METROFLOW

THE NY/NJ FLOW CYTOMETRY USERS GROUP

October 18th, 2016

An Effort Toward Calibrating and Standardizing Measurements of EVs: A Tri-Society Endeavor

ISAC International Society for Advancement of Cytometry

ISEV INTERNATIONAL SOCIETY FOR EXTRACELLULAR VESICLES

ISTH International Society on Thrombosis and Haemostasis

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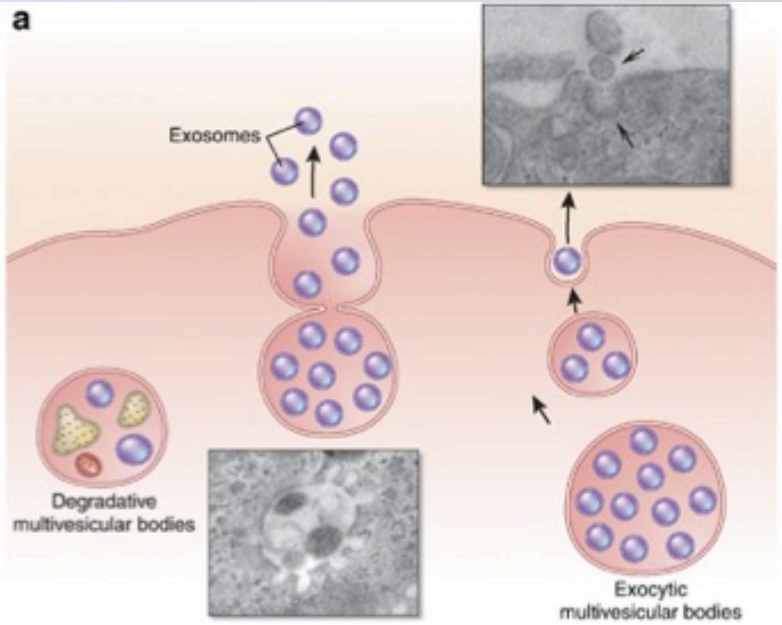
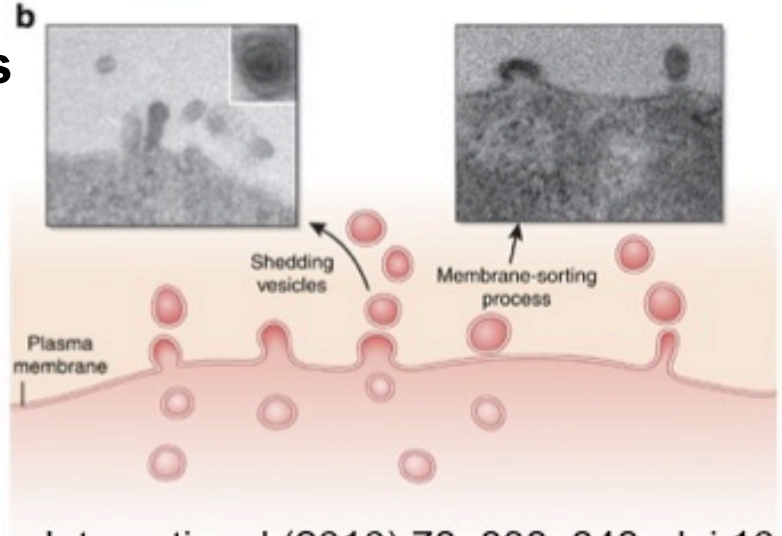
UNIVERSITY of VIRGINIA SCHOOL OF MEDICINE

What are Extracellular Vesicles (EVs)?

