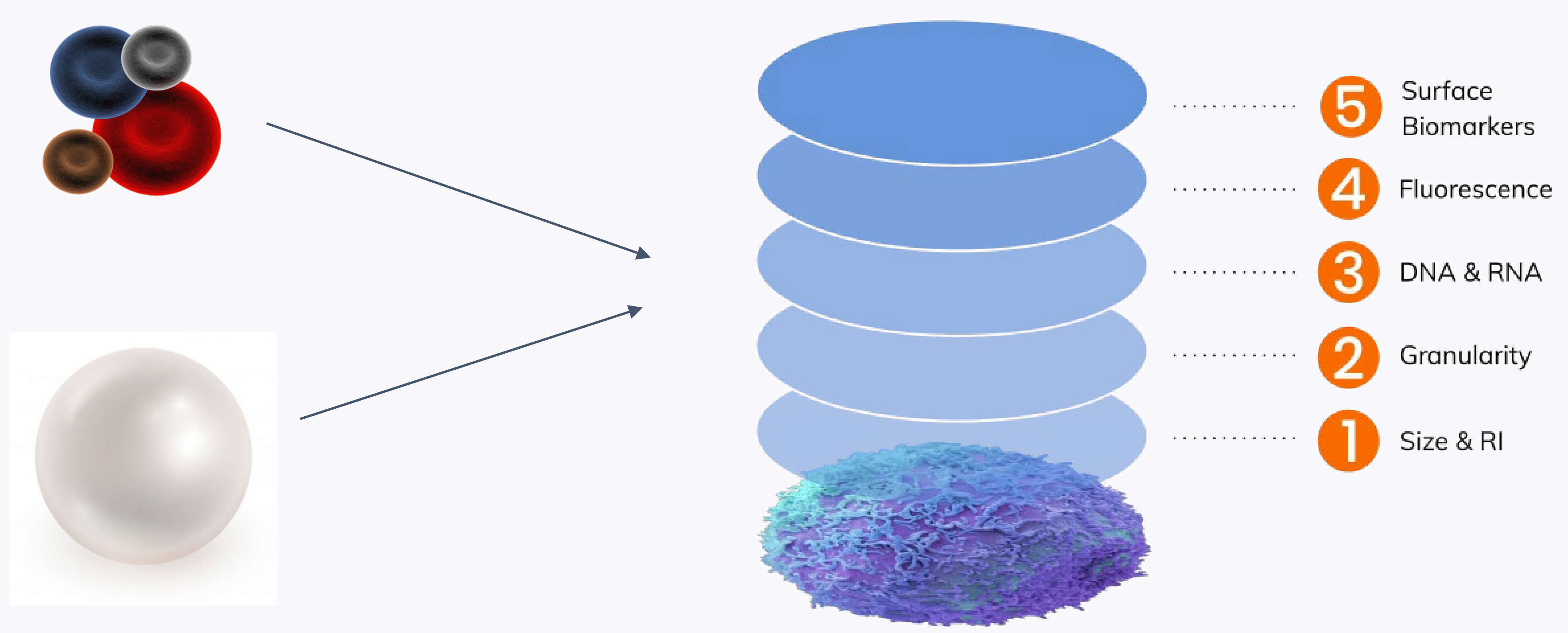
Introducing: FlowCytes™ - Synthetic Cells

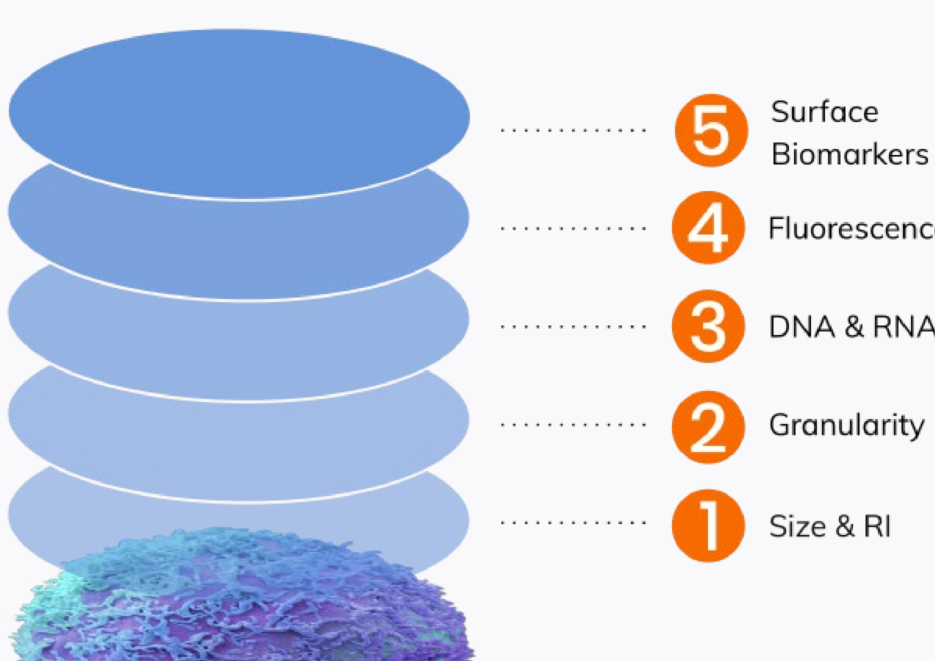


The World's First On-Demand, Synthetic Cell Platform

FlowCytes combine the best features of cells and polymers to address key market opportunities in cellular controls and reagents.

Confidential. Do not reproduce or redistribute.

Introducing: FlowCytes™ - Key Features



Fully Customizable Properties

Instantly tuned (within hours) to match any cell type, rare malignancy, cellular property

Rapid, Consistent, Microprocessor-Based Manufacturing

Real-time development time, 1 week scale up (1E12 cells/hr/rig)

Rock Solid Stability

Stable at room temp in aqueous solution for >10 years. No cold chain requirements

Superior Optical and Biophysical Properties

100x binding capacity, cell-like autofluorescence, time-release payload

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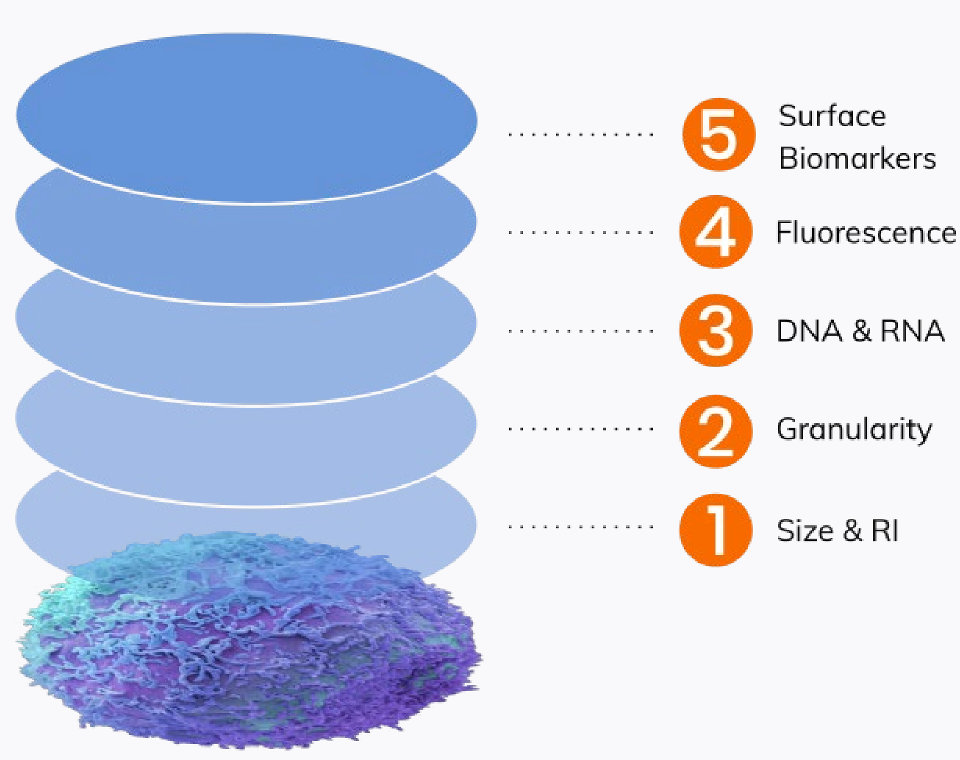
Introducing: FlowCytes™ - Parameter Space



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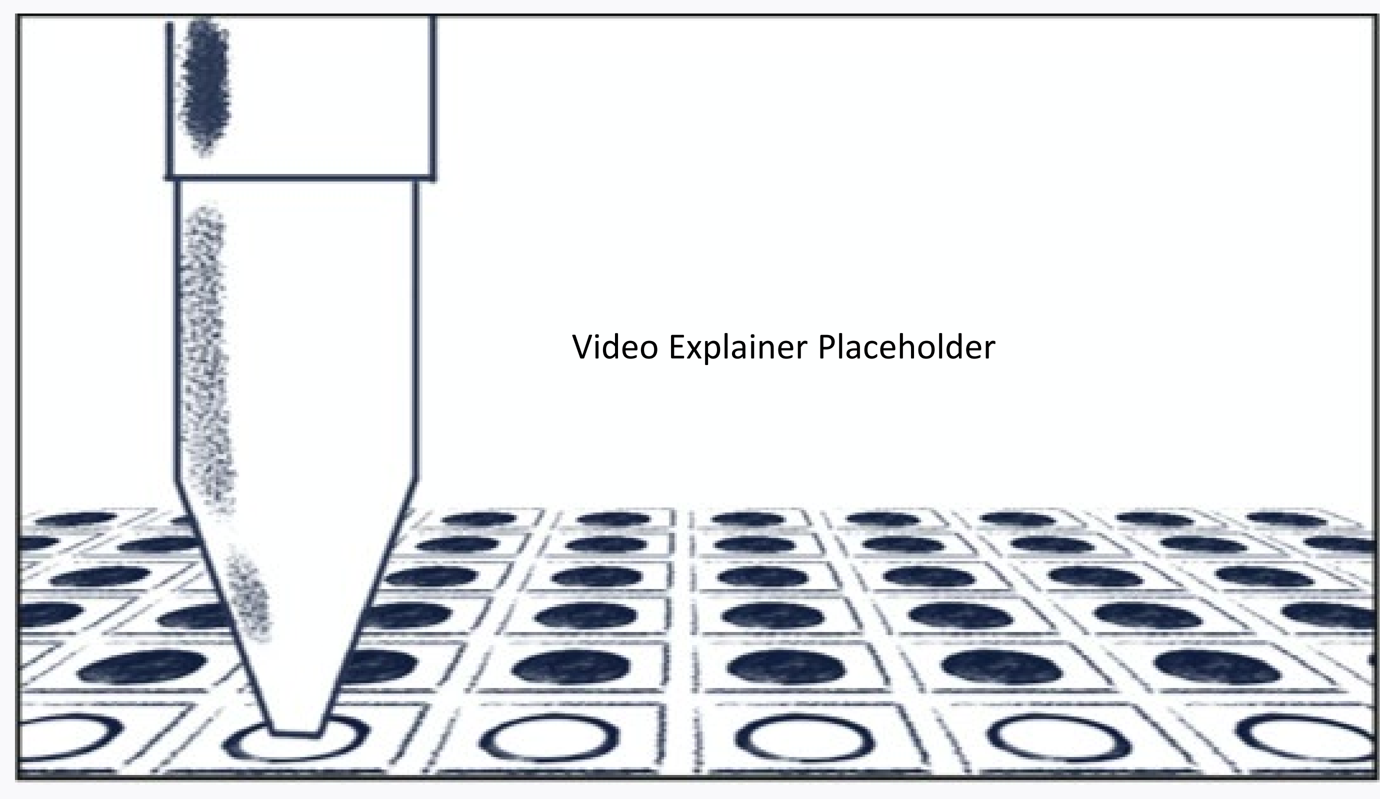
50nm - >1mm

Breakthrough Scalable Microfluidic Manufacturing Process

- Developed patented system that can "print" up to 1 trillion cells/hr/rig, individually, with extremely high precision.

- Can meet global demand in our current footprint for high volume components.



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Overview

Product Highlight

Applications

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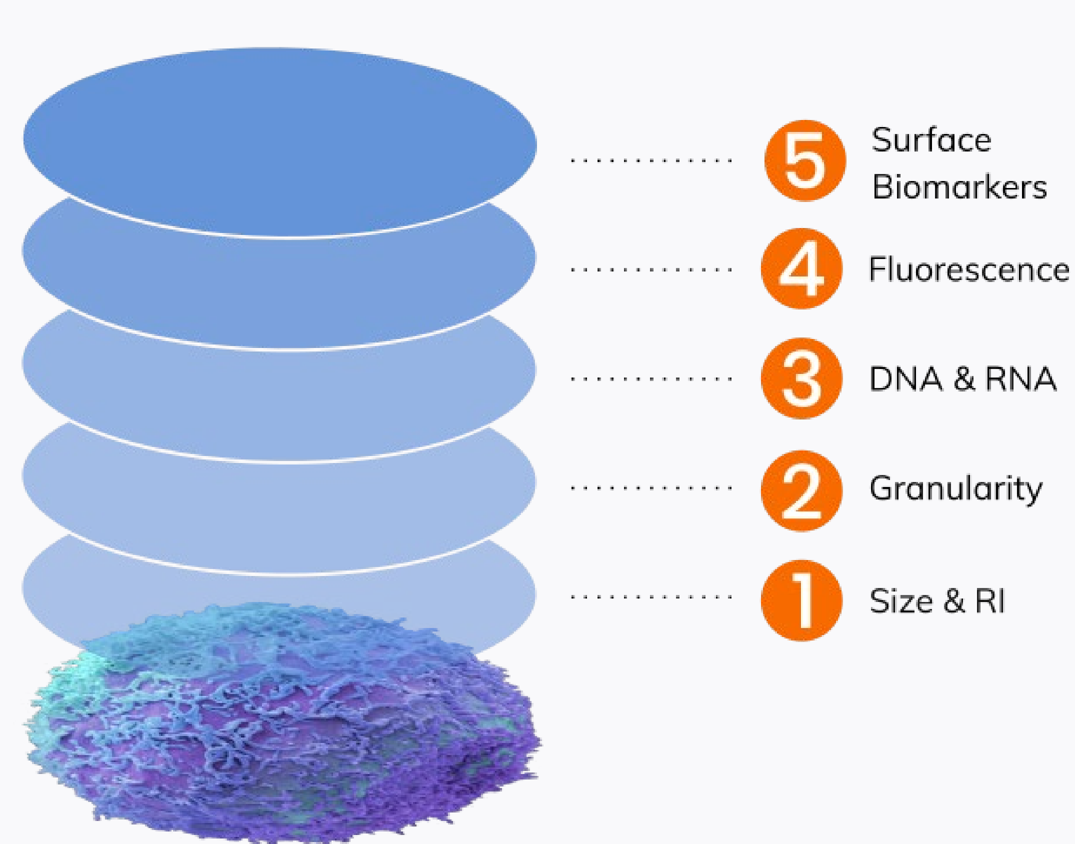
FlowCytes are a Platform for Flow Reagents and More



Traceability Standards

SpectraComp Compensation Beads

Viability Compensation Beads

Single-stain Controls (FC beads)

Antigen Density \ MESF\MEFL Biomarker Beads

FMO Beads....and more

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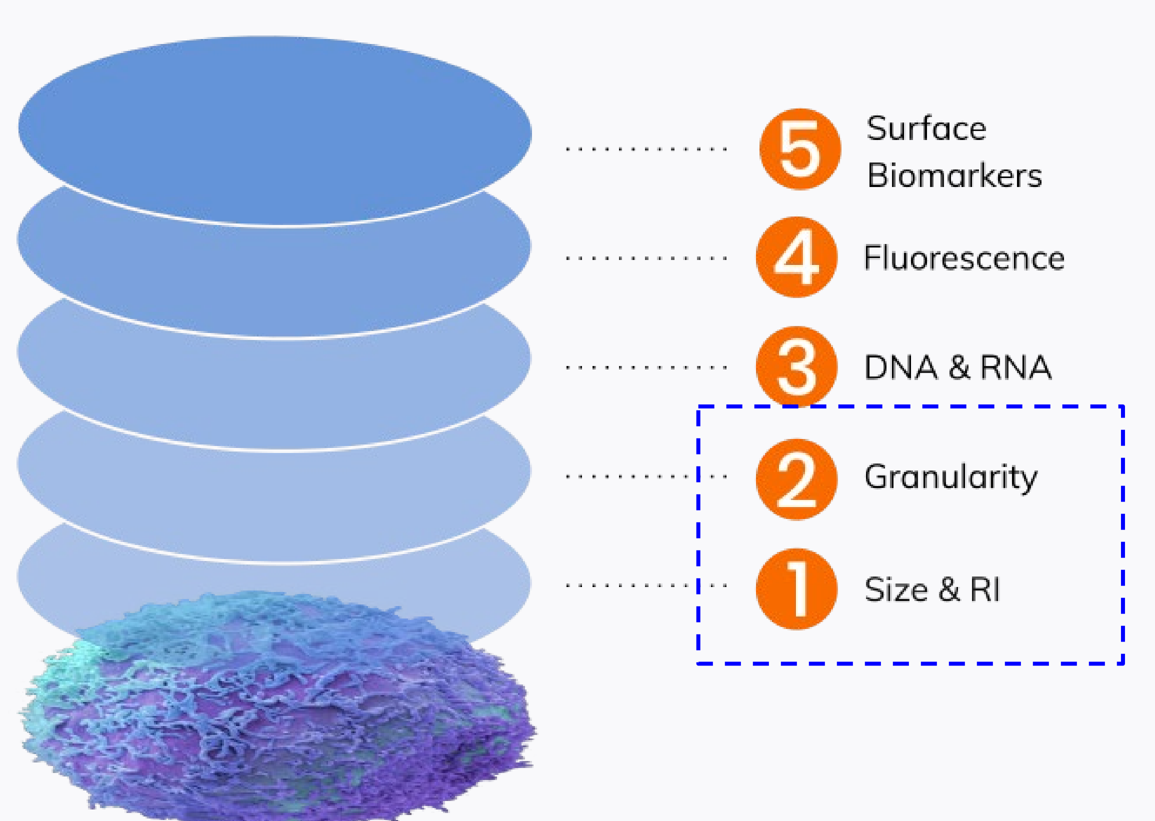
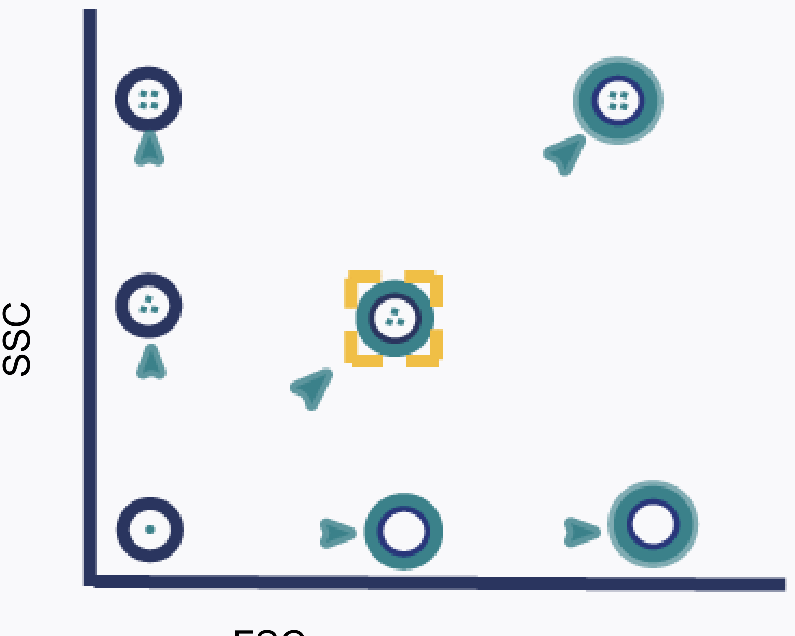
FlowCytes Can Optically Mimic Any Cell Subtype



Traceability Standards



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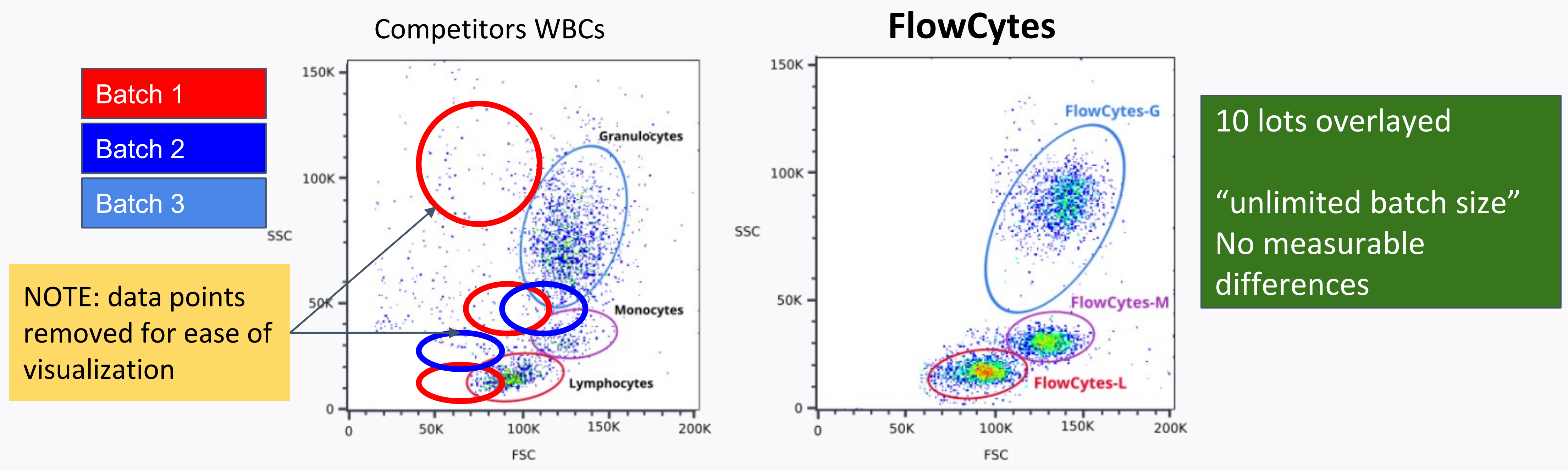
FlowCytes Can Optically Mimic WBC's (stable/traceability control)



- **KEY TAKEAWAY - Eliminate operator and site-to-site variability with the ultimate traceability control for initial set-up. Standardize consortium instrument settings, normalize data.**

- Optimal calibration control for scatter detection and laser optics. Build consistency between manufacturing, multi-site and longitudinal deployments

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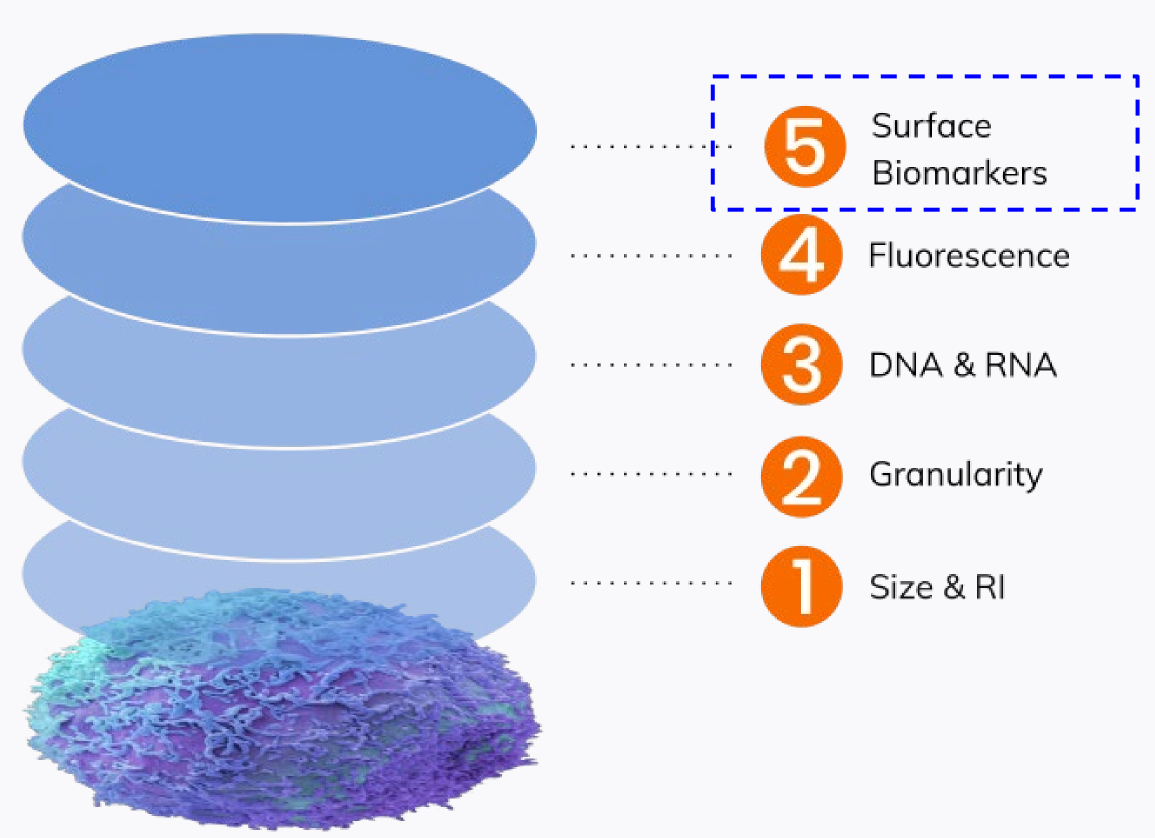
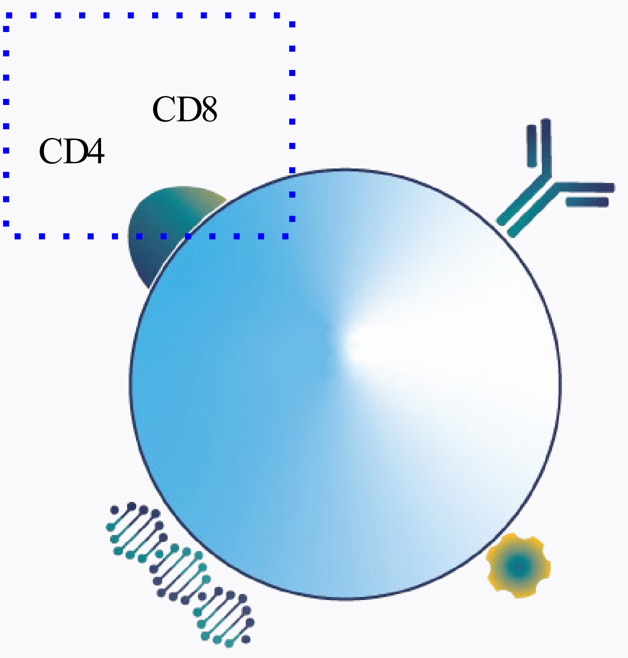
FlowCytes Can be Modified with Biomarkers