Exosomes
50-100nm

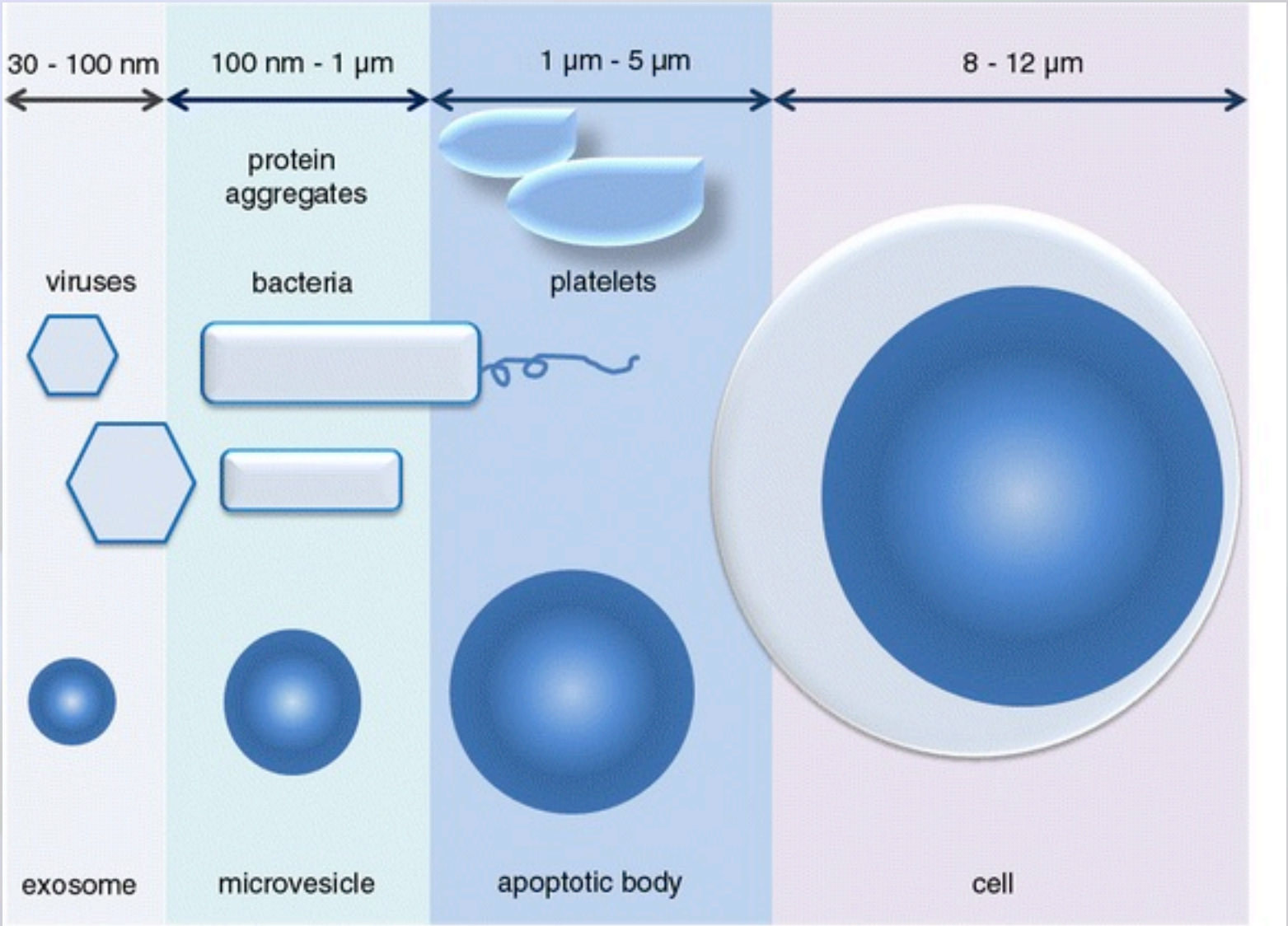


Microparticles
200-1000nm



Camussi et.al. Kidney International (2010) 78, 838–848; doi:10.1038/ki.2010.278

Size Range



Gyorgy et.al. Cell Mol Life Sci. 2011 Aug;68(16):2667-88

Why Does Everyone Want to Measure Them??