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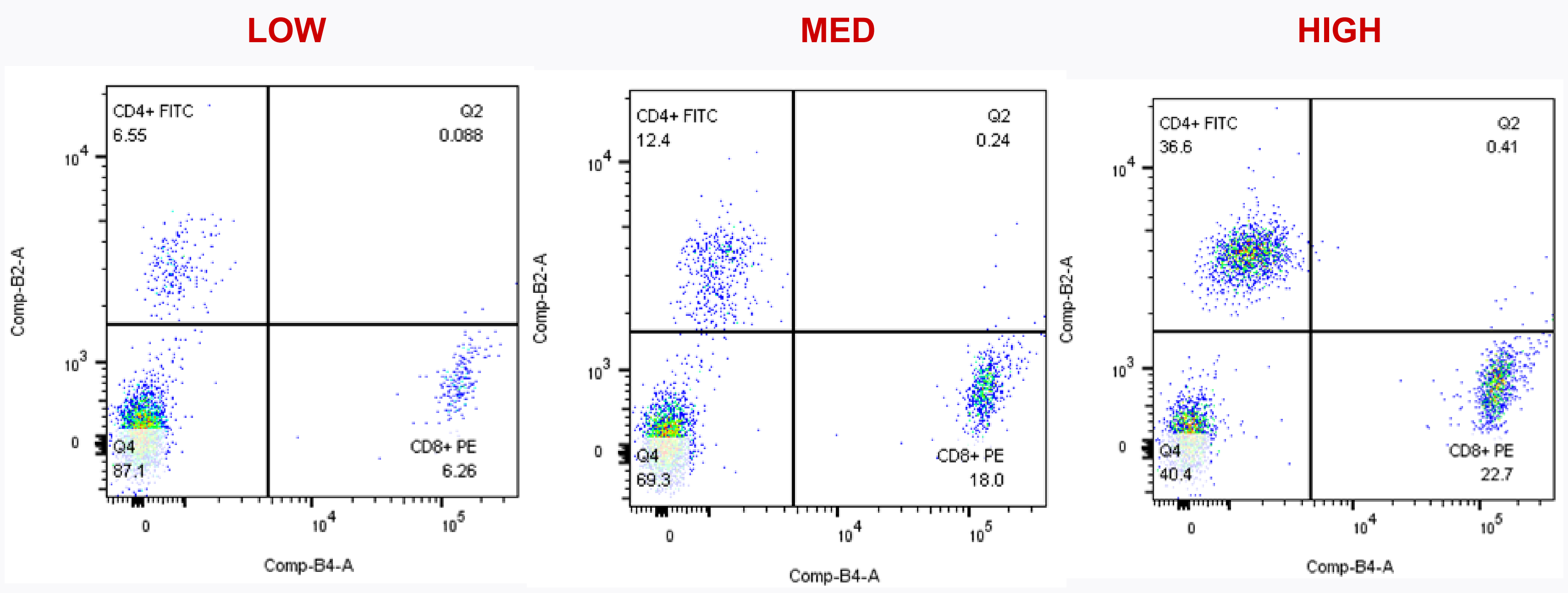
FlowCytes Can be Modified with Biomarkers

CD4 & CD8 lymph beads



*BD MultiTest using aCD4 FITC and aCD8 PE

- **KEY TAKEAWAY - Instantly c**reate multi-level standards for any biomaker(s). Skip biobanks, get consistent material, on-demand. Better signal separation than any competing product, more stable, lower cost, wider range of biomarkers.

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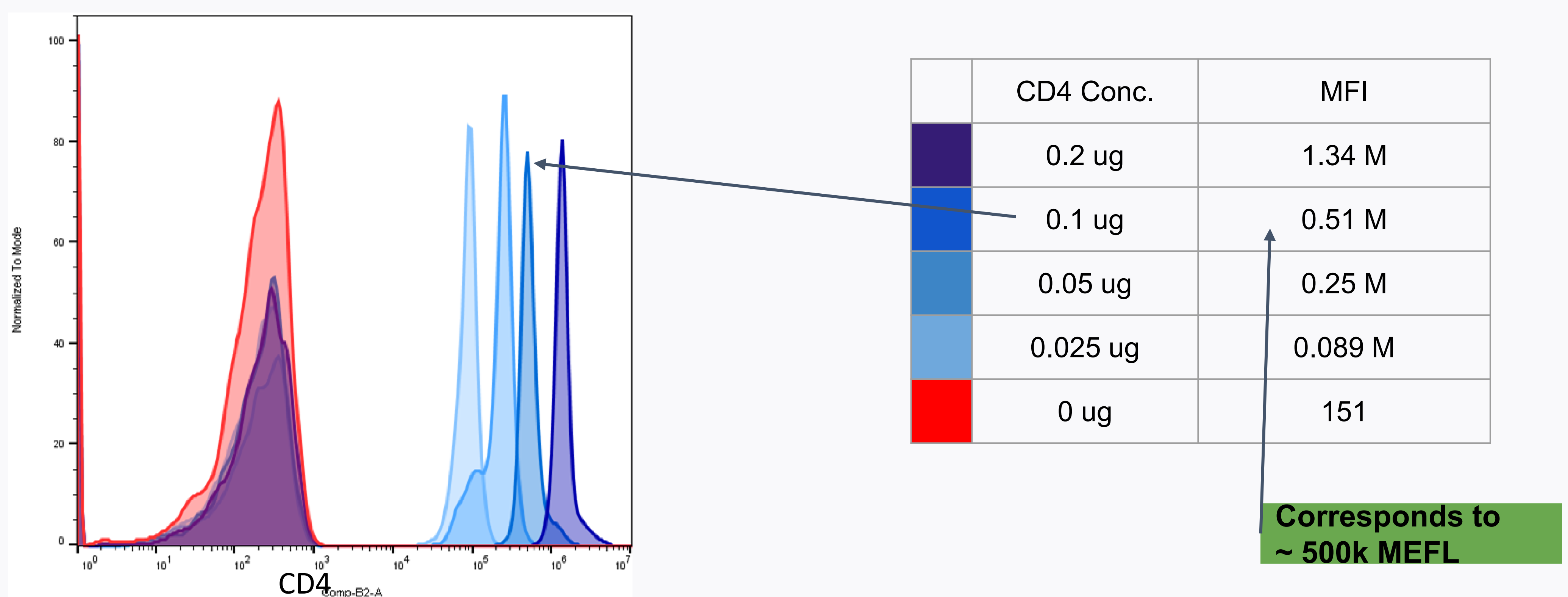
FlowCytes Can be Quantitatively Modified with Biomarkers



	CD4 Conc.	MFI
	0.2 ug	1.34 M
	0.1 ug	0.51 M
	0.05 ug	0.25 M
	0.025 ug	0.089 M
	0 ug	151

- KEY TAKEAWAY - Biomarker “expression” can be tuned to match poorly-expressed markers. First-in-class product enables CAR-T manufacturing controls and disease-specific biobanking controls.

- **MEFL/MESF** FlowCytes can be generated at precise concentrations for any biomarker.

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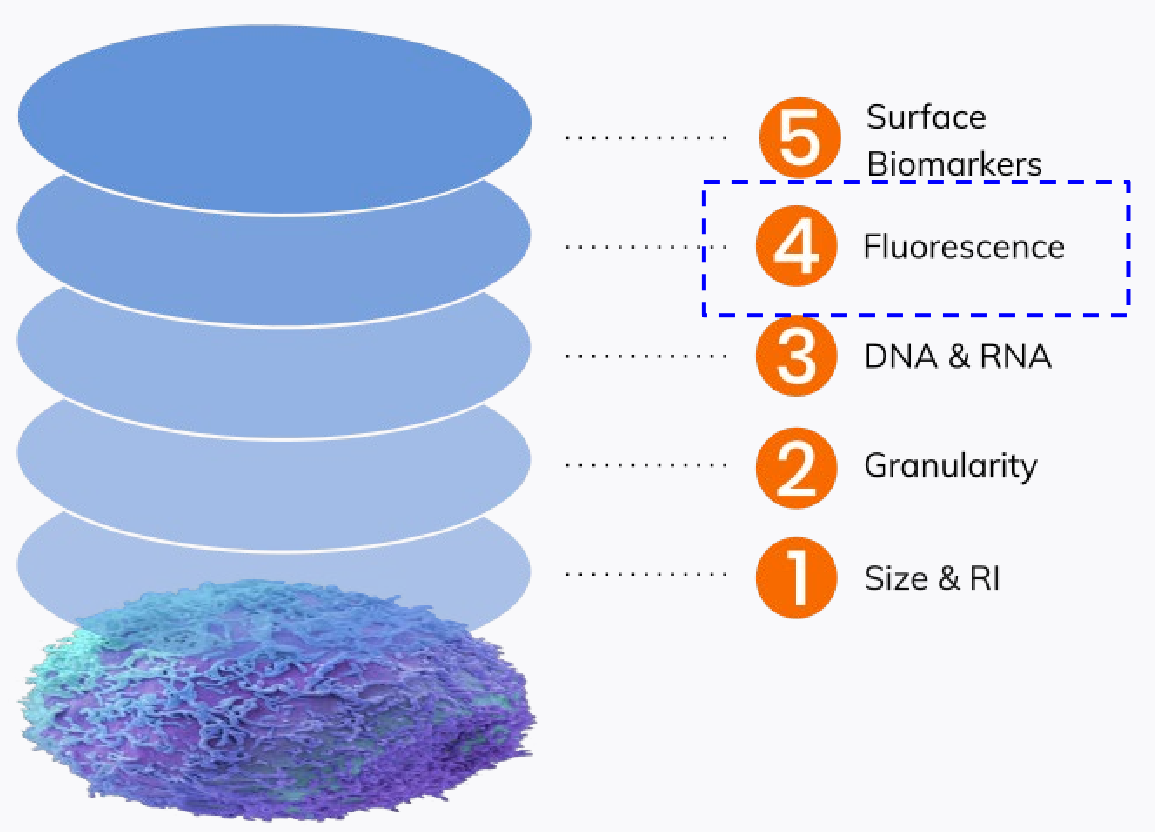
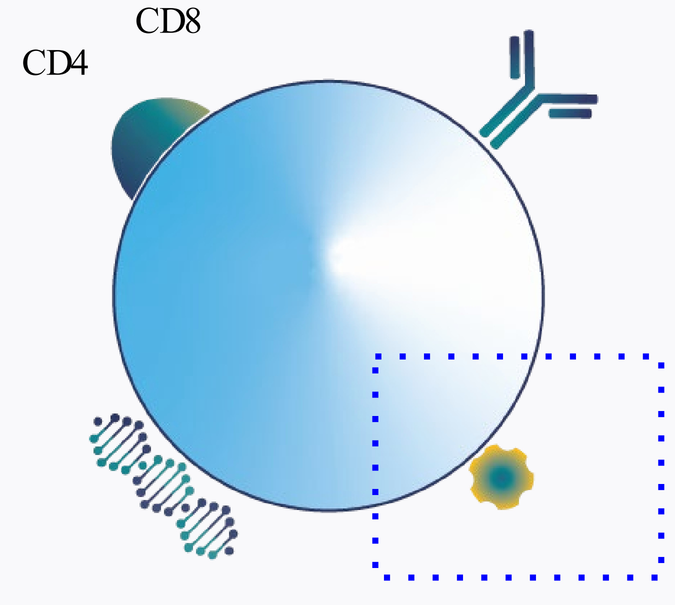
FlowCytes Can be Modified with Fluorophores/chromes





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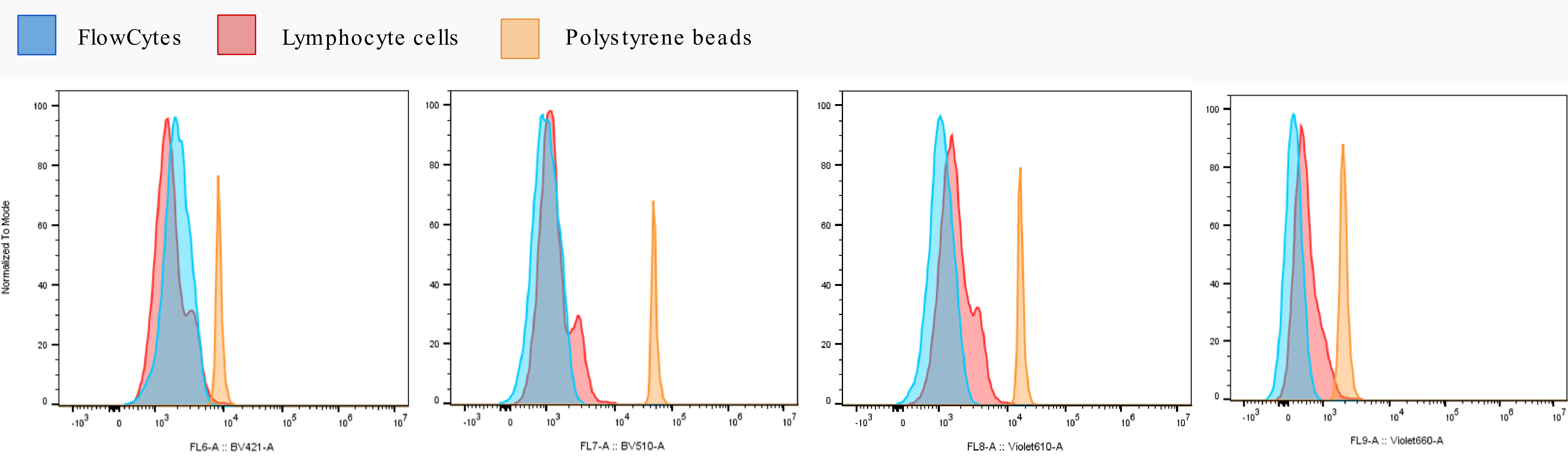
FlowCytes Have Superior Autofluorescence vs. Polystyrene



KEY TAKEAWAY - FlowCytes have lower AF in UV and violet spectrum and are more Cell-Like

- High signal-to-noise that allows for better detection of poorly expressed or “dim” biomarkers LLOD.
- Better baseline fluorescence response for tandem dye panel compatibility and more.

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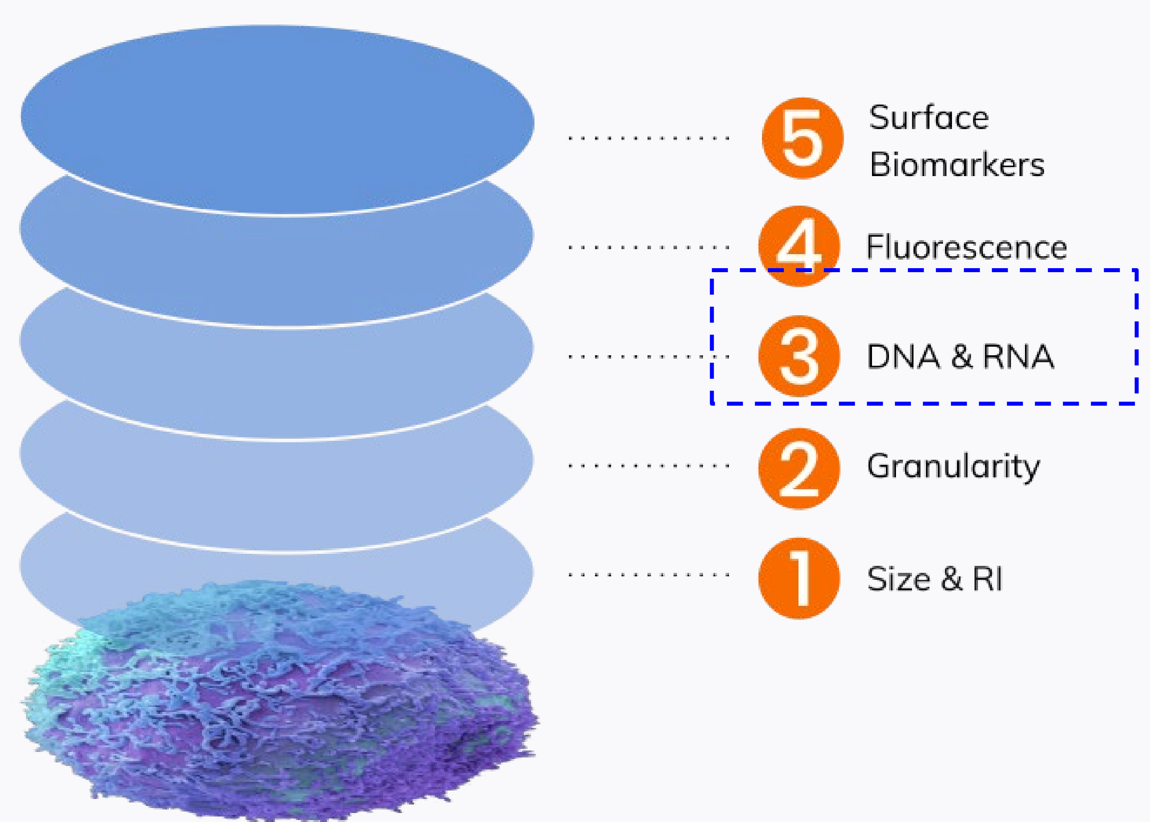
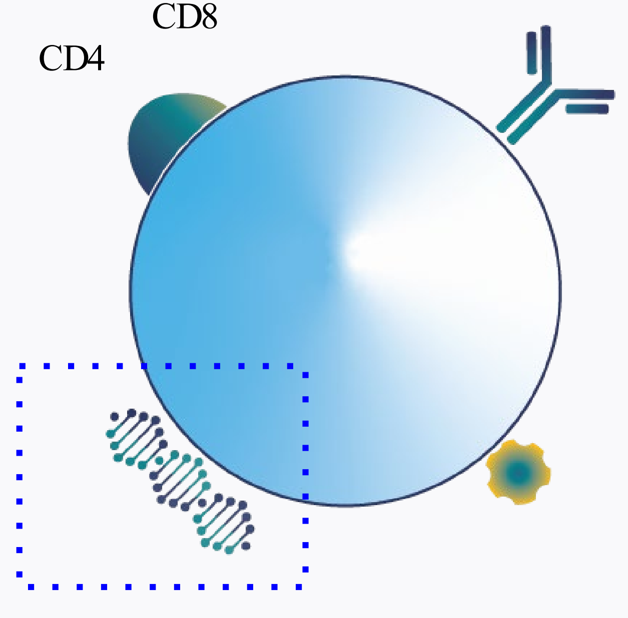
FlowCytes Can be Modified with Nucleic Acids





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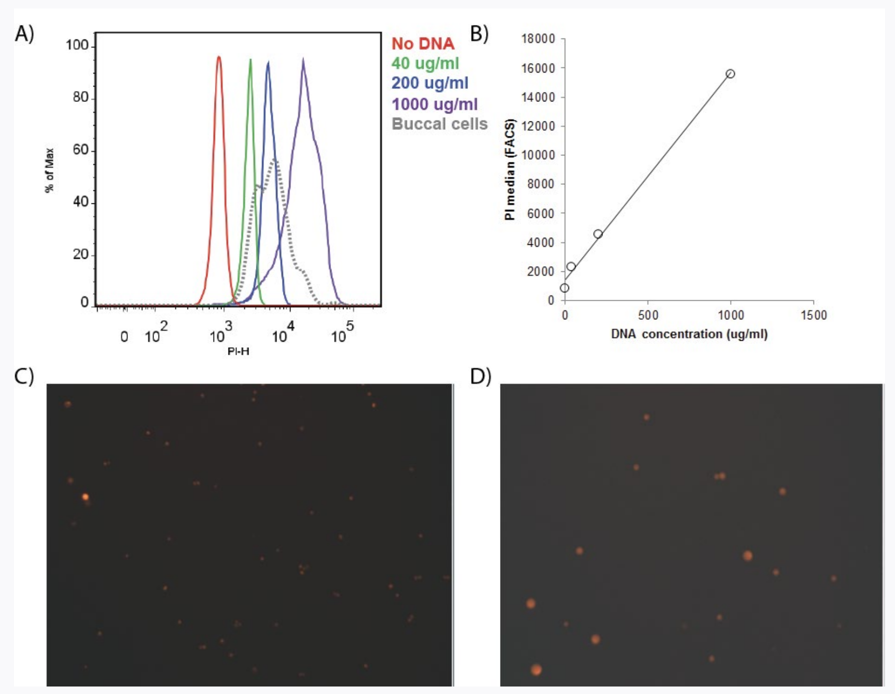
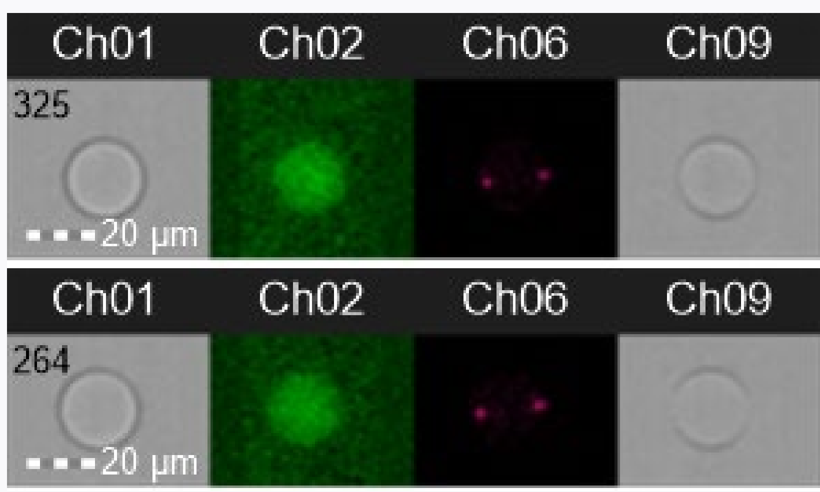
FlowCytes Can be Modified with Nucleic Acids





KEY TAKEAWAY - FlowCytes can be modified quantitatively with nucleic acids (localized)

- Localized staining for imaging controls
- Quantitative response (qPCR, copy-number validated)

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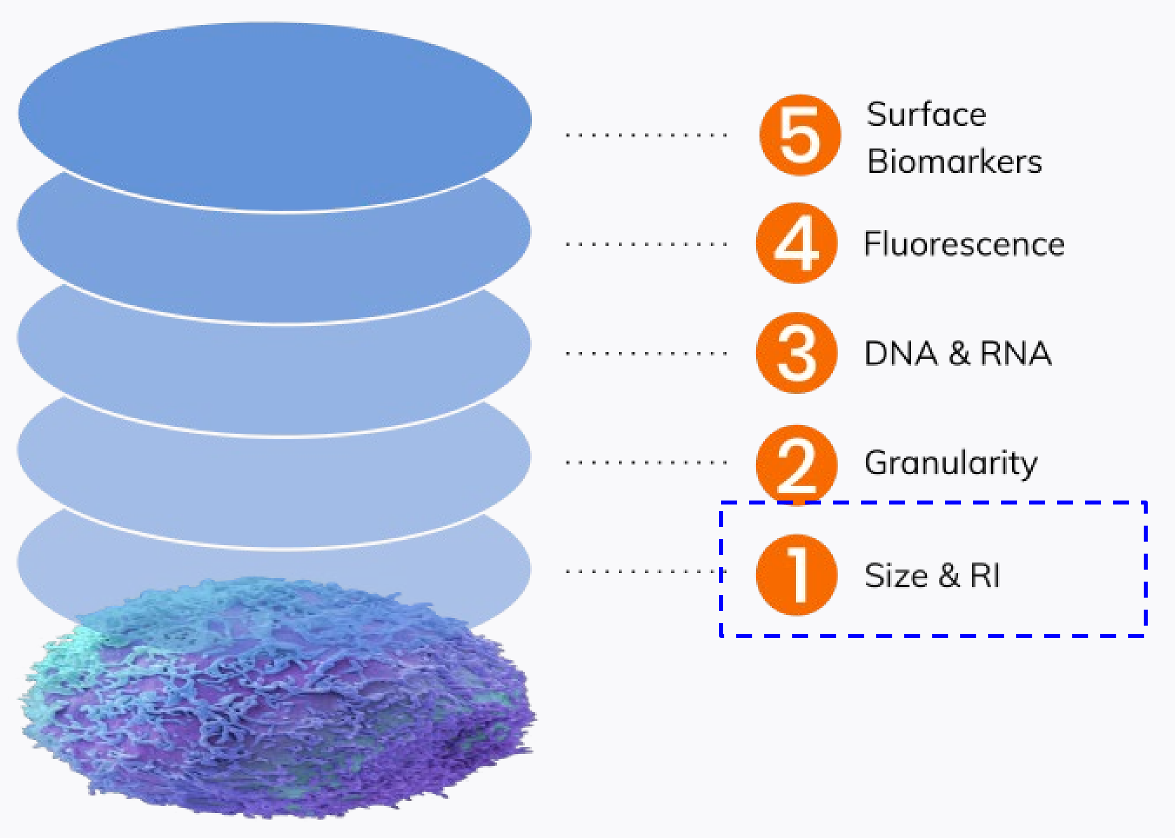
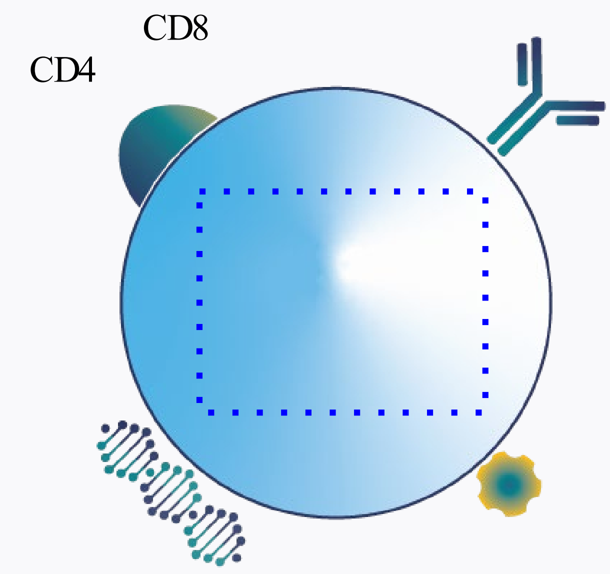
FlowCytes Can be Modified with Nucleic Acids and More





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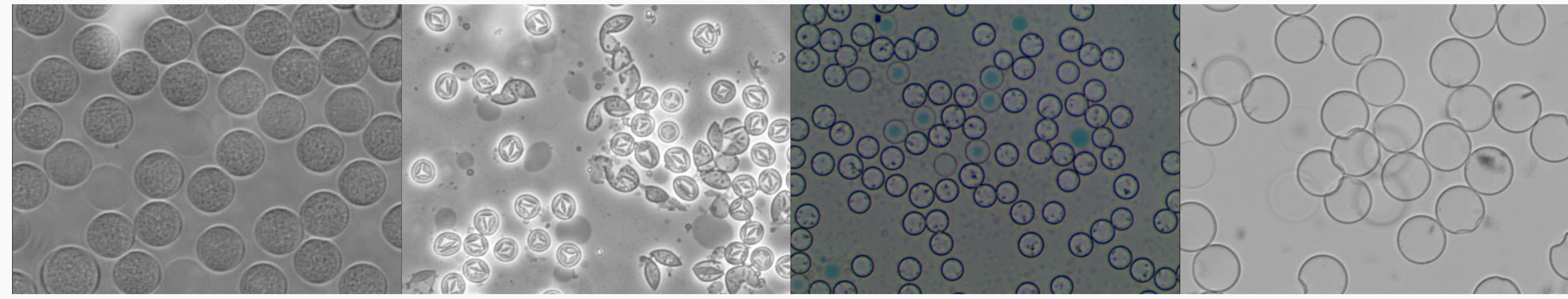
FlowCytes Can be Made in any Shape or Size



KEY TAKEAWAY - FlowCytes can be formed into unique shapes/sizes to usher in controls for next-generation image-based cytometry systems (label-free, machine learning)

- Unlimited capacity to alter size/shape and sub-cellular morphology to generate image segmentation controls for Cell Manufacturing and beyond.

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Slingshot Bio Customers and Partners

Versatility

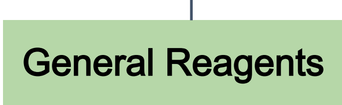
Biobanked disease control market + clinical trials

SLiNGSHOT

Manufacturing Speed

Cell-based healthy control market (FC/HA)

General Reagents



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WBC Traceability standards will be provided, FREE OF CHARGE, to NIST Consortium participants.

Please inquire at www.slingshotbio.com for more information or contact us at ops@slingshotbio.com for partnership opportunities.

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AmberGlass Technology: Enabling Standardization of Complex Multiparametric Flow Assays

February 2021

Framing the Problem

"Process is the product" is no longer just a slogan but is a critical current need.

This requires standardization of the process.

Repeatability is a fundamental requirement prior to standardization.

Repeatable process analytics is the way to ensure process repeatability

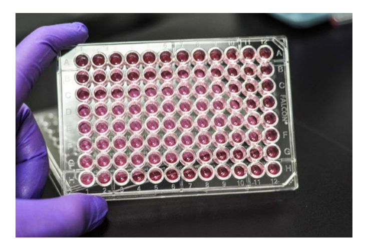
Multiparameter, highly multiplexed flow assays (process analytics) involve pipetting many small volume reagents – making repeatability a challenge.

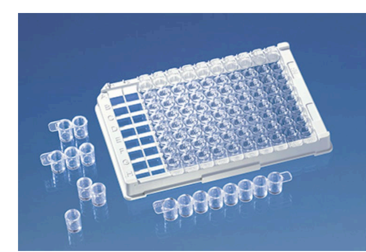
AmberGlass enables elimination of these pipetting steps (major source of variability) to improve repeatability.

AmberGlass is not "the standard" but it enables the development of "standards"

AmberGlass Technology

AmberGlass Delivery Format





AmberGlass technology permits a master mix of flow reagents to be unitized and dried at the bottom a tube or well. Addition of the sample reconstitutes the AmberGlassified reagents.

Built on a well validated foundational technology of over 30 years

Forms an amorphous, thin "glass" layer that adheres to the surface of the container

Encapsulates the pre-formulated master mix of reagents, while preserving the conformational integrity of the reagents in the dry state

Demonstrated equivalency to liquid reagents

Phenotyping Leukapheresis – a recent example

Dried Down Reagents

CD4 / Time — Time 99.0
FSC-H / FSC-A — Singlets 98.3
SSC-A / FSC-A — Leukocytes 59.5
SSC-A / CD45 — CD45 99.1
CD45 / CD19 — CD19 13.3
Live / CD3 — Live CD45 98.6
CD45 / CD3 — CD3- 45.6, CD3+ 54.4
CD4 / CD8 — CD4 34.5, CD8 55.9
CD14 / FSC-A — CD14+ 22.7, CD14- 77.3
CD56 / CD3 — CD3-CD56+ 6.42, CD3+CD56+ 9.35

Fresh Prepared Reagents

CD4 / Time — Time 99.0
FSC-H / FSC-A — Singlets 96.9
SSC-A / FSC-A — Leukocytes 72.5
SSC-A / CD45 — CD45 99.3
CD45 / CD19 — CD19 8.93
Live / CD3 — Live CD45 98.1
CD45 / CD3 — CD3- 42.0, CD3+ 58.0
CD4 / CD8 — CD4 35.7, CD8 55.2
CD14 / FSC-A — CD14+ 23.9, CD14- 76.1
CD56 / CD3 — CD3-CD56+ 5.85, CD3+CD56+ 8.73

Classes of Reagents AmberGlassified

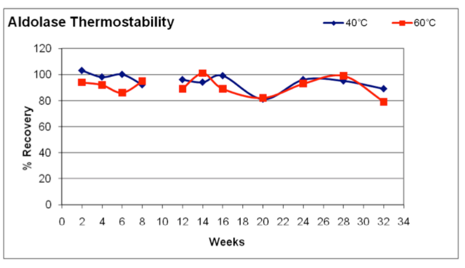
- Antibodies – agnostic to type of label on the antibody
- Enzymes (including PCR master mix with primers)
- Nucleic Acid – both DNA and RNA
- Fixed Mammalian Cells
  - Prelabeled and crosslinked to serve as reference standard.
  - Membrane protein confirmation conserved to act as targets for functional assays
- Functional assay reagents – combination of targets and detection reagents for reporter cell signaling molecules

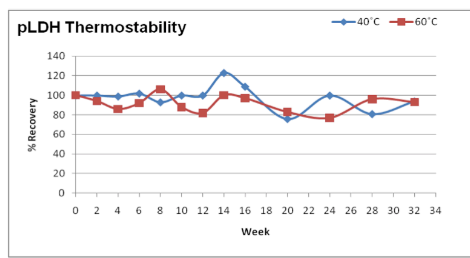
Changing the Paradigm

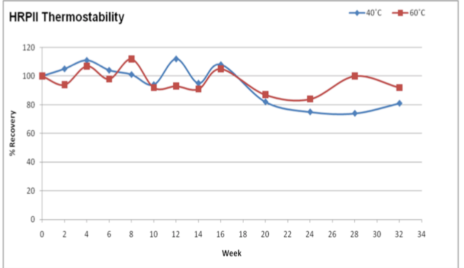
- Unitized and standardized reagents in a ready-to-use formats – automating workflow.
- Ability to deliver ready-to- use single color controls, using the same components that make up the master – more precise compensation matrix.
- Many years of room temp storage – Single well-characterized batch of reagents to be used across not only in multi-center but also for multi-year long clinical trials.

Examples of Thermal Stability

PCWs were tested with commercially available standard ELISA kits







Malaria PCW reagents are stable at 40ºC and 60ºC for 32 weeks

Looking to the Future

What could you do with AmberGlass that you could not do before?

For example - how do we use AmberGlass technology to deliver fully normalized flow data that is independent of the platform or the location?

- *Thank you*

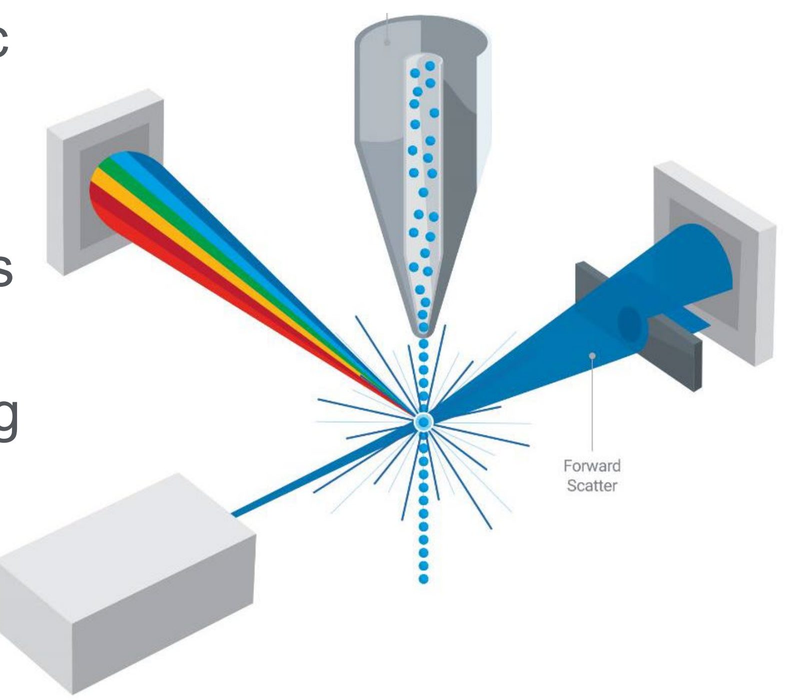
Standardization of Flow Cytometry Instrumentation and Methodology for Assessment of Absolute Count Accuracy

NIST - Flow Cytometry Standards Consortium Workshop

Garret Guenther, PhD
Agilent Technologies, Cell Analysis

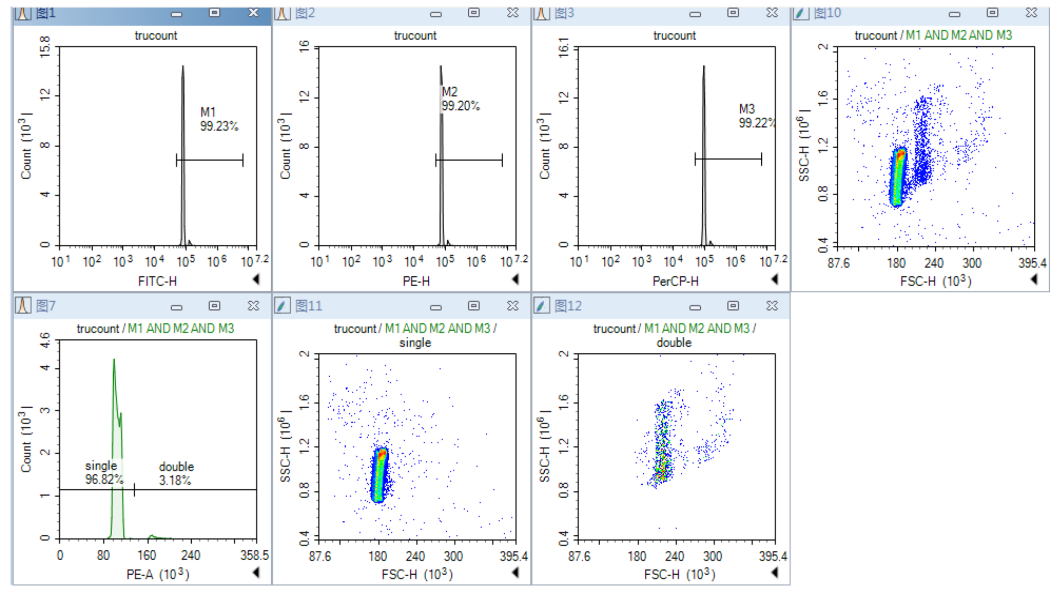
Need for standardization of flow cytometric absolute counting

- Flow cytometry is becoming a useful resource for absolute counting
- New instrumentation is utilizing precise syringe pumps for accurate volumetric measurements
- Need standardized methods to assess accuracy
- Need traceability of reference counting sample concentration

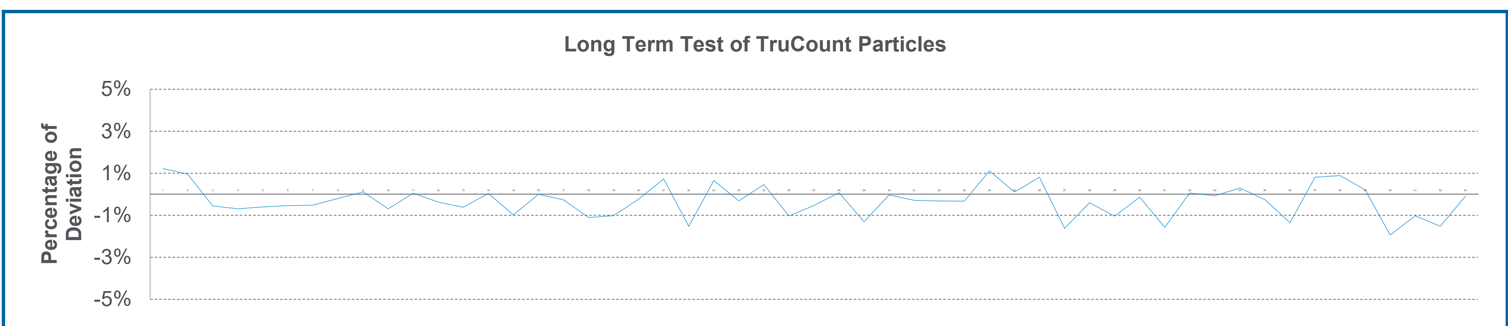


Platform validation

TruCount™ Tubes contains a lyophilized pellet of fluorescent beads in a single-use tube. The number of particles contained inside each tube is certified by the manufacturer.

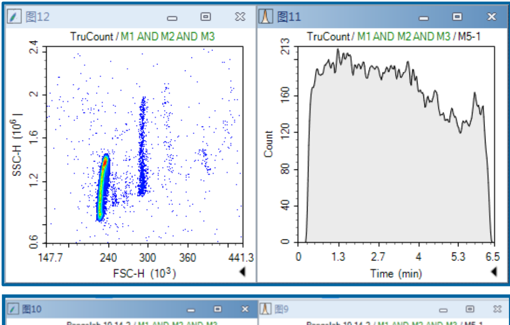


Percentage of Deviation of the particles counts (target number: 47,000) of TruCount beads (Lot # 16096) tested for 53 days.
Average of Percentage of Deviation: -0.31%
CV of Percentage of Deviation: 0.75%

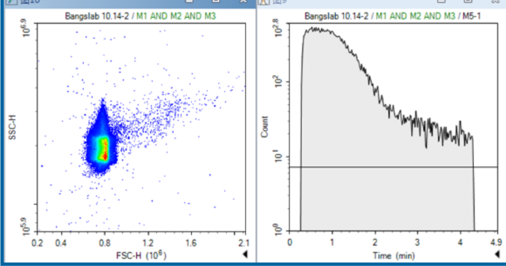


Variations in particle characteristics between vendors

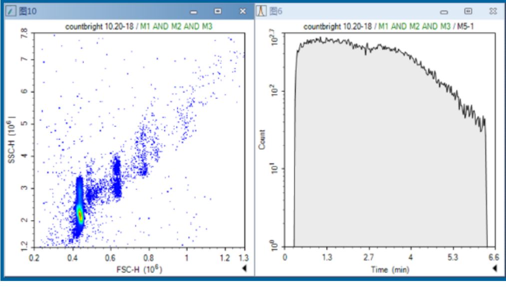
- Different characteristics of counting particles are shown
- Sedimentation/adherence as they travel through the sample lines
- FSC/SSC profile variations



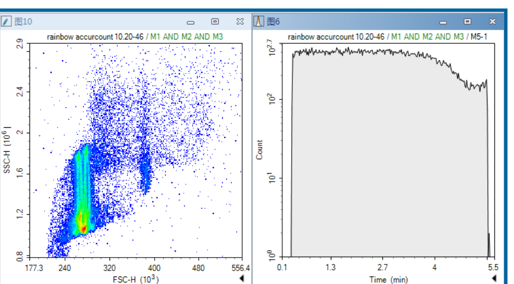
Vendor 2



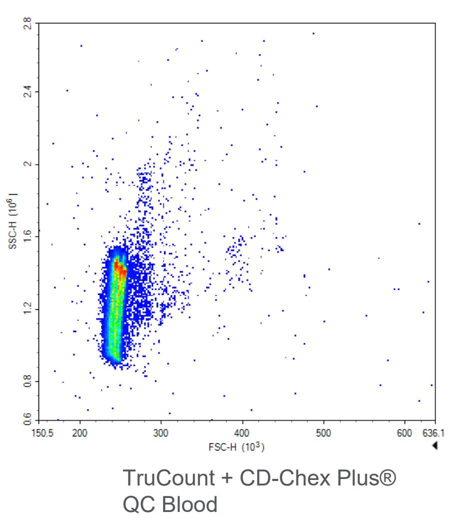
Vendor 3



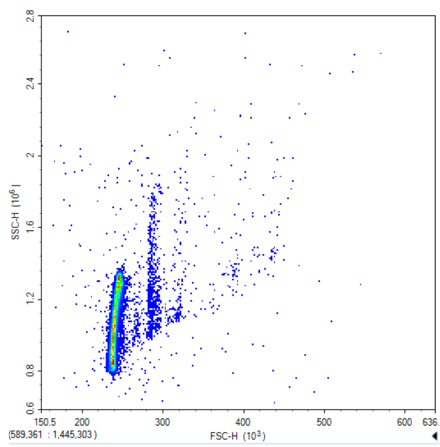
Vendor 4



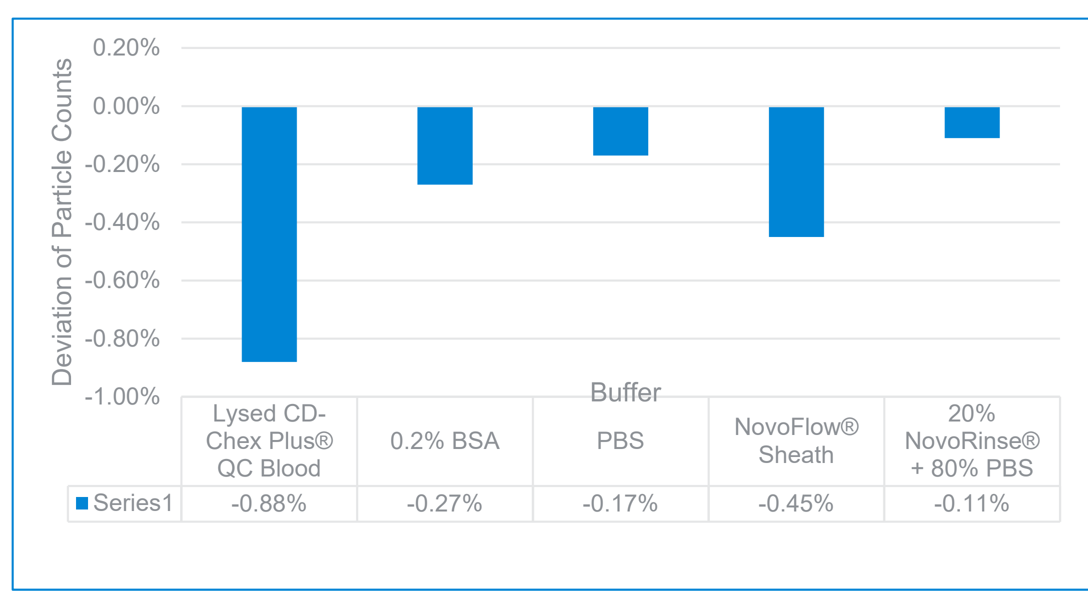
Comparison of Different Dilution Buffer



TruCount + CD-Chex Plus® QC Blood



TruCount + PBS (or 0.2% BSA, NovoFlow® Sheath, 20% NovoRinse® + 80% PBS)



	Lysed CD-Chex Plus® QC Blood	0.2% BSA	PBS	NovoFlow® Sheath	20% NovoRinse® + 80% PBS
Series1	-0.88%	-0.27%	-0.17%	-0.45%	-0.11%

On the modified positive-pressure driven platform, TruCount beads are diluted with different buffer. The number of TruCount beads being rinsed out at each time varies by different buffer. Therefore, the buffer used to dilute the sample also plays a role in the accuracy of the absolute counting results.

Counting variations observed between vendor samples with the same instrument

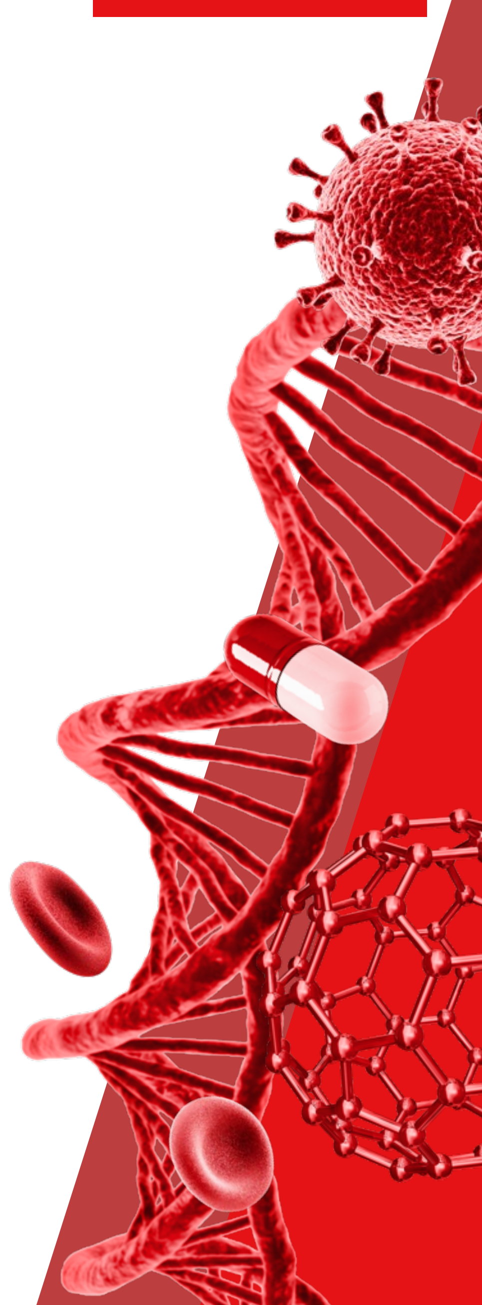
- Variations in physical properties of counting particles (adherence/sedimentation)
- Variations in dilution buffer
- Variations in accuracy
- Variations with respect to reference laser*

Deviation	
	1:1 Dilution
Vendor/Lot 1	-2.67%
Vendor/Lot 2	5.66%
Vendor/Lot 3	-0.59%
Vendor/Lot 4	0.38%
Vendor/Lot 5	-10.68%
Vendor/Lot 6	6.98%
Vendor/Lot 7	8.03%
Vendor/Lot 8	17.20%
Vendor/Lot 9	-0.9%
Vendor/Lot 10	-1.9%
Vendor/Lot 11	-2.7%
Vendor/Lot 12	-1.4%

Thank you!

Fluorescent Microspheres for Flow Cytometry Calibration and Standardization

Yu-Zhong Zhang, Ph.D.
Chemistry R&D Team, Protein and Cell Analysis
Thermo Fisher Scientific



The world leader in serving science

Longstanding Collaboration between the National Institute of Standards and Technology and the Protein and Cell Analysis (PCA) Business Unit of Thermo Fisher Scientific

20+ year collaboration - Developing fluorescence intensity reference materials and microsphere calibration standards for flow cytometry.

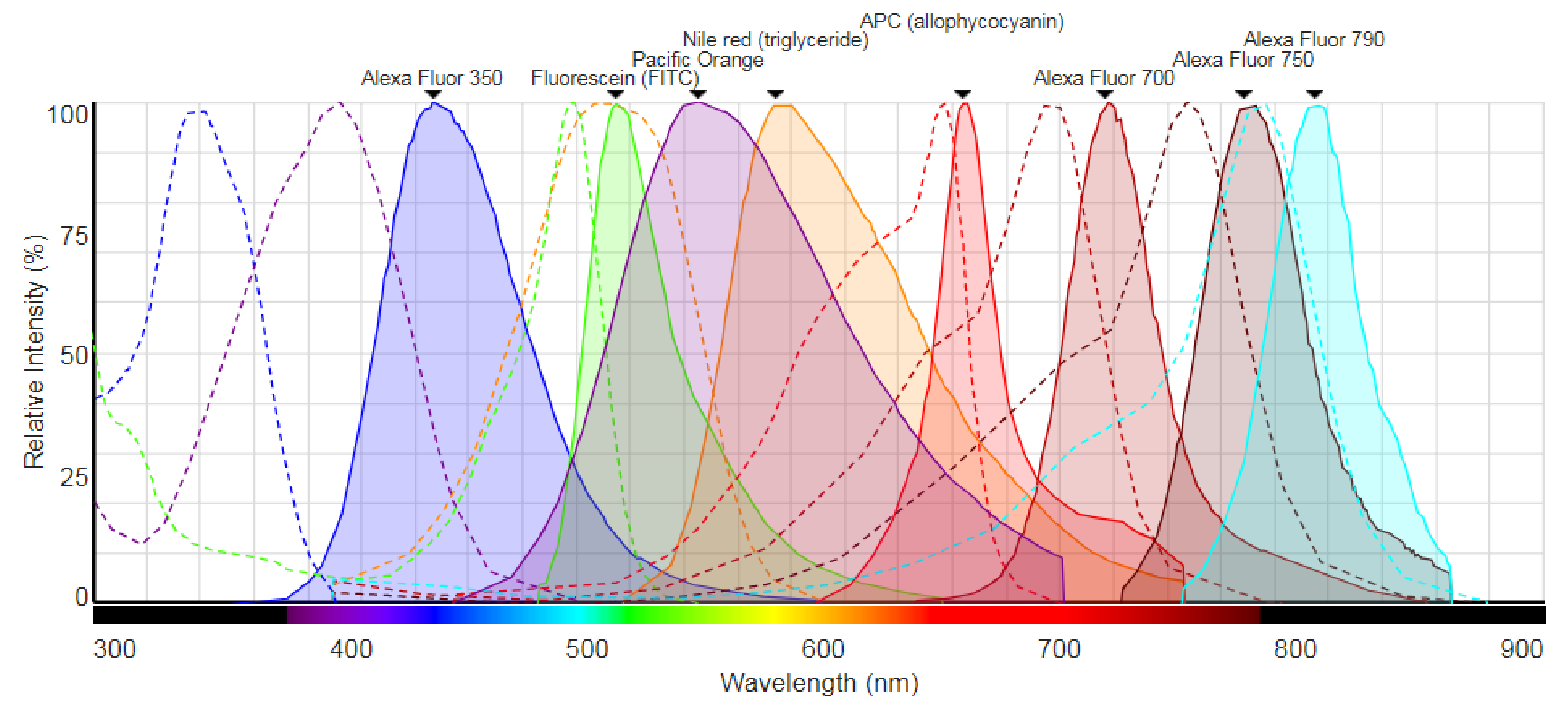
1998 – NIST/PCA CRADA contract. Fluorescein Dye for Quantitative Flow Cytometry (SRM 1932)

2016 – NIST/PCA CRADA contract. Fluorescent Dyes for Quantitative Flow Cytometry (Visible Spectral Range) (SRM 1934)

2020 – PCA/NIST CRADA contract. ERF intensity assignments of cell-sized fluorescent microparticles for quantitative flow cytometry.

Longstanding Collaboration between NIST and PCA

Provide High Purity and Quality Fluorescent Dyes for REF Assignment



Thermo Fisher SCIENTIFIC

Develop Better Calibration Standards for Quantitative Flow Cytometry

AccuCheck ERF Reference Particles

AccuCheck ERF Reference Particles have NIST assigned/traceable ERF values for 26 flow cytometry filter set channels

- Quantitation of fluorescence staining
- Antibody binding capacity
- Level of biomarker expression
- Check data accuracy
- Determine traceability of measurement
- Inter- and intra-lab instrument data comparison
- Will help address increasing level of regulations requiring reproducible experimental results

Excitation laser	Emission filter set	ERF value			NIST reference fluorophore
		Low intensity	Medium intensity	High intensity	
405 nm	440/50	7.01×10^4	4.24×10^6	2.09×10^7	Coumarin 30
	512/25	3.54×10^4	1.29×10^6	8.86×10^6	Coumarin 30
	603/48	1.3×10^4	4.56×10^5	1.24×10^7	Pacific Orange
	615/24	1.13×10^4	4.00×10^5	1.37×10^7	Pacific Orange
	670/30	8.00×10^3	3.98×10^5	1.61×10^7	Pacific Orange
	710/50	2.51×10^4	5.03×10^5	2.30×10^7	Pacific Orange
	720/60	2.63×10^4	5.16×10^5	2.38×10^7	Pacific Orange
488 nm	525/35	1.85×10^3	6.34×10^4	4.62×10^5	Fluorescein
	530/30	2.38×10^3	8.03×10^4	6.17×10^5	Fluorescein
	574/26	1.25×10^5	4.50×10^6	3.12×10^7	Nile Red
	593/52	3.88×10^4	1.39×10^6	1.08×10^7	Nile Red
	590/40	4.09×10^4	1.52×10^6	1.17×10^7	Nile Red
	695/40	1.37×10^4	4.66×10^5	5.91×10^6	Nile Red
	780/60	1.49×10^4	5.47×10^5	9.46×10^6	Nile Red
561 nm	585/16	4.54×10^4	1.34×10^6	8.85×10^6	Nile Red
	620/15	2.45×10^4	7.26×10^5	5.37×10^6	Nile Red
	670/30	2.61×10^4	8.08×10^5	6.54×10^6	Nile Red
	695/40	2.59×10^4	8.18×10^5	7.32×10^6	Nile Red
	720/60	2.62×10^4	8.41×10^5	7.99×10^6	Nile Red
	780/60	3.75×10^4	1.09×10^6	1.15×10^7	Nile Red
	789/78	3.75×10^4	1.09×10^6	1.15×10^7	Nile Red
640 nm	660/20	1.32×10^3	3.91×10^4	2.00×10^5	APC
	670/14	2.08×10^3	6.32×10^4	3.49×10^5	APC
	670/30	1.93×10^3	5.88×10^4	3.27×10^5	APC
	720/30	2.96×10^4	8.51×10^5	6.14×10^6	Alexa Fluor™ 700
	780/60	1.82×10^4	5.47×10^5	4.09×10^6	Alexa Fluor™ 700

Additional Reagents Developed by Thermo Fisher Scientific for Instrument Calibration Standardization and Compensation

- UltraComp Compensation Beads
- UltraComp Plus Compensation Beads
- AbC Total Compensation Beads
- ArC Amine Reactive Compensation Bead Kit
- GFP BrightComp Compensation Beads
- CountBright Cell Counting Beads (7 µm diameter)
- CountBright Plus Cell Counting Beads (4 µm diameter)
- AccuCheck ERF Reference Particles

UltraComp Plus eBeads

Recombinant Human IgG1 (CD20)
Polyclonal Rabbit IgG Isotype
Mouse IgG1, kappa (CD38)
Mouse IgG2a, kappa (CD8)
Mouse IgG2b, kappa (CD4)
Mouse IgM, kappa (CD160)
Rat IgG1, kappa Isotype
Rat IgG2a, kappa Isotype
Rat IgG2b, kappa (CD45)
Rat IgG2c, kappa (Ly-6D)
Rat IgM, kappa (CD49b)
Rat IgG2a, lambda (CD21/CD35)
Syrian Hamster IgG (CD3e)
Armenian Hamster IgG (CD3e)

Single Vesicle Flow Cytometry (vFC™): Rigorous and Reproducible Extracellular Vesicle (EV) Measurements

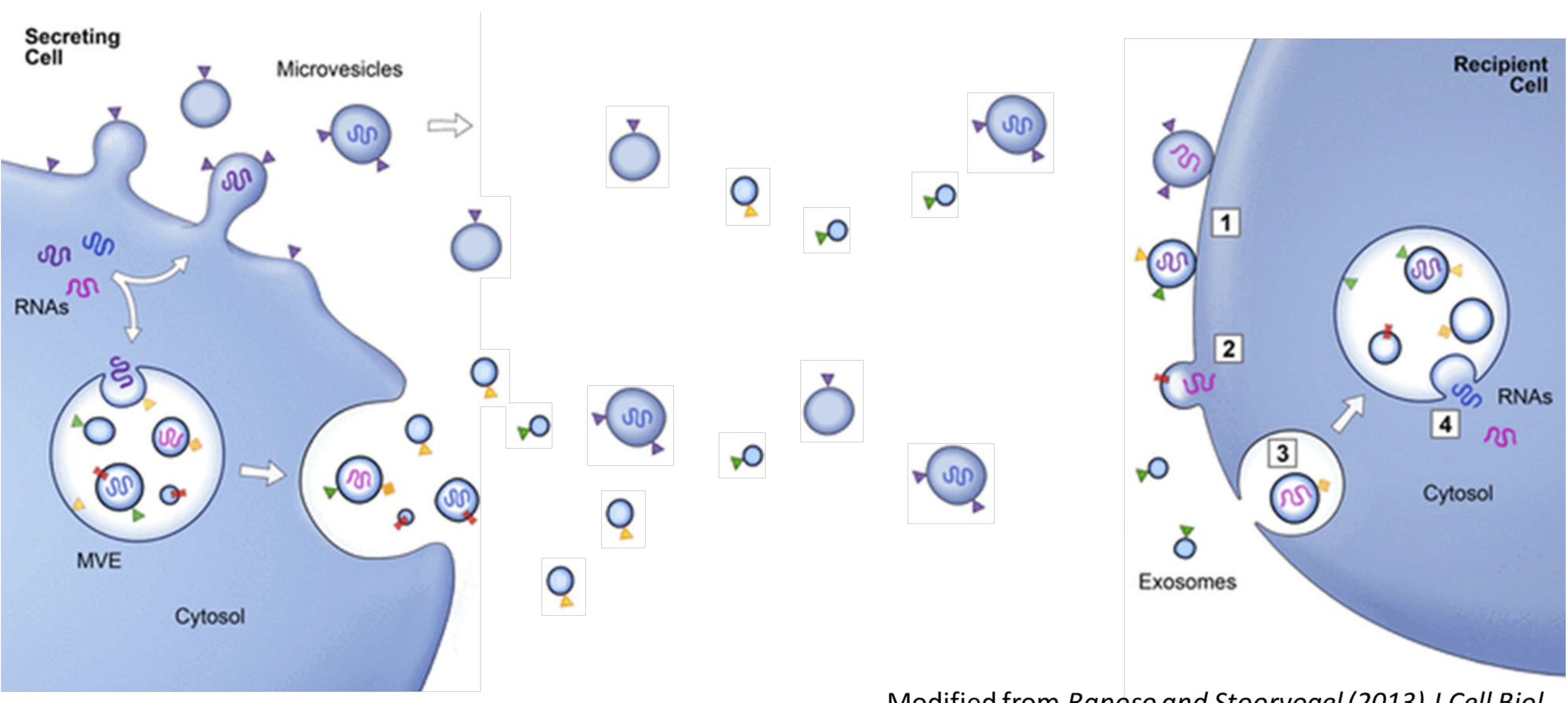
NIST FLOW CYTOMETRY STANDARDS CONSORTIUM WORKSHOP

FEBRUARY 17, 2021

JOHN P NOLAN PHD

CELLARCUS BIOSCIENCES, INC

Extracellular Vesicles (EVs)

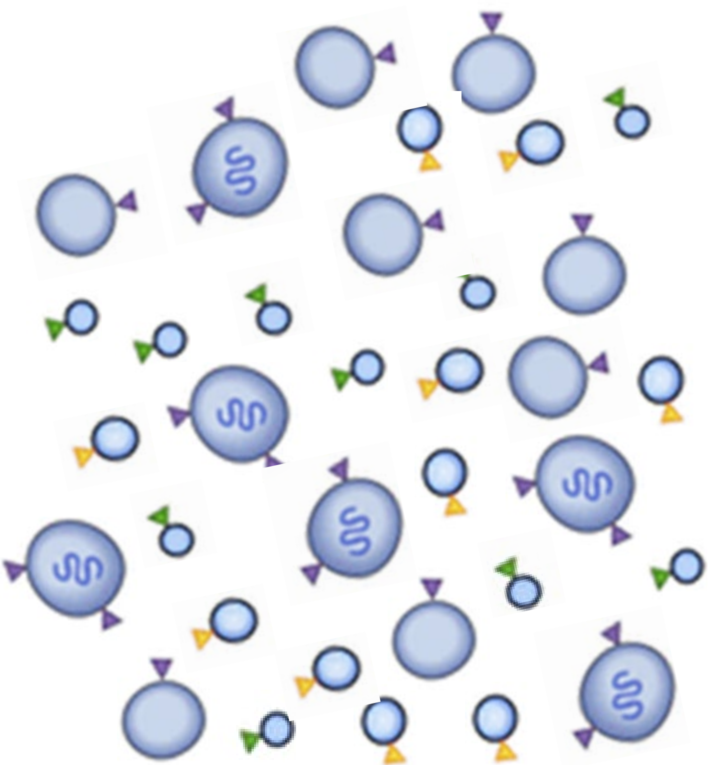


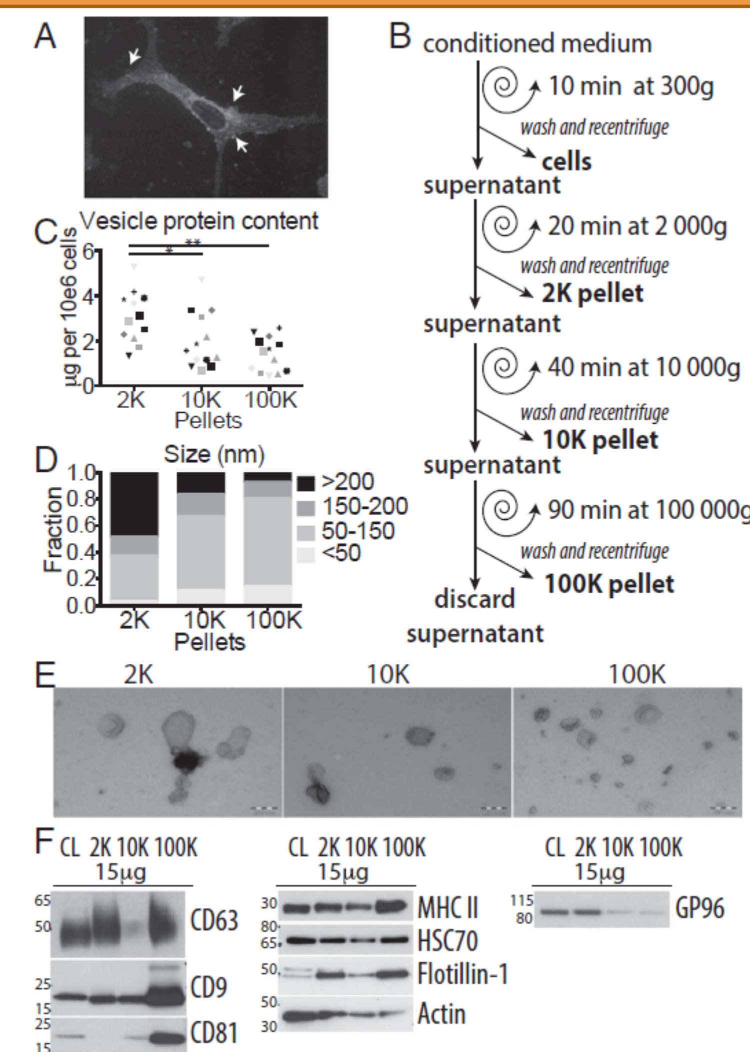
Modified from *Raposo and Stoorvogel (2013) J Cell Biol*

Conventional EV Analysis: Spin and Blot

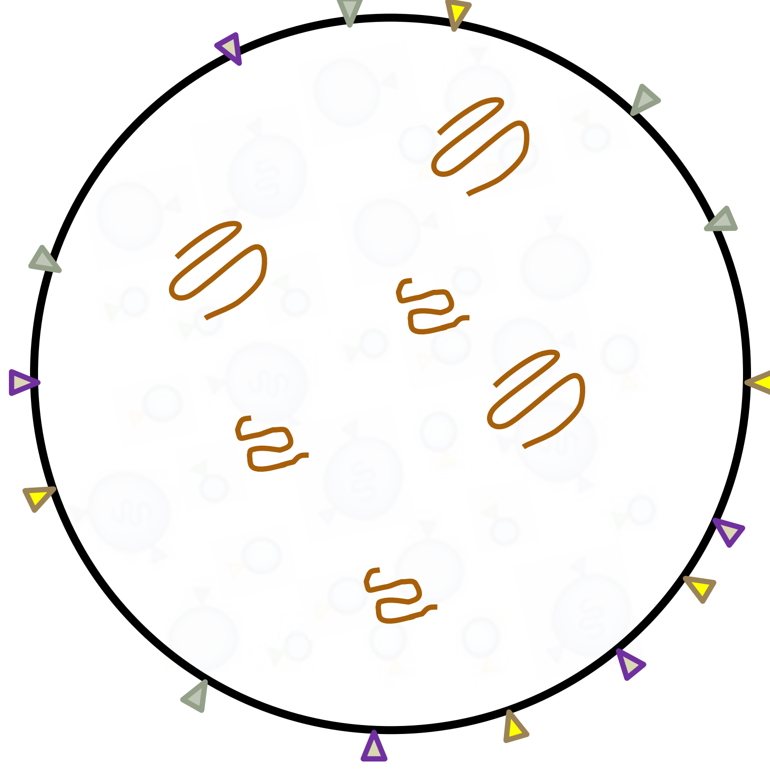
Conventional Bulk Analysis

Vesicle sample





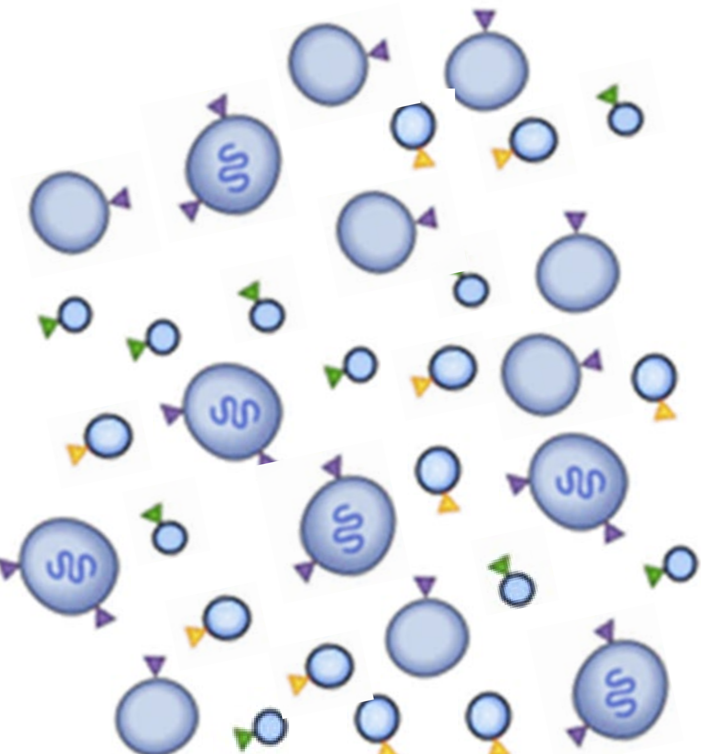
"Average" Vesicle



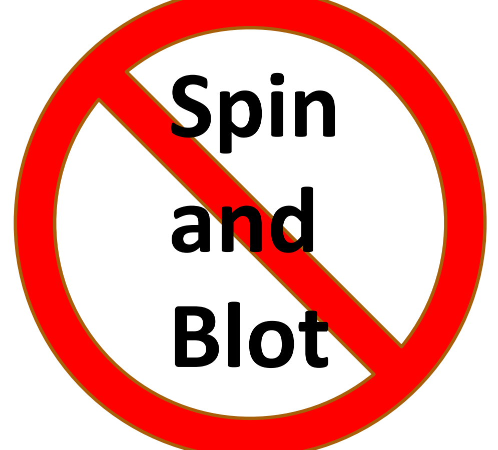
Single Vesicle Biochemical Analysis

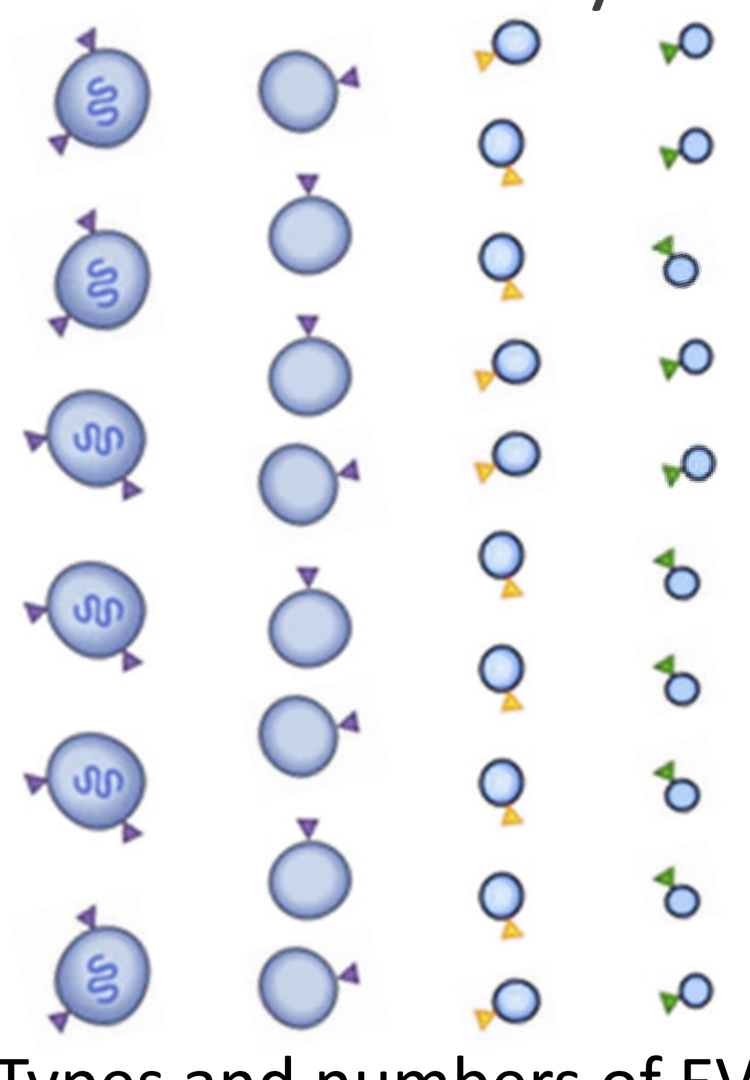
Single Vesicle Analysis

Vesicle sample



EV number
EV size and
EV cargo
for each vesicle





Types and numbers of EVs

Flow Cytometry for Single EV Analysis

Why?

- Sensitive measurement of individual particles
- Standards and calibrators for quantitative analysis
- Homogeneous assays, automation-compatible
- Widely employed in academic, pharma, and clinical labs

Why not?

Conventional instruments lack sensitivity

Conventional assays lack specificity

Key method details often not reported

Key controls and calibration omitted

Poor reproducibility

ISEV-ISAC-ISTH EV FC Working Group

People

Marca Wauben, Ger Arkesteijn, EstefaniaLozano Andres (Utrecht)
Rienk Nieuwland, Edwin van der Pol (Amsterdam)
John Nolan, Erika Duggan (San Diego)
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James Higgenbotham, Bob Coffey (Vanderbilt)
An Hendrix, Oliver de Wever (Ghent)
Xiaomei Yan (Xiamen)

Assays and instruments

Nano-FACS
Nanoscale flow cytometry
Nano-FCM
Nano-flow
Nano-flow cytometry
Imaging flow cytometry
Dedicated flow cytometry
Dark field flow cytometry
Flow exometry
Flow virometry
FAVS
Vesicle flow cytometry (vFC™)

ISEV-ISAC-ISTH EV FC Working Group

JOURNAL OF EXTRACELLULAR VESICLES
2020, VOL. 9, 1713526
https://doi.org/10.1080/20013078.2020.1713526

Welsh et al 2020 – ***EVFlowcytometry.org***

MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments

MIFlowCyt-EV | MISEV-Flow: Reporting Framework

1	Experimental design & preanalytical variables	1.1. Report preanalytical variables conforming to MISEV guidelines 1.2. Report experimental design according to MIFlowCyt guidelines
2	Sample Preparation	2.1. Sample staining 2.2. Sample wash steps 2.3. Sample dilution
3	Assay Controls	3.1. Unstained controls 3.2. Isotype controls 3.3. Buffer alone 3.4. Buffer with reagents 3.5. Procedural controls 3.6. Serial dilutions 3.7. Detergent treatment
4	Instrument data acquisition & calibration	4.1. Trigger channel(s) and threshold(s) 4.2. Flow rate & volumetric quantification (µL min^{-1} / µL) 4.3. Fluorescence Calibration (MESF/ERF units) 4.4. Light Scatter Calibration (nm^2)
5	EV characterization	5.1. EV diameter approximation 5.2. EV refractive index approximation 5.3. Epitope number approximation
6	Reporting FCM data	6.1. Complete MIFlowCyt checklist 6.2. Calibrated channel detection range 6.3. EV number/concentration 6.4. EV brightness
7	Sharing FCM data	7. Share data to public repository (e.g. FlowRepository)

Assay

Instrument

ORIGINAL ARTICLE

MIFlowCyt: The Minimum Information About a Flow Cytometry Experiment

Jamie A. Lee,[1†] Josef Spidlen,[2†] Keith Boyce,[3] Jennifer Cai,[1] Nicholas Crosbie,[4] Mark Dalphin,[5]

EDITORIAL

Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles

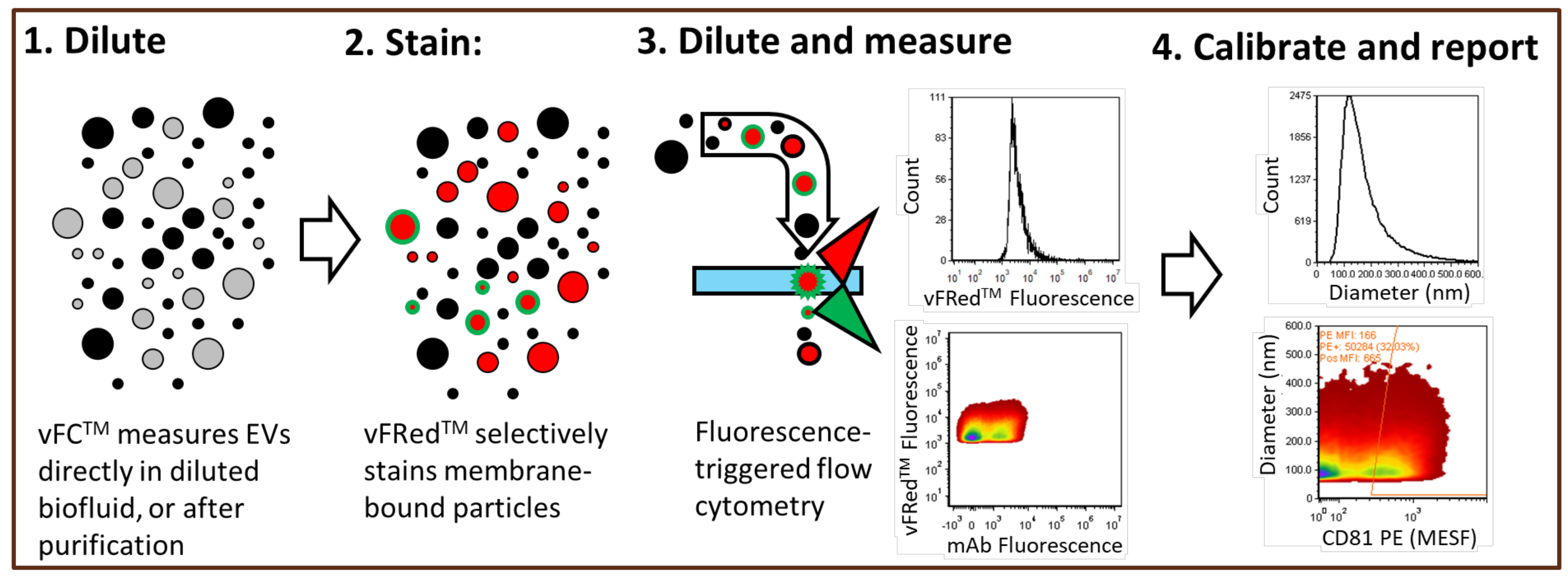
228 | VOL.14 NO.3 | MARCH 2017 | NATURE METHODS

EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research

EV-TRACK Consortium*

We argue that the field of extracellular vesicle (EV) biology needs more transparent reporting to facilitate interpretation and replication of experiments. To achieve this, we describe EV-TRACK, a crowdsourcing knowledgebase (http://evtrack.org) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice.

Vesicle Flow Cytometry (vFC™)



- Membrane probe provides specificity
- Measures EVs directly in biofluid: no isolation/purification required
- Homogeneous assay: no wash steps
- Plate-based assays, robot-compatible

- Uses commercial flow cytometers
  - Beckman CytoFlex
  - Luminex CellStream

- Sensitive and specific detection: vesicle size to ~50 nm, cargo to <10 molecules
- Calibrated measurements for inter-lab, longitudinal, cross-platform comparisons

Instruments and Assays for EV analysis

Instruments

Assay

- Instrument, calibrated
- Reagents
- Sample preparation protocols
- Data analysis protocols
- Validation and reporting

<u>Beckman Coulter CytoFlex</u>

Sensitive APD array detectors
Efficient high NA light collection
High resolution light scatter
Multiple lasers
Plate loader

<u>Luminex CellStream/ImageStream</u>

Sensitive CCD detector
TDI-based signal integration
Image-based object detection
Multiple lasers
Plate loader

<u>Cellarcus Vesicle Flow Cytometry (vFC^{TM})</u>

Membrane selective detection
Fluorescence-based size estimate
No-wash, plate-based assay
Calibrators and standards
Standardized protocols

vFC™: Standardized Sample Prep

CELLARCUS BIOSCIENCES

Vesicle Flow Cytometry Analysis Kit

FOR VESICLE COUNTING AND SIZING

Protocol 2-1: Measuring EV Surface Cargo Demo

Purpose

This Protocol demonstrates vFC by counting, sizing, and measuring surface marker (tetraspanin) expression of an EV standard.

Kit Component	Size	Store	Notes
vFRed™, membrane stain (100x)	1 x 50 uL	2-8°C	
Lipo100™ Standard (10x)	2 x 50 uL	2-8°C	
EV Lysing Solution (1000x)	1 x 25 uL	2-8°C	
vFC™ Staining Buffer (1x)	1 x 100 mL	2-8°C	
Anti-Tetraspanin (TS) Mix [PE] (10x)	1 x 20 tests	2-8°C	
PLT EV standard (10x)	1 x 50 uL	2-8°C	

Materials to be provided by User

Gloves
Microwell plate (Sartstedt 82.1583.001)
Pipettes (5 uL – 300 uL)
Pipette tips

Protocol 2-1: Measuring EV Surface Cargo

Materials

a. Gloves
b. Microwell plate
c. vFRed™ Membrane Stain (100x)
d. VFC Staining Buffer, 2 mL
e. Lipo100™ Standard (10x)
f. EV standard (10x)
g. Fluorescent antibody (FL mAb, 10x)
h. EV lysing solution

Procedure

Prepare Working Solutions

1. Prepare 200 uL 10x vFRed™ working solution (5 uL per well) by adding 20 uL vFRed™ (100x) to 180 uL VFC Staining Buffer (for 4 samples plus controls)
2. Prepare 10x Vesicle Lysing Solution by adding 5 uL to 495 uL Staining Buffer (500 uL)

Prepare Samples

3. Dilute sample to between ~$1x10^6$ and $1x10^8$/uL in VFC Staining Buffer in a microfuge tube and mix well.

Note: For new samples with unknown concentrations, see Protocol 1.

4. Place 35 uL of VFC Staining Buffer into individual wells (see Protocol 2 Plate Map).
5. Add 5 uL of FL mAb (or buffer for no mAb samples)
6. Add 5 uL of diluted samples and standards to designated wells.
7. Add 5 uL of 10x vFRed™ to each well (expect row H), mix by pipetting up and down.
8. Incubate for 60 minutes in the dark at RT.

Dilute and Read

9. Place 145 uL Staining Buffer into wells in Columns 5-8, and 291 ul into wells in Columns 9-12.
10. Transfer 5 uL of stained sample from wells in Columns 1-4 into wells in Columns 5-8 and mix by pipetting up and down (Dilution 1).
11. Transfer 9 uL of Dilution 1 into the well in Columns 9-12 and mix (Dilution 2).
12. Run on Dilution 2 on CytoFlex for fixed time (120 seconds) at fixed flow rate (High, 60 uL/min).

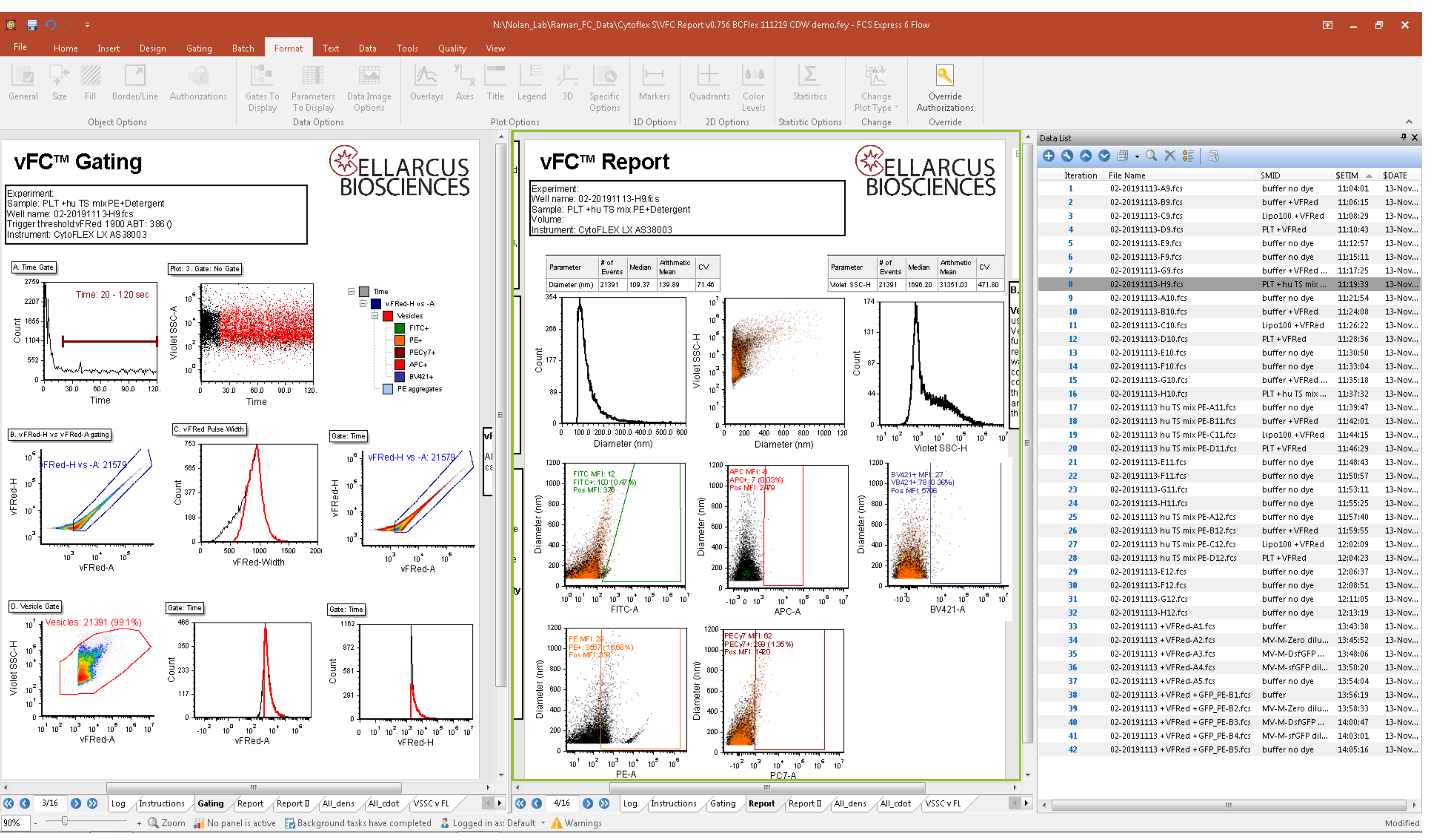
Detergent Sensitivity

1. Following the first post-stain dilution (Step 10), add 5 uL 10x Vesicle Lysing Solution to desired Staining Wells (eg wells A1-D4) and incubate 10 minutes.
2. Dilute and read as above.

Protocol 2-1 Plate Map

	Staining wells No mAb FL		Staining wells + mAb FL		Dilution 1 Don't run				Dilution 2 Run on cytometer			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Buffer	Buffer	Buffer	Buffer	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
B	Buffer + vFRed™	Buffer + vFRed™	Buffer + TS PE mix + vFRed™	Buffer + TS PE mix + vFRed™	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
C	Lipo100+ vFRed™	Lipo100+ vFRed™	Lipo100+ TS PE mix + vFRed™	Lipo100+ TS PE mix + vFRed™	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
D	EV Std+ vFRed™	EV Std+ vFRed™	EV Std + TS PE mix + vFRed™	EV Std + TS PE mix + vFRed™	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	5 uL into 145 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL	9 uL into 291 uL
E												
F												
G												
H												

vFC™: Guided Data Analysis



EV Measurement: Essential Quantities

Quantity	Instrument	Units	Standard	Calibration	Units
Concentration	Count	Number	Counting bead	Flow rate	EVs/mL
Size	Arbitrary Intensity	Photons	Vesicle size standard Diameter/Refractive index standards	Fluorescence/nm^2 Scattering x-section	EV Diameter (nm) EV Surface area (nm^2) EV Volume (nm^3)
Cargo abundance	Intensity	Photons			Molecules/EV
			Intensity standard	Fluorescence/EV	Fluorophores/EV
			Ab binding standard	Antibodies/EV	Antibodies/EV
Refractive index	Intensity	Photons	Diameter/Refractive index standards		EV Refractive index

Cellarcus Standards for vFC™

	Count	**Size**	**Abs/EV**	Fluors/EV	Ag/EV	Photons/EV
vCal™ nanoRainbow beads	●	○	○	○	○	●
Lipo100™		●				
vCal™ Ab cap beads			●	○		
MESF beads			○	●		
Ag beads					●	
LED Pulser						●
AccuCount	●					

Cellarcus Products

vFC™ Vesicle Analysis Kits
- EV count and size
- EV cargo marker

vTag ™ antibodies and multicolor panels
- Tetraspanin profile, mix and panel
  - Profile each of seven major TS molecules
  - Measure total amount of CD9 + CD63 + CD81
  - Multicolor measurement of each of CD9, CD63, and CD81
- Blood cell EV panel
  - PLT and RBC EVs
- Integrin profile and panel
- Neuronal marker EV panel
- MSC-derived EV panel
- Cancer-associated EV panel

vCal™ Calibrators and Standards
- nanoRainbow Beads
- Antibody Capture Beads
- Lipo100TM Vesicle Standard
- EV Standard Reference Preparations

vPlex™ EV Capture and Isolation Kits
- vPlex EV Immumoassay
- vCap™ EV Isoltation and Depletion kits

Cellarcus CRO Services

Vesicle Analytics

Single vesicle flow cytometry (vFC™)

Multiplexed EV immunoassay (MEVI)

Nanoparticle tracking analysis (NTA)

Resistive Pulse Sensing (RPS)

Cryo-electron microscopy (cEM)

Protein and lipid determination

Biofluid Fractionation and EV Isolation

Centrifugal ultrafiltration

Polymer precipitation

Ultracentrifugation

Size Exclusion Chromatography

Preparative Immunoisolation

EV Production and Engineering

Cell culture and engineering

EV labeling and loading

Cellarcus Biosciences

Capabilities
vFC™
MEVI™
mRPS
NTA
cryoEM
Spectroscopy
Flow cytometry
EV Production
EV Enrichment (SEC, UC, UF, vIC™)
HTS
Custom assay development and validation
Reagent development and validation

Products
Assays
Antibodies
EV Reference Preps
Size standards
Intensity standards
Antibody binding standards

Influence and Leadership
ISEV
ISAC/CYTO
ISTH
EV FC Working Group
NIH ERCC
Cytometry Development Workshop
Journals (JEV, Current Protocols in Cytometry, Cytometry Part A)

Intellectual Property
Issued patents
- US 10,429,302
- Related divisional and continuations

Patent applications
- Additional applications on related technologies

Formulations and protocols
- Optimized and validated over >50,000 analyses

Funding
R43 DA046616-01 - $150,000
R44 DA046616-02 - $747,646
R44 DA046616-03 - $695,433
R44 GM136165-01 - $755,406
R44 GM136165-02 - $890,919

Extra slides

Cellarcus vFC^{TM} Calibration

		Count	Size	Abs/EV	Fluors/EV	Photons/EV
Protocol 0.1	**vCal™ nanoRainbow beads**	●	○	○	○	●
Protocol 0.2	**Lipo100™**		●			
Protocol 0.3	**vCal™ Ab cap beads**			●	○	
	MESF beads			○	●	
	LED Pulser					●
	Accucount	●				

Comparison: Single EV Analysis Methods

Method Principle	Vendor	Size range	Antigen detection	Specificity	Speed Samples/hr
EM Electron density	FEI, Hitachi, JOEL, Thermo, Zeiss	5 - 500 nm	1 - 2 markers Immunogold	Moderate	2
NTA Light scatter/diffusion	Malvern NanoSight, ParticleMetrix,	70 - 500 nm	No	None	8
TRPS Impedance	Izon qNano, Spectradyne	50 - 300 nm	No	None	4
Conventional FC Light scatter	Apogee, Beckman Coulter, Becton Dickenson	300 nm+	1-10+ markers Immunofluorescence	None	4-30
vFC™ +CytoFLEX® **Fluorescence**	**Cellarcus**	**~70 - 1000 nm**	**1-10+ markers** **Immunofluorescence**	**Membrane**	**30**

EV Detection by Flow Cytometry

Trigger Parameter	Advantages	Disadvantages
Forward angle light scatter (FSC)	Strong scatter from particles larger than laser wavelength	High background, non-specific Difficult to estimate particle size from intensity
Orthogonal light scatter (SSC)	Lower background compared to FSC	Non-specific, difficult to estimate particle size from intensity
Ligand fluorescence (FL-mAb)	High specificity for target, Quantitative, sensitive	Only detects particles bearing detectable amounts of target
Membrane fluorescence	Membrane particles selectivity Calibration to estimate size	Some membrane dyes can result in unwanted background
Volume fluorescence	Can stain EVs	Staining depends on enzymatic activity, may also stain other particles.

Opportunity

Sales to existing EV researchers

- 2000+ researchers
- Supplant NTA, RPS, SP-IRIS instrumentation (install base of ~1200 instruments) and legacy cytometers and create active user base.

Sales to Broader Research Markets

- EV analysis as ubiquitous as cell analysis ➟ driven by observations of unique signaling mechanisms in culture models and growing at CAGR >25% over past decade.

Biomarkers/Diagnostic Reagents

- Patient stratification according to tumor marker expression
- Response to therapy
- Monitoring disease progression
- Screening for multiple tumor EV biomarkers

Future Instrumentation

- EVs down to 30nm
- Single molecule sensitivity

Tactics

Co-marketing/sales to support initial EV research target

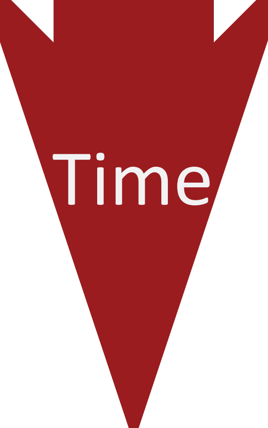
- Sync approaches and messaging by funding project to develop holistic method incorporating scatter and fluorescence-based methods.
- Use Beckman's instrumentation and marketing resources to leverage Cellarcus' influence within KOL groups and societies.
- Develop sales team to leverage approach and commercial assays and services to create users and intelligence.

Product development to speed adoption amongst existing researchers

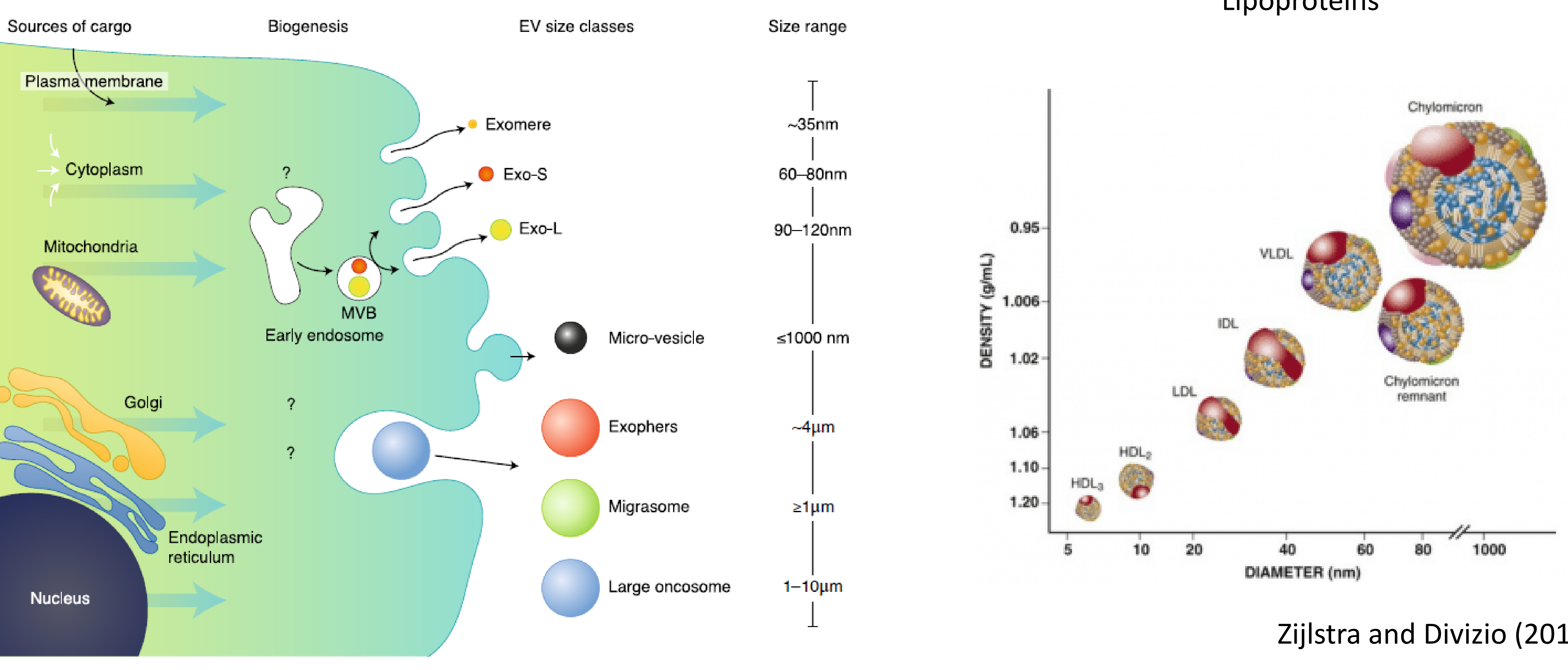
- Modifications to existing CytoFLEX® to achieve better sensitivity and a base configuration to drive immediate adoption.
- Development of data analysis and sharing capabilities on Cytobank

Extract additional revenues and expand into larger markets

- Support development of future instrumentation – Related IP
- Further development of related, supporting methods – Related IP
- Expansion to clinical markets (solid tumors, neuro) – Related IP



Single cells to single molecules



Zijlstra and Divizio (2018)

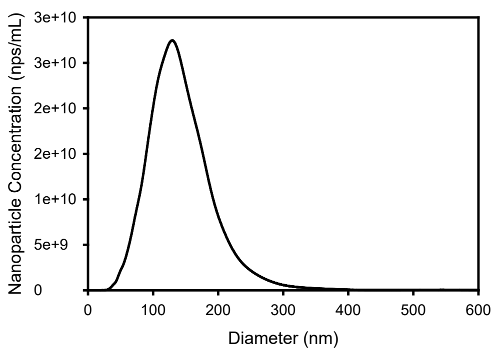
Nanoparticle Tracking Analysis (NTA)

Nanosight.com *Particle-metrix.de* *mantainc.com*

Individual particles are detected via laser light scatter

Brownian motion tracked

Size estimated from diffusion coefficient



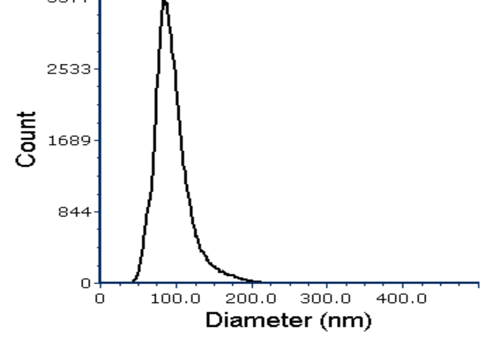
Pros
- Estimates diameter
- Label free

Cons
- Non-specific
- Sensitivity limited by light scatter
- Can't measure cargo

Resistive Pulse Spectroscopy (RPS)

www.izon.com *Particleanalyzer.com*

- Coulter principle using nanopores
- Particles block current when they enter pore
- Impedance is proportional to size



Diameter (nm)

Pros
- Estimates volume
- Label free

Cons
- Non-specific
- Sensitivity, dynamic range limited by pore size
- Can't measure cargo

Key Challenges for EV Research

EVs in biofluids are heterogeneous

- Many types of EVs from many different cells

Conventional biochemical analysis report population averages

- e.g. Western, ELISA, mass spec, PCR
- Signals for low abundance, low frequency EVs are lost in noise

Single EV analysis can resolve heterogeneity, but

- EVs are too small and dim for existing methods
- Improved tools for analysis of individual EVs are needed

Comparison: Single EV Analysis Methods

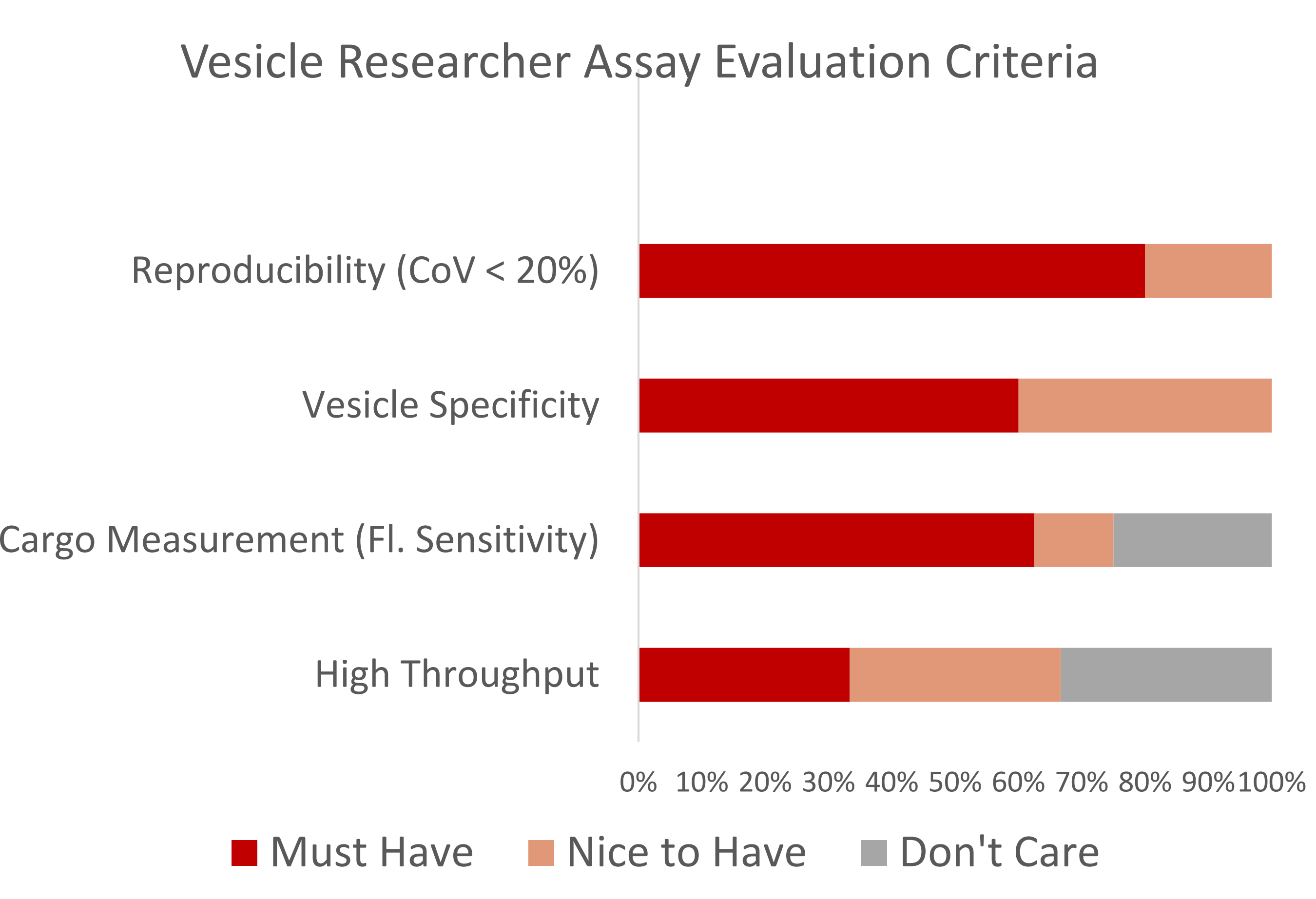
Method Principle	Vendor	Size range	Antigen detection	Specificity	Speed Samples/hr
EM Electron density	FEI, Hitachi, JOEL, Themo, Zeiss	5 - 500 nm	1 - 2 markers Immunogold	Moderate	2
NTA Light scatter/diffusion	Malvern NanoSight, ParticleMetrix,	70 - 500 nm	No	None	8
TRPS Impedance	Izon qNano, Spectradyne	50 - 300 nm	No	None	4
Conventional FC Light scatter	Apogee, Beckman Coulter, Becton Dickenson	300 nm+	1-10+ markers Immunofluorescence	Low (immunofluor)	4-30
SP-IRIS Interferometry	ExoView	50-200 nm	1-3 markers Immunofluorescence	Low (immunocapture)	4

How to Displace Existing Methods and Emerge as the Dominant Approach for Vesicle Analysis

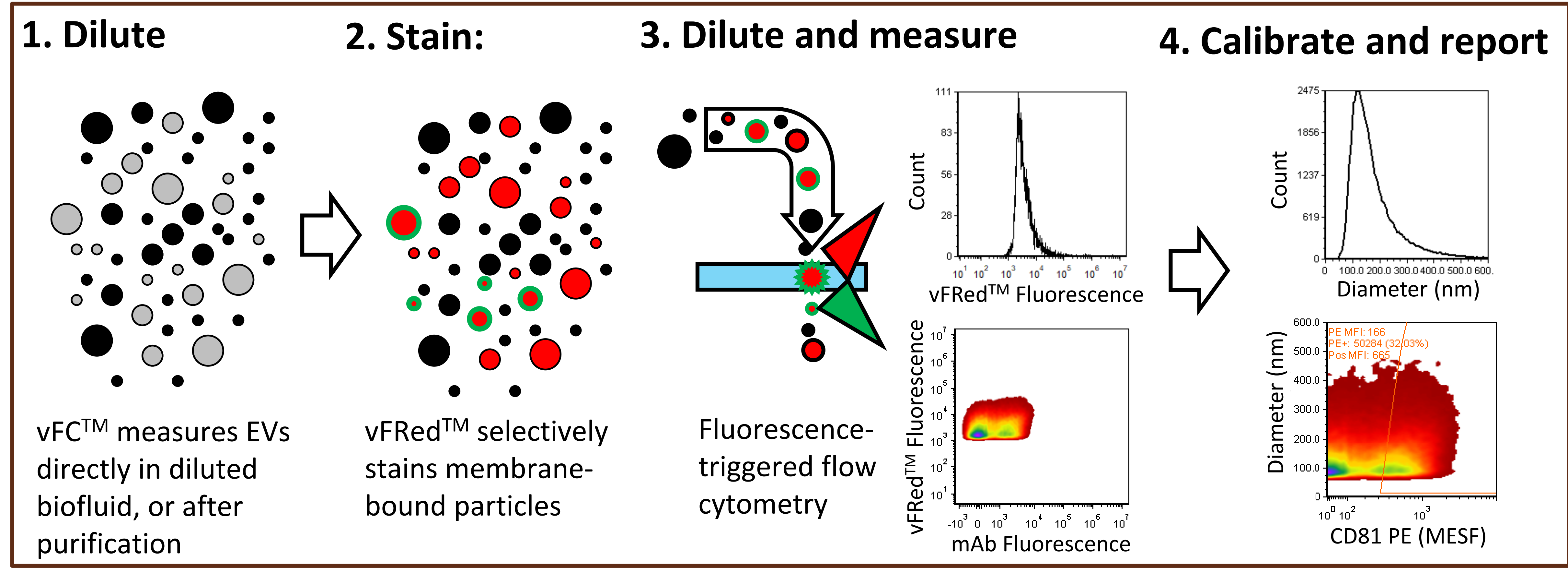
Develop an assay that is more specific, reproducible, and possibly also high throughput and easy to use.

Enable analysis of vesicle subsets via cargo.

Leverage standards and reference preps to demonstrate assay performance against backdrop of ISEV push for better methods.



Vesicle Flow Cytometry (vFC™)



- Membrane probe provides specificity
- Measures EVs directly in biofluid: no isolation/purification required
- Homogeneous assay: no wash steps
- Plate-based assays, robot-compatible

- Uses commercial flow cytometers
  - Beckman CytoFlex
  - Luminex CellStream

- Sensitive and specific detection: vesicle size to ~50 nm, cargo to <10 molecules
- Calibrated measurements for inter-lab, longitudinal, cross-platform comparisons

Instruments and Assays for EV analysis

Instruments

Beckman Coulter CytoFlex
Sensitive APD array detectors
Efficient high NA light collection
High resolution light scatter
Multiple lasers
Plate loader

Assay

- Instrument, calibrated
- Reagents and standards
- Sample preparation protocols
- Data analysis protocols
- Validation and reporting

Cellarcus Vesicle Flow Cytometry (vFC™)
Membrane selective detection
Fluorescence-based size estimate
No-wash, plate-based assay
Calibrators and standards
Standardized protocols

Comparison: Single EV Analysis Methods

Method **Principle**	**Vendor**	**Size range**	**Antigen detection** **Principle**	**Speed** **Samples/hr**
EM Electron density	FEI, Hitachi, JOEL, Themo, Zeiss	5 - 500 nm	1 - 2 markers Immunogold	2
NTA Light scatter/diffusion	Malvern NanoSight, ParticleMetrix, Manta	70 - 500 nm	No	8
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vFC™ +CytoFLEX® **Fluorescence**	**Cellarcus**	**~70 - 1000 nm**	**1-10+ markers** **Immunofluorescence**	**30**

Existing Methods with are limited

Cellarcus Biosciences' Vesicle Flow Cytometry vs Competing Technologies				
Feature	NTA	TRPS	SP-IRIS	Conventional FC
EV detection principle	Light scatter	Impedance across a nanopore	Interferometry	Light scatter
Specificity	Low	Low	High	Low
EVs Measured	All	All	Marker +	All
Sample volume	300 uL	300 uL	50 uL	50 uL
EV size range	80 - 500 nm	50-300 nm or 200-500 nm	>50nm	80 - 2000 nm+
EV surface marker measurement (LOD)	No	No	Yes	Yes
Automation/High throughput	No/limited	No	No	96 well plates
Commercial Offering	Malvern, ParticleMatrix	Izon, Spectradyne	Nanoview	Becton Dickenson, Beckman Coulter, Luminex/Amnis, Acea, and others

Opportunity

Biomarkers/Diagnostics

- Patient stratification according to tumor marker expression
- Response to therapy
- Monitoring disease progression
- Screening for multiple tumor EV biomarkers

Therapeutics

- Development of targeted delivery systems
- Nanoparticle characterization and quality control

Research Use Only products to support both

Cellarcus Solutions

Cellarcus offers tools and services to study EVs:

- **High-resolution single vesicle analysis**
  - **Counting and sizing down to ~50 nm, cargo to <10 molecules/vesicle**
- Multiplexed vesicle immunoproteomics
  - Efficient and sensitive immunocapture array technology
- High resolution analysis of molecular cargo
  - Extending multiplexed analysis to EV genomics, proteomics, and lipidomics
- Standards and calibrators to support these tools
  - Vesicle size standards
  - Calibration standards for EV immunofluorescence
  - Highly characterized cell-specific EVs

Cellarcus Solutions

Cellarcus offers tools and services to study EVs:

- **Single Vesicle Flow Cytometry (vFC™)**
  - **Counting and sizing down to ~50 nm, cargo to <10 molecules/vesicle**
- Multiplexed vesicle immunoproteomics (vPlex™)
  - Efficient and sensitive immunocapture array technology
- High resolution analysis of molecular cargo
  - Extending multiplexed analysis to EV genomics, proteomics, and lipidomics
- Standards and calibrators to support these tools
  - Vesicle size standards
  - Calibration standards for EV immunofluorescence
  - Highly characterized cell-specific EVs

Cellarcus Intellectual Property

Issued patents

- US 10,429,302
- Related divisional and continuations

Patent applications

- Additional applications on related technologies

Formulations and protocols

- Optimized and validated over >50,000 analyses

Cellarcus Funding

Service revenue

Kit and reagent revenue

Grant revenue

- R44 DA046616-01 - $150,000
- R44 DA046616-02 - $747,646
- R44 DA046616-03 - $695,433
- R44 GM136165-01 - $755,406
- R44 GM136165-02 - $890,919

Flow Cytometry of Vesicles

Single EV measurements are required, but individual EVs are small, dim, and hard to measure:

- EVs are ~100X smaller in diameter compared to cells
- EVs have ~10,000x less surface area than cells (surface cargo detection)
- EVs have ~1,000,000 less volume than cells (internal cargo detection)

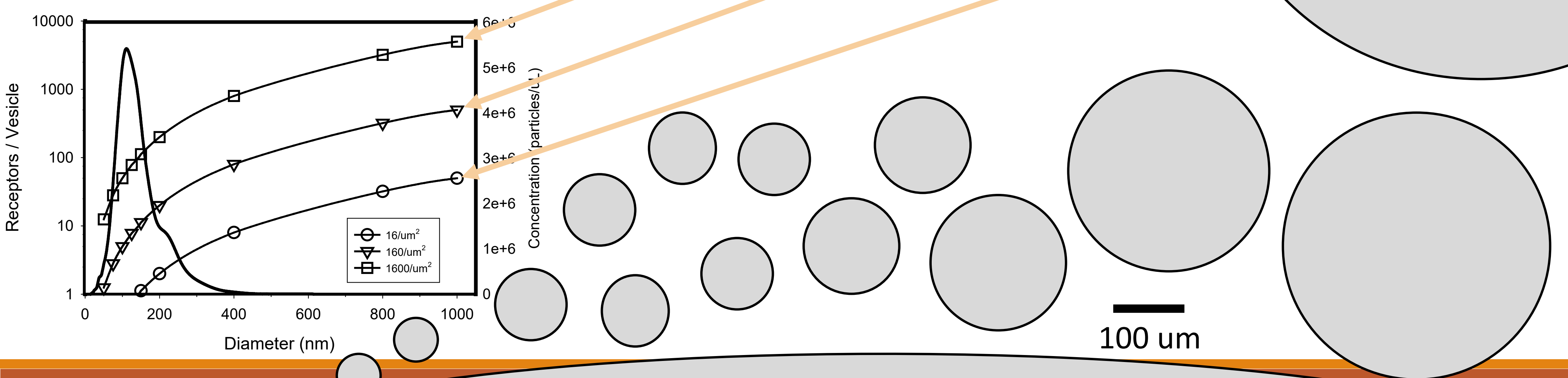
Cell

10 um diameter

High abundance: 500,000 copies (~1600/um²)

Medium abundance: 50,000 copies (160/um²)

Low abundance: 5,000 copies (16/um²)



Vesicle Flow Cytometry (vFC™)

1. Dilute

vFC measures EVs directly in diluted biofluid, or after purification

2. Stain:

vFRed™ stains membrane-bound particles, bright mAbs stain cargo

3. Detect and measure

Fluorescence-triggered flow cytometry

Membrane Fluor

mAb Fluorescence

4. Calibrate and report

Diameter (nm)

CD81 PE (MESF)

- Membrane probe provides specificity
- Homogeneous assay: no wash steps
- Measures EVs directly in biofluid: no isolation/purification required
- Uses commercially-available flow cytometers
- Lab automation-compatible
- Sensitive and specific detection: vesicle size to ~50 nm, cargo to <10 molecules
- Calibrated measurements for inter-lab, longitudinal, cross-platform comparisons

VFC Assay Specificity

Essential Control Experiments

- Specificity
  - Buffer only sample shows low level of background events
  - Detergent lability – confirms vesicular nature of detected particles
- Serial Dilution
  - Number of detected particles decreases in proportion to dilution
  - Particle brightness does not change with dilution: consistent with measurement of individual vesicles

Analysis of Individual Extracellular Vesicles by Flow Cytometry

Chapter 5

Teresa S. Hawley and Robert G. Hawley (eds.), *Flow Cytometry Protocols*, Methods in Molecular Biology, vol. 1678, DOI 10.1007/978-1-4939-7346-0_5, © Springer Science+Business Media LLC 2018

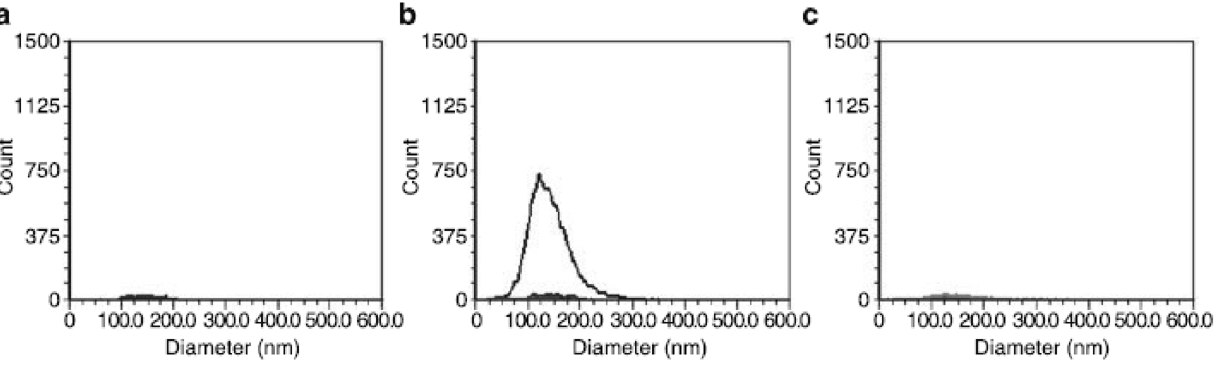


Fig. 2 VFC of RBC EVs. Fluorescence histograms of (**a**) buffer plus vesicle stain, (**b**) RBC EVs plus stain, and (**c**) RBC EVs plus stain plus detergent

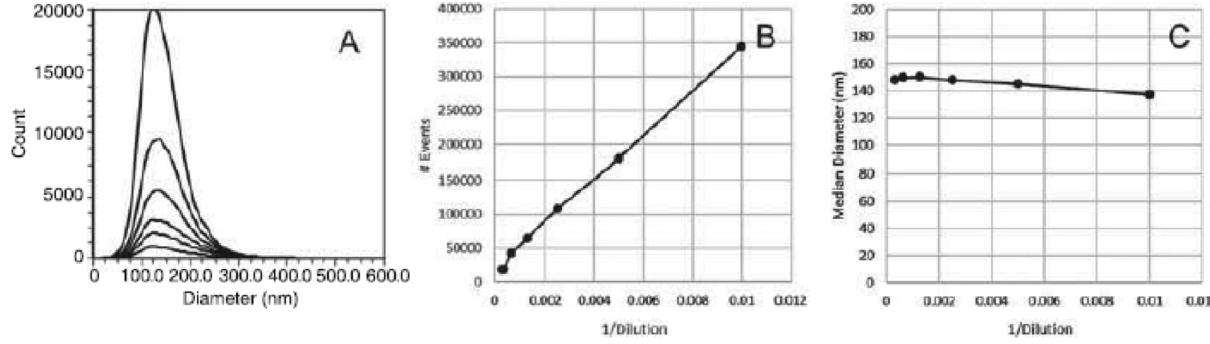
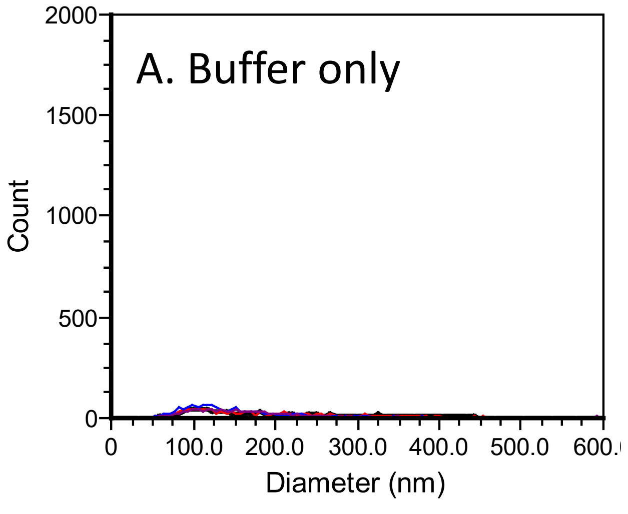
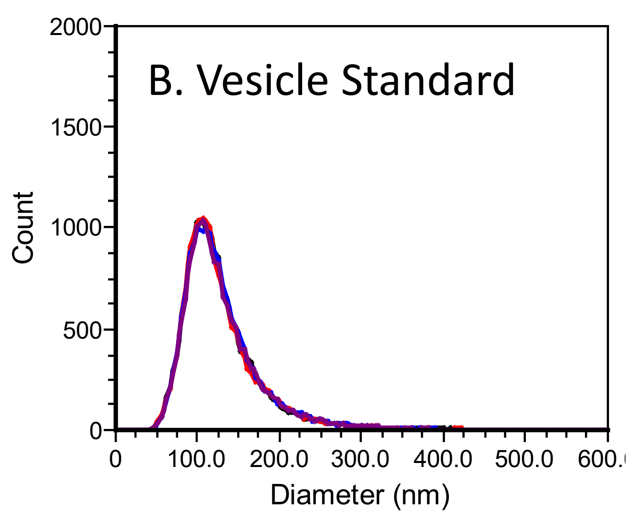
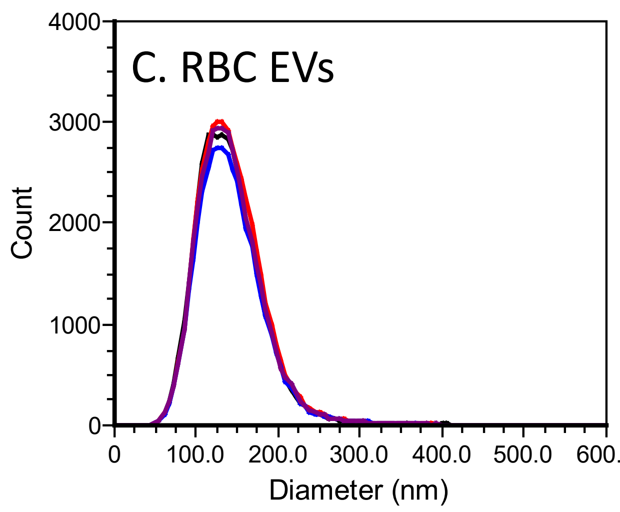


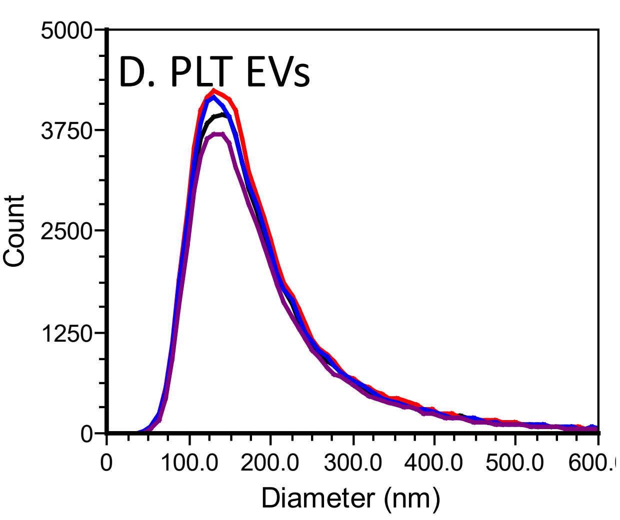
Fig. 4 Dilution of stained sample to demonstrate lack of coincidence. (**a**) Fluorescence intensity histograms of twofold serially diluted stained RBC EVs. (**b**) Plot of Event Number vs reciprocal dilution. (**c**) Plot of fluorescence Intensity-derived Median Diameter vs reciprocal dilution

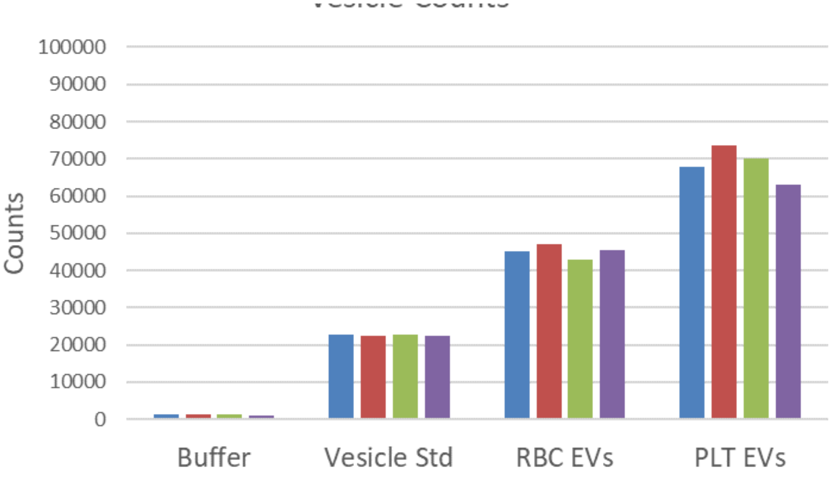
VFC Assay Reproducibility

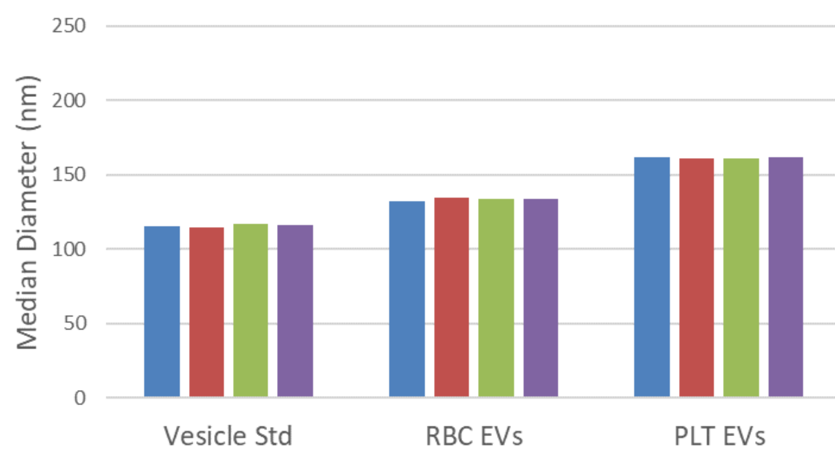




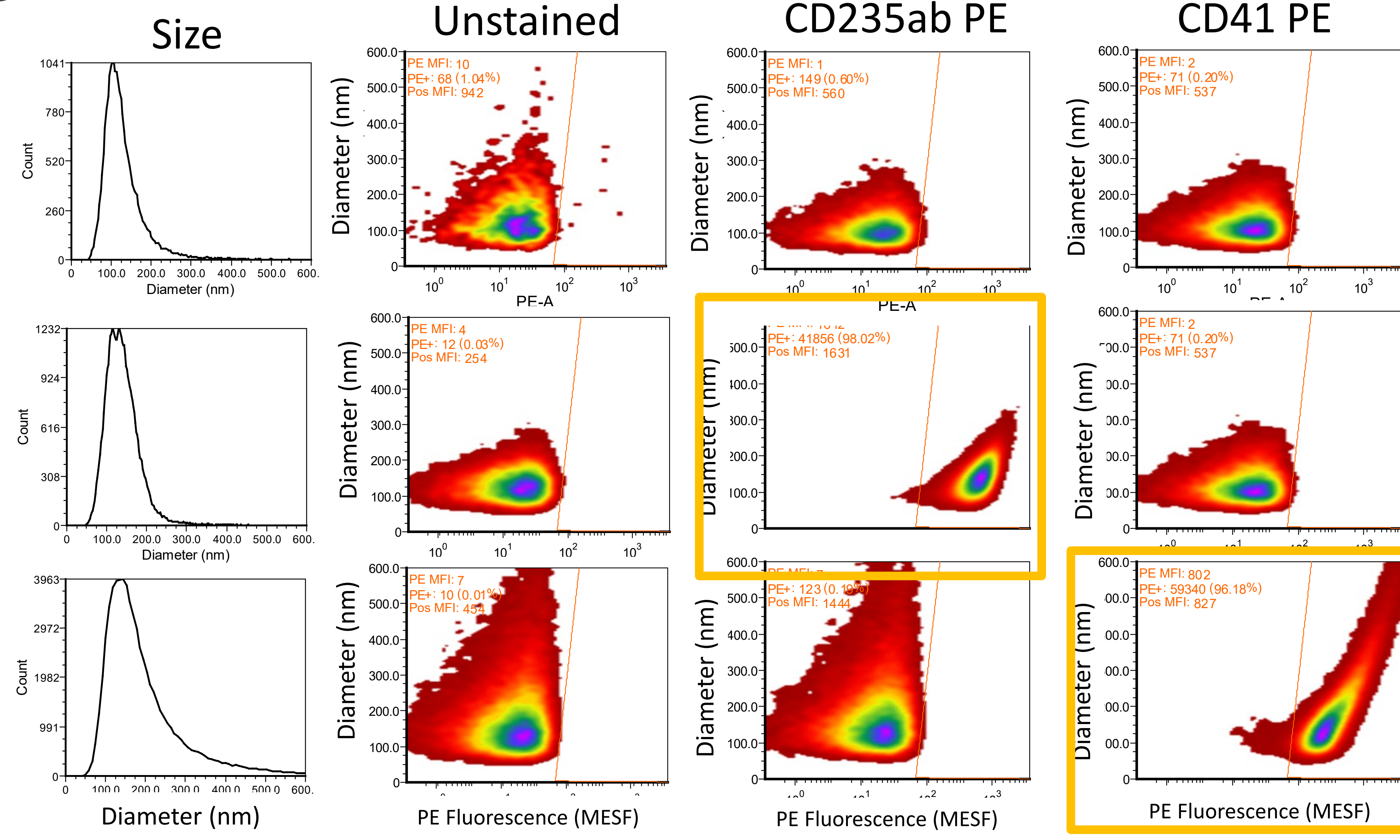








VFC Cargo Immunofluorescence

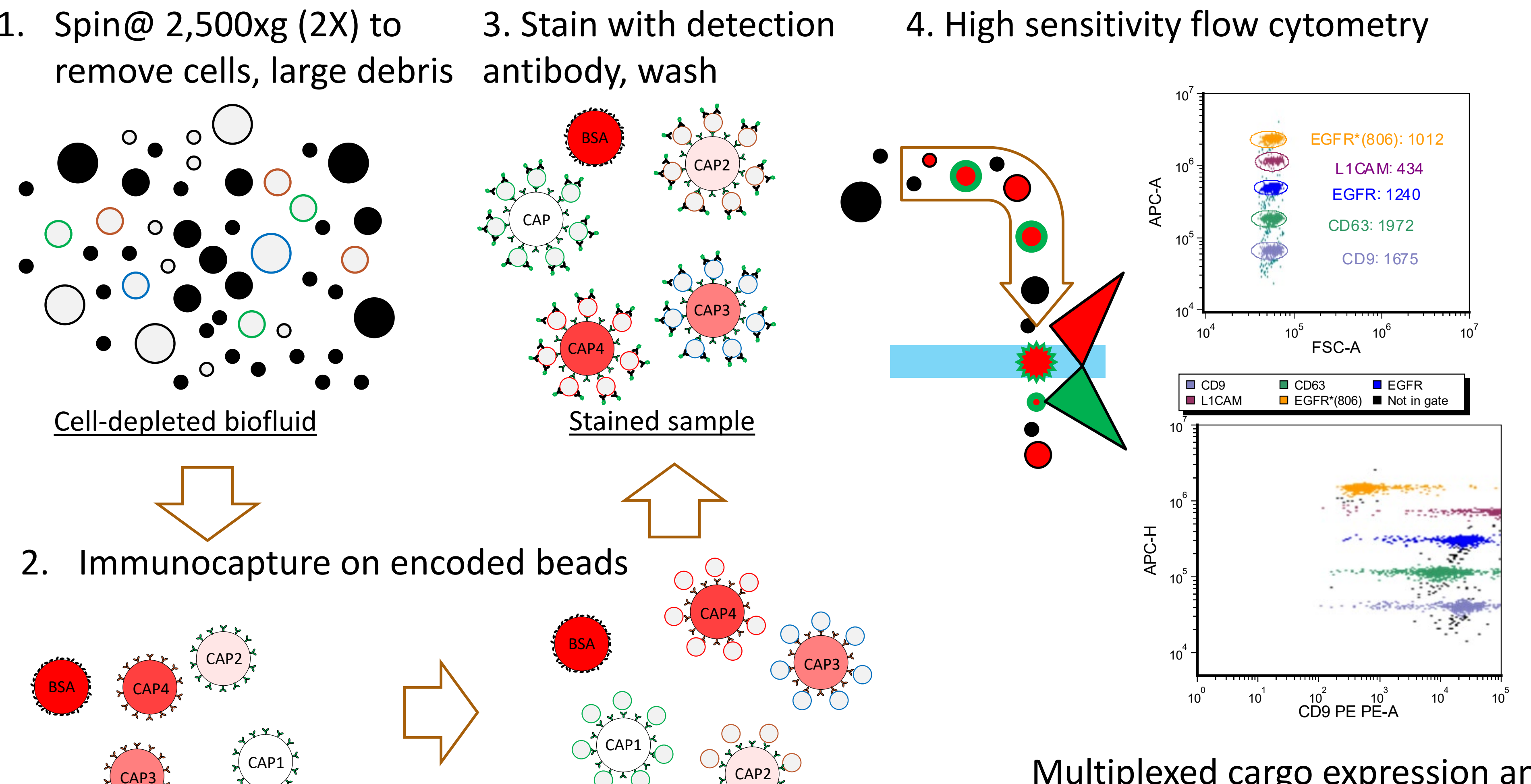


Cellarcus Solutions

Cellarcus offers tools and services to study EVs:

- High-resolution single vesicle analysis
  - Counting and sizing to ~75 nm, cargo to ~30 molecules/vesicle
- **Multiplexed vesicle immunoproteomics**
  - **Efficient and sensitive immunocapture array technology**
- High resolution analysis of molecular cargo
  - Extending multiplexed analysis to EV genomics, proteomics, and lipidomics
- Standards and calibrators to support these tools
  - Vesicle size standards
  - Calibration standards for EV immunofluorescence
  - Highly characterized cell-specific EVs

Multiplex EV Immunoassay (MEVI)



Multiplexed cargo expression and co-expression
Microscale analysis: >25 uL of biofluid
Quantitative reporting: molecules of cargo

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- **Standards and calibrators to support these tools**
  - **Vesicle size standards**
  - **Calibration standards for EV immunofluorescence**
  - **Highly characterized cell-specific EVs**

vFC™: Standards for EV Analysis

Measurement	Standard	Uses	Data
Vesicle size	Lipo100™ : synthetic vesicle, extruded through nanopore filters, extensively characterized	Calibrate VFC measurements, Immunofluorescence negative control	Lipo100™ Vesicle Size Standard
Fluorescence intensity	vCal™ nanoRainbow and MESF calibration beads: Polymer beads (800 nm) with calibrated fluorescence	Calibrate fluorescence (MESF units) Enable cross-platform fluorescence measurements	vCal™ MESF calibration beads
Antibody binding	vCal™ mAb binding beads: Polymer beads (800 nm) with calibrated mAb capture capacity	Qualify antibody conjugates, Calibrate antibody binding, Enable cross-platform measurements	vCal™ mAb capture beads stained with PE-anti-CD41
Cell-derived EVs	EVs prepared from specific cell types expressing characteristic cargo	Cargo expression positive control, size and concentration standard, enable cross-platform measurements	vCal™ RBC EVs staining with PE-anti-CD235ab

Standards for EV Analysis

Standard Type	Description	Use	Example
Vesicle size standard	Phospholipid vesicle prepared by extrusion and characterized by a suite of particle sizing methods	Calibrate VFC size measurements	100 nm Vesicle Size Standard
Fluorescence intensity	Polymer beads with calibrated levels of fluorescence	Calibrate fluorescence, enable cross-platform fluorescence measurements	PE MESF calibration beads
Antibody binding	Polymer beads with calibrated mAb capture capacity	Calibrate and standardize EV immunofluorescence measurements, enable cross-platform measurements	mAb capture beads stained with PE-anti-CD235ab
Cell-derived EVs	EVs prepared from specific cells types expressing characteristic cargo	Cargo expression std, size and concentration standard, enable cross-platform measurements	RBC EVs staining with PE-anti-CD235ab

Cellarcus Services and Products

EV Analysis Services

VFC High Resolution EV Counting and Sizing
- EV concentration (EVs/uL)
- EV size distribution

VFC EV Cargo Measurement
- Antigen molecules/EV
- Antigen+ EV concentration

EV Standards Development and mAb Validation
- Species specificity, marker specificity

Custom Assay Development and Validation
- Multiplexed immunocapture
- EV production, isolation and characterization
- Antibody validation

EV Analysis Products

EV Analysis Kits

Vesicle Size Standards

Fluorescence intensity standards

Antibody binding standards

Cell-specific EV preparations
- RBC EVs
- PLT EVs
- Cell line EVs (inquire)

More info: *www.cellarcus.com*

VFC Publications

Vesicle Flow Cytometry

Stoner SA, Duggan E, Condello D, Guerrero A, Turk JR, Narayanan PK, Nolan JP. High sensitivity flow cytometry of membrane vesicles. Cytometry Part A. 2016;89(2):196-206. doi: 10.1002/cyto.a.22787.

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Saugstad JA, Lusardi TA, Van Keuren-Jensen KR, Phillips JI, Lind B, Harrington CA, McFarland TJ, Courtright AL, Reiman RA, Yeri AS. Analysis of extracellular RNA in cerebrospinal fluid. Journal of Extracellular Vesicles. 2017;6(1):1317577.

Brooks MB, Turk JR, Guerrero A, Narayanan PK, Nolan JP,Besteman EG, Wilson DW, Thomas RA, Fishman CE, Thompson KLE-Z, J.B. Pierson, A. Paulman, A.Y. Chiang, A.E. Schultz. Non-Lethal Endotoxin Injection: A Rat Model of Hypercoagulability. PloS ONE. 2017;12(1):e0169976.

Narayanan PK, Shen L, Curtis BR, Bourdon M, Nolan JP, Zhou F, Christian B, Gupta S, Schaubhut JL, Greenlee S, Hoffmaster C, Burel S, Witztum JL, Engelhardt JA, Henry SP. Investigation into the mechanism(s) that leads to platelet decreases in cynomolgus monkeys during administration of ISIS-104838, a 2'-MOE-modified antisense oligonucleotide. Toxicological Sciences. 2018:kfy119-kfy. doi: 10.1093/toxsci/kfy119.

Nolan JP, Duggan E. Analysis of Individual Extracellular Vesicles by Flow Cytometry. Flow Cytometry Protocols: Springer; 2018. p. 79-92.

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Nolan JP, Stoner SA. A trigger channel threshold artifact in nanoparticle analysis. Cytometry Part A. 2013;83A:301-5.

Nolan JP. Flow Cytometry of Extracellular Vesicles: Potential, Pitfalls, and Prospects. Curr Protoc Cytom. 2015;73:13.4.1-.4.6. Epub 2015/07/02. doi: 10.1002/0471142956.cy1314s73.

Nolan JP, Jones JC. Detection of platelet vesicles by flow cytometry. Platelets. 2017:1-7. doi: 10.1080/09537104.2017.1280602.

Coumans FA, Brisson AR, Buzas EI, Dignat-George F, Drees EE, El-Andaloussi S, Emanueli C, Gasecka A, Hendrix A, Hill AF, Lacroix R, Lee Y, van Leeuwen TG, Mackman N, Mäger I, Nolan JP, van der Pol E, Pegtel DM, Sahoo S, Siljander PRM, Sturk G, de Wever O, Nieuwland R. Methodological Guidelines to Study Extracellular Vesicles. Circulation research. 2017;120(10):1632-48.

EVs for Liquid Biopsies

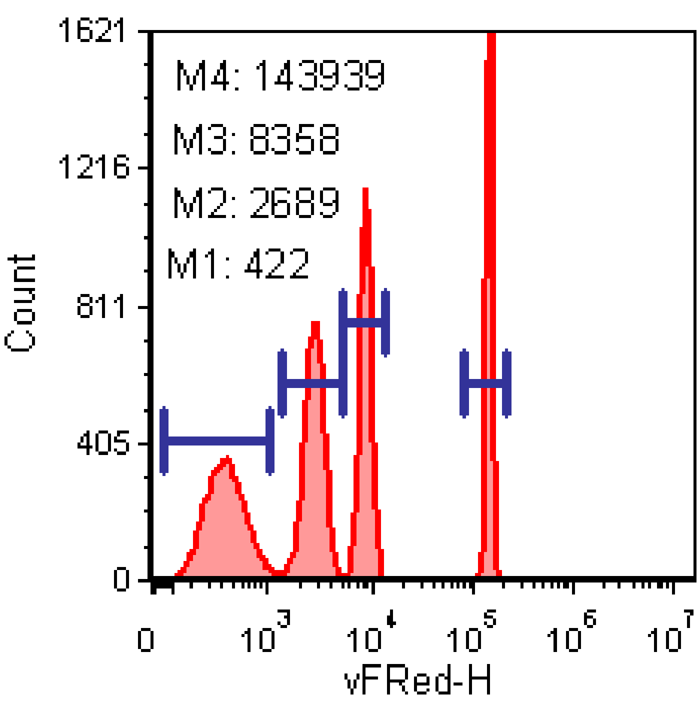
Target	Advantages	Disadvantages
ctDNA	Released from dead/dying cells - potentially abundant	High non-tumor DNA background May not correlate with tumor status
CTCs	Released from growing tumors Carry multiple tumor markers (nucleic acids, proteins)	Rare, low abundance May not reflect tumor heterogeneity
EVs	Tumor cells release many EVs Carry tumor markers (protein, nucleic acids) in context	Small, heterogeneous and difficult to measure

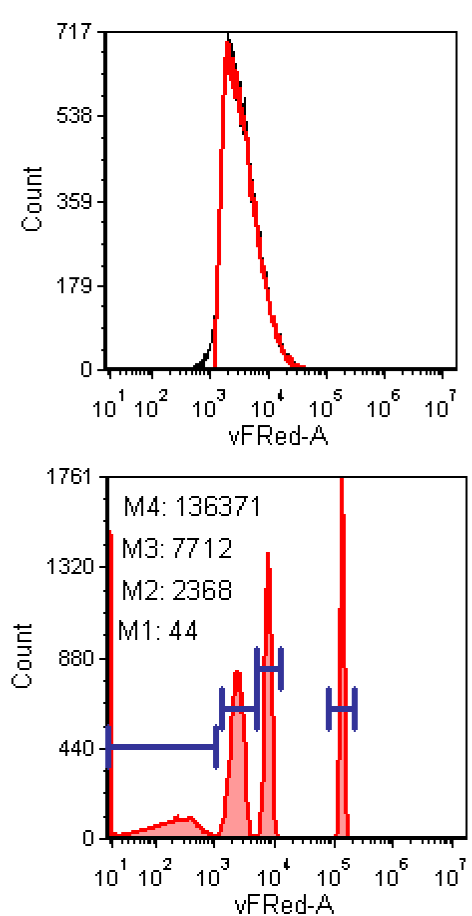
Comparison: EV Fractionation Methods

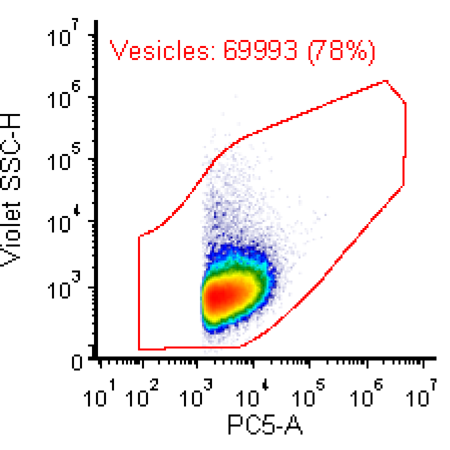
Method **Principle**	**Principle**	**Resolution**	**Volume range**	**Scalability**
Ultra-centrifugation	Density	Low	0.1-10 mL	Low
Gradient Ultracentrifugation	Density	Moderate-high	0.1-1 mL	Low
Size Exclusion Chromatography	Diameter	Low-moderate	0.1-1 mL	Moderate
Filtration	Diameter	Low	0.1 – 1L	High
vIC™ **Immunocapture**	**Antigen specificity**	**High**	**0.1- 1 L**	**High**

vCal™ nanoRainbow beads

vFRed Calibration

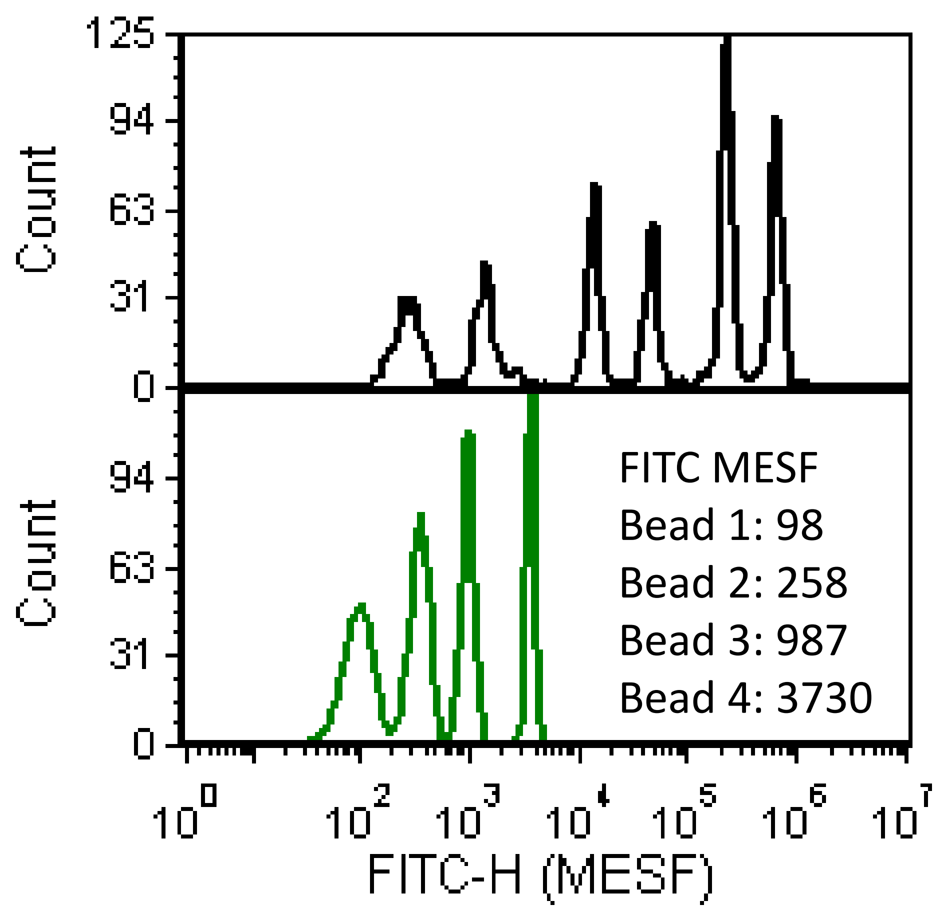


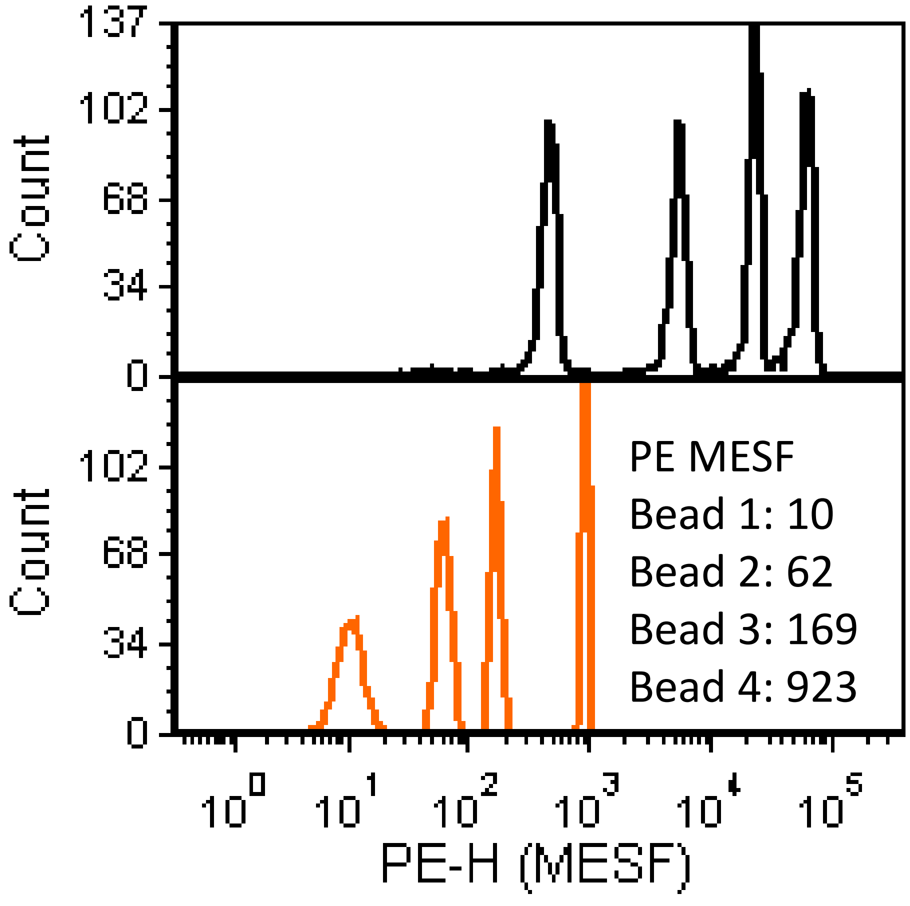




vCal™ nanoRainbow beads

MESF Cross Calibration





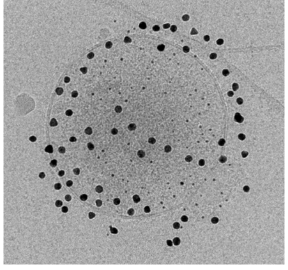
EV Standards: Blood cell-derived EVs

Liposome

No antigens

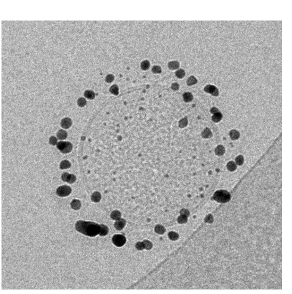
RBC EVs

- CD235
- AnnV



PLT EVs

- CD41
- AnnV



	No mAb	PE-Annexin V	PE-anti-CD235ab	PE-anti-CD41
Liposome	PE MFI: 10 PE+: 68 (1.04%) Pos MFI: 942	PE MFI: 1088 PE+: 43471 (98.27%) Pos MFI: 1099	PE MFI: 1 PE+: 149 (0.60%) Pos MFI: 560	PE MFI: 2 PE+: 71 (0.20%) Pos MFI: 537
RBC EVs	PE MFI: 4 PE+: 12 (0.03%) Pos MFI: 254	PE MFI: 142 PE+: 14855 (33.08%) Pos MFI: 410	PE MFI: 1612 PE+: 41856 (98.02%) Pos MFI: 1631	PE MFI: 2 PE+: 71 (0.20%) Pos MFI: 537
PLT EVs	PE MFI: 7 PE+: 10 (0.01%) Pos MFI: 454	PE MFI: 1065 PE+: 73456 (90.23%) Pos MFI: 1162	PE MFI: 7 PE+: 123 (0.1[illegible]%) Pos MFI: 1444	PE MFI: 802 PE+: 59340 (96.18%) Pos MFI: 827

Axes (all plots): Diameter (nm), 0–600.0; PE-A, 10^0–10^3

Unitized flow cytometry antibody panels for standardized multi-site studies

Eda Holl, Ph.D., RAC

Global Medical and Scientific Affairs

21.02.871.FLOW

Beckman Coulter Life Sciences

Mission: Empowering those seeking answers to life's important scientific and healthcare questions

Vision: Accelerating Answers

LIFE SCIENCES	DIAGNOSTICS	ENVIRONMENTAL & APPLIED SOLUTIONS

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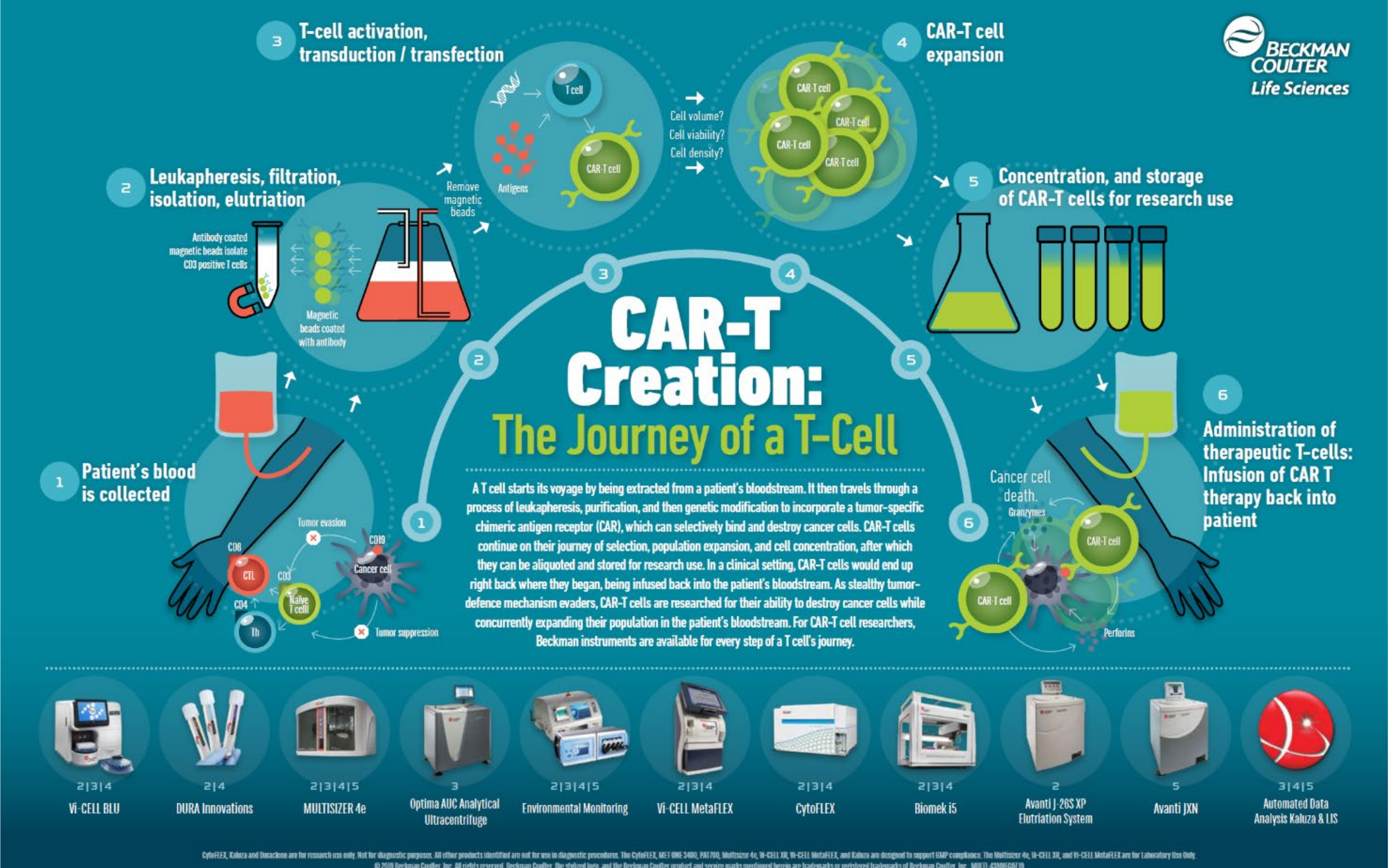
Supporting the CAR-T journey



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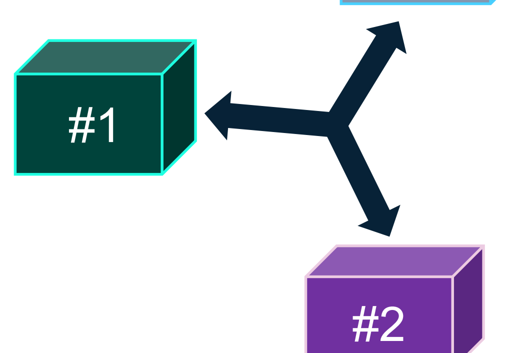
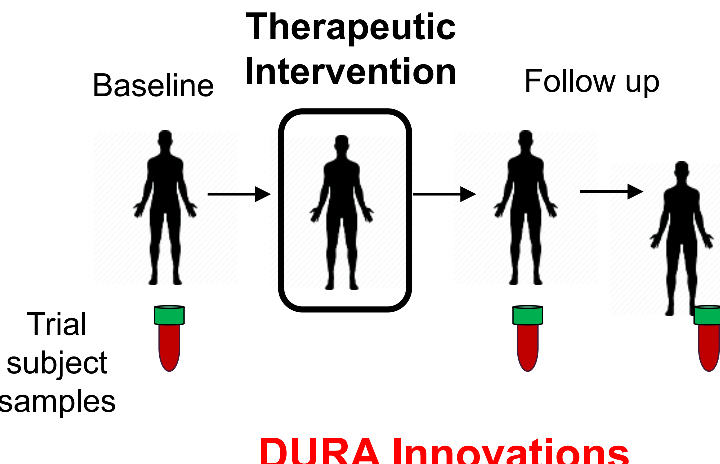
BECKMAN COULTER Life Sciences

Cancer Research Studies

- Multi-center
- Sample transport
- Timely processing post collection
- Standardization and reproducibility across sites
- Reduced error
- Efficient data collection and analysis
- Reduced bias

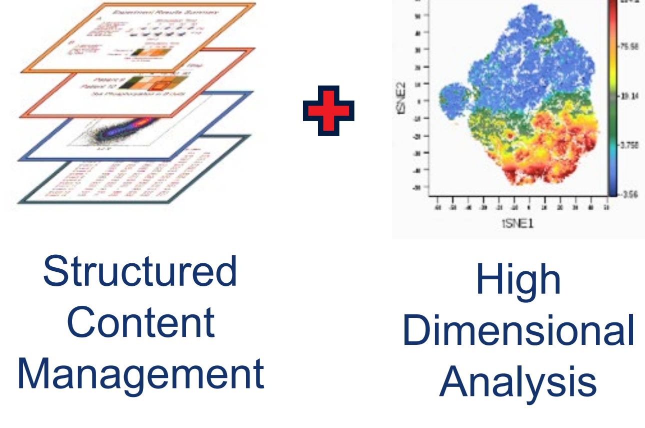


- Longitudinal
- Patient recall
- Disease changes
- Staff changes
- Instrumentation drift



DURA Innovations

- General Concerns
- Data storage guidelines
- Time to drug approval



Structured Content Management

High Dimensional Analysis

Cytobank

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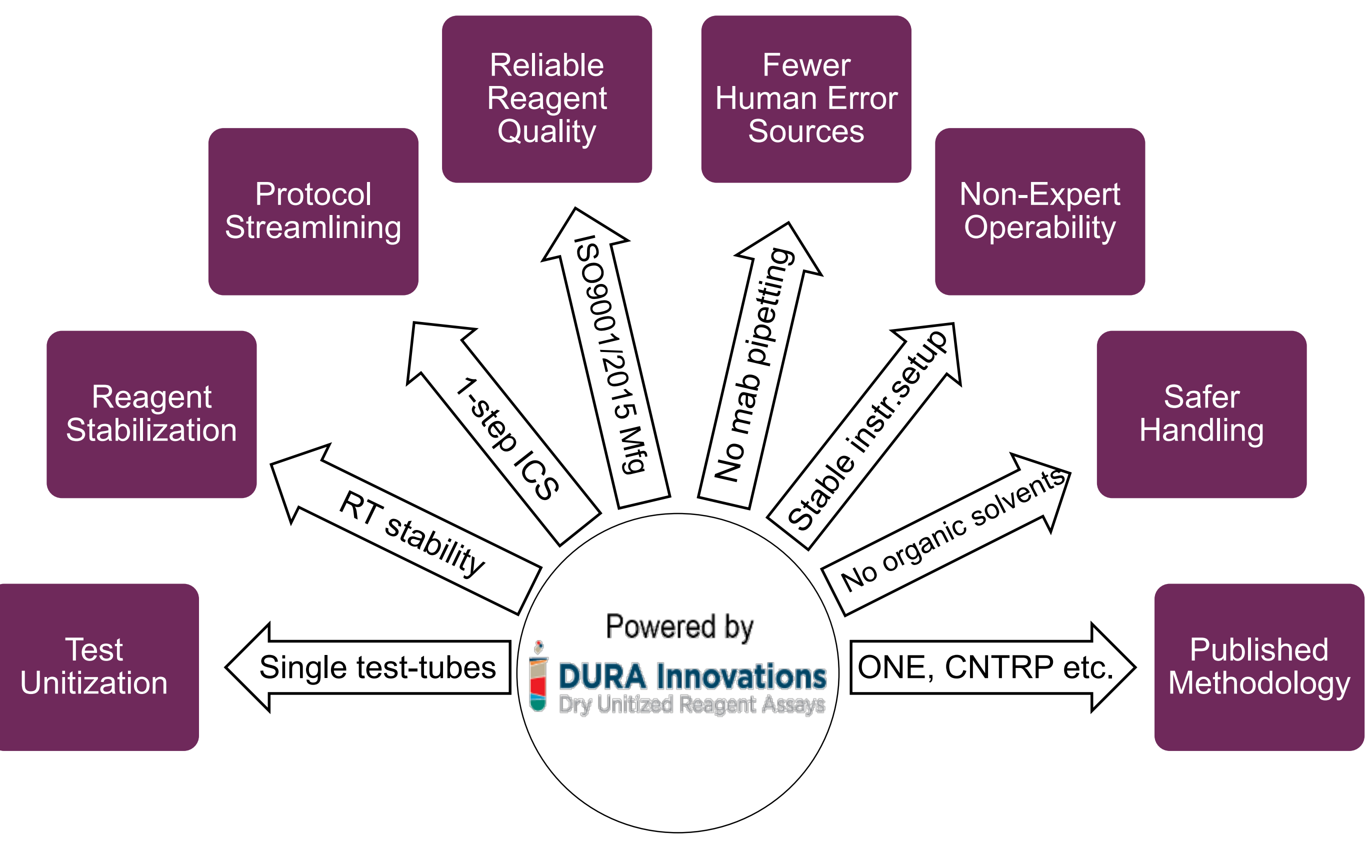
Experimental Rigor Through Dry Reagent Formulation

Dry
- Uniform reagent layer at tube bottom
- Ship & store at room temperature

Unitized
- Pre-formulated for 1 test
- Just add sample!

Reagent
- Known Beckman antibody quality
- Enhanced tandem dye stability

Assays
- Optimized panel configurations
- Up to 11 colors, beads optional
- Catalogue and custom design available



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The ONE Study: Standardizing Flow Cytometry To Advance Immune Therapies

Streitz et al. Transplantation Research 2013, 2:17
http://www.transplantationresearch.com/content/2/1/17

TRANSPLANTATION RESEARCH

RESEARCH — Open Access

Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study

Mathias Streitz[1], Tewfik Miloud[2], Michael Kapinsky[3], Michael R Reed[3], Robert Magari[3], Edward K Geissler[4], James A Hutchinson[4], Katrin Vogt[1], Stephan Schlickeiser[1], Anders Handrup Kverneland[1], Christian Meisel[1], Hans-Dieter Volk[1,5] and Birgit Sawitzki[1,5*]

2013

THE LANCET

ARTICLES | VOLUME 395, ISSUE 10237, P1627-1639, MAY 23, 2020

Regulatory cell therapy in kidney transplantation (The ONE Study): a harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials

Prof Birgit Sawitzki, PhD • Paul N Harden, FRCS • Prof Petra Reinke, MD • Aurélie Moreau, PhD • James A Hutchinson, MD • David S Game, FRCP • et al. Show all authors

Published: May 23, 2020 • DOI: https://doi.org/10.1016/S0140-6736(20)30167-7 • Check for updates

2020 (IF=59)

- Recognized as the golden reference in multi-site standardization
- 1st unified approach to characterize cellular tolerance induction
- Complete flow cytometry solution
- Trial results published in The Lancet

ONE STUDY

FLOW-7453CP08.20

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For research use only. Not for use in diagnostic procedures

Published Methodology

Examining Peripheral and Tumor Cellular Immunome in Patients With Cancer

Eda K. Holl, Victoria N. Frazier, Karenia Landa, Georgia M. Beasley, E. Shelley Hwang and Smita K. Nair**

Department of Surgery, Duke University, Durham, NC, United States

JCI insight

A standardized immune phenotyping and automated data analysis platform for multicenter biomarker studies

Sabine Ivison,[1,2] Mehrnoush Malek,[3] Rosa V. Garcia,[1,2] Raewyn Broady,[4] Anne Halpin,[5] Manon Richaud,[6] Rollin F. Brant,[2] Szu-I Wang,[5] Mathieu Goupil,[6] Qingdong Guan,[7] Peter Ashton,[8] Jason Warren,[9] Amr Rajab,[10] Simon Urschel,[5] Deepali Kumar,[8] Mathias Streitz,[11] Birgit Sawitzki,[11] Stephan Schlickeiser,[11] Janetta J. Bijl,[6] Donna A. Wall,[7] Jean-Sebastien Delisle,[6] Lori J. West,[5] Ryan R. Brinkman,[3,12] and Megan K. Levings[1,2]

First published December 6, 2018 - More info

"The development of rapid, reliable, and reproducible monitoring of the cellular immunome is required for immune biomarker development. We will use the analysis presented in this study in the planned clinical studies in patients with recurrent glioblastoma (NCT02986178), breast cancer (NCT03564782), and melanoma (NCT03712358).

*"Testing of the automated pipelines on an independent data set revealed **the power of standardization** and enabled direct comparison of data from different studies and/or centers, collected over different time intervals."*

Published Methodology

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Thank you!

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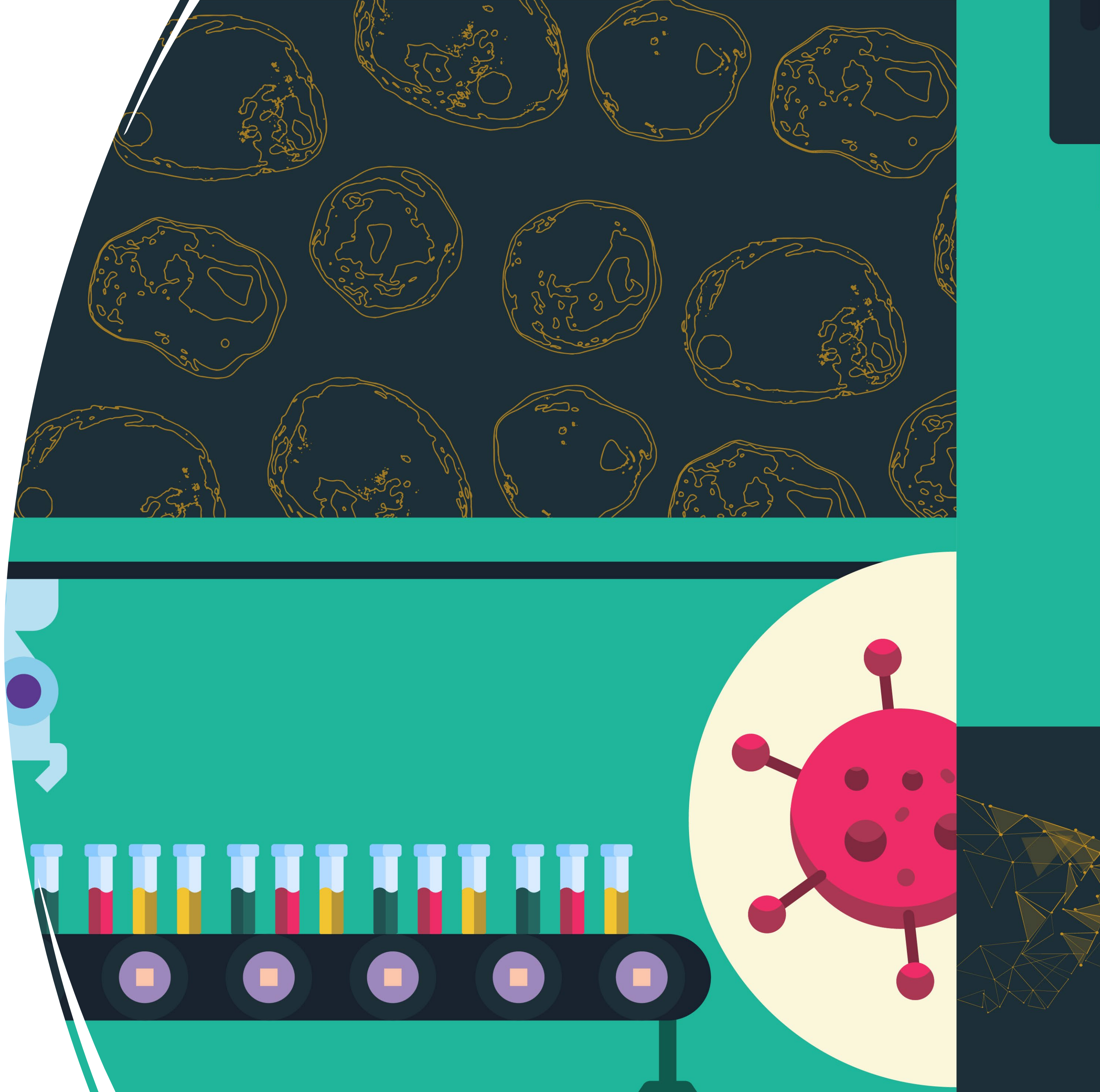
BECKMAN COULTER Life Sciences

NIST Flow Cytometry Standard Consortium

https://www.nist.gov/programs-projects/nist-flow-cytometry-standards-consortium

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

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



Why a NIST Consortium

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 Goals

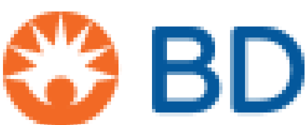
- Develop **reference standards** including **reference materials**, **reference data**, **reference methods**, and **measurement service** for assigning the **Equivalent Number of Reference Fluorophores** (ERF) to calibration microspheres and assessing the associated uncertainties and utilities.
- Develop **candidate reference standards** including biological reference materials, reference data, reference methods
- **Design interlaboratory studies** based on candidate reference materials to support the development of best practices and standard methods

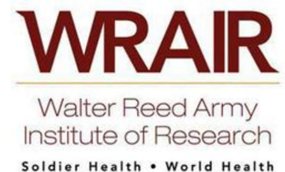
Anticipated Impact

- Shared measurement assurance tools and standards for flow cytometry measurement confidence
- Data from interlaboratory studies to support development of best practices and standard methods
- Improved flow cytometry measurement capabilities

Consortium Members

Mojave Bio, Inc





Consortium Membership

MEMBER BENEFITS

- Access to a neutral forum to address pre-competitive needs
- Participation in the development of reference materials, methods and protocols, and interlaboratory studies
- Access to tools developed by the Consortium ahead of public release
- Institutional representation on Consortium steering committee

BECOME A MEMBER

- Complete the Letter of Interest Form
- Participants will sign a Cooperative Research and Development Agreement (CRADA); Federal agencies may join under a Memorandum of Understanding (MOU)
- Annual fee of $25,000 or in-kind support of equivalent value

Monthly consortium meetings will be held only with the consortium members. The first closed meeting is tentatively scheduled for March 18, 2021