Important Biomarkers

- Cardiovascular diseases
- Cancer
- Infectious diseases
- Liver diseases
- Autoimmune diseases

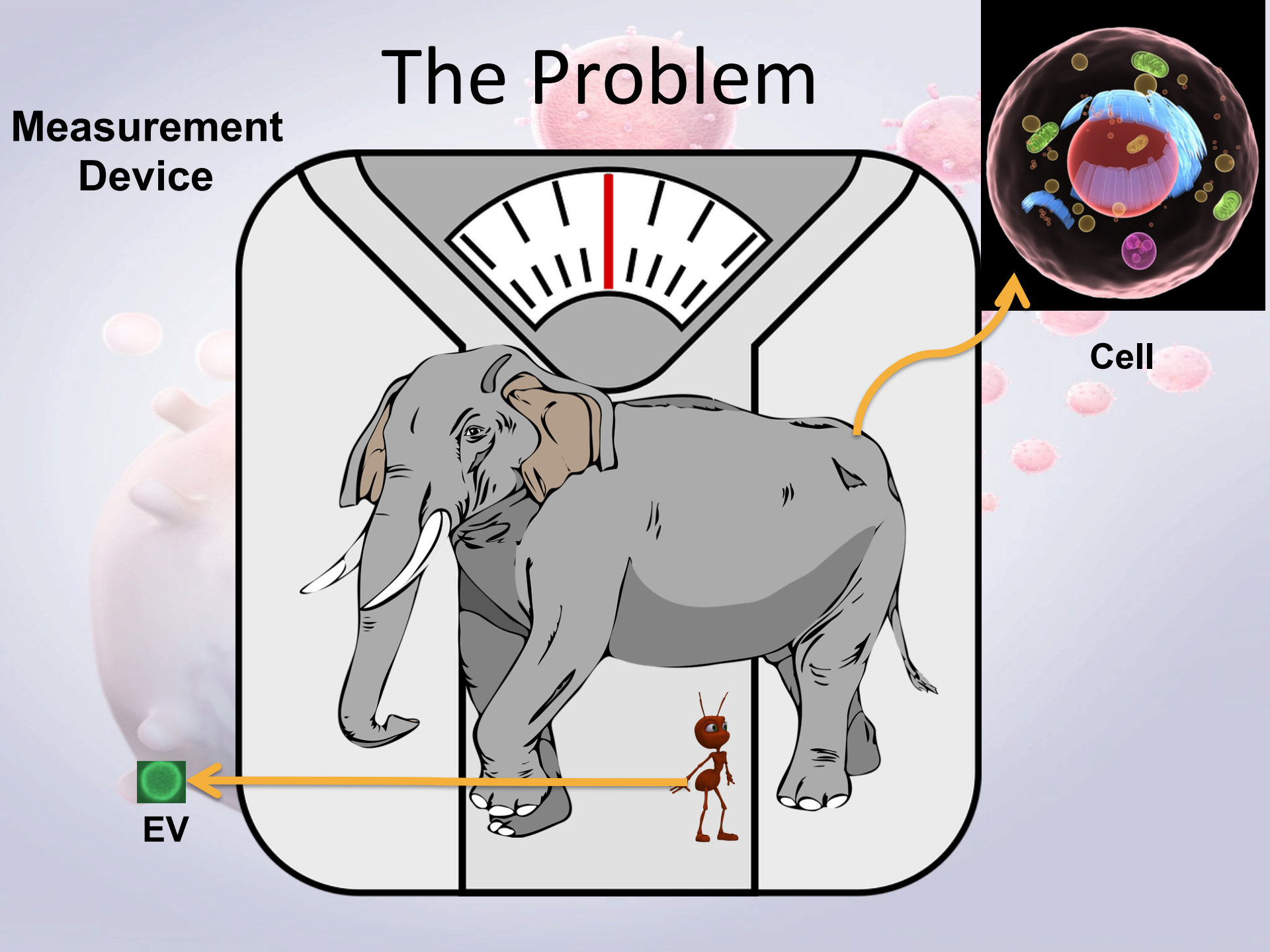
Involved in Pathogenesis

- Coagulopathies
- Inflammation
- Immunosuppression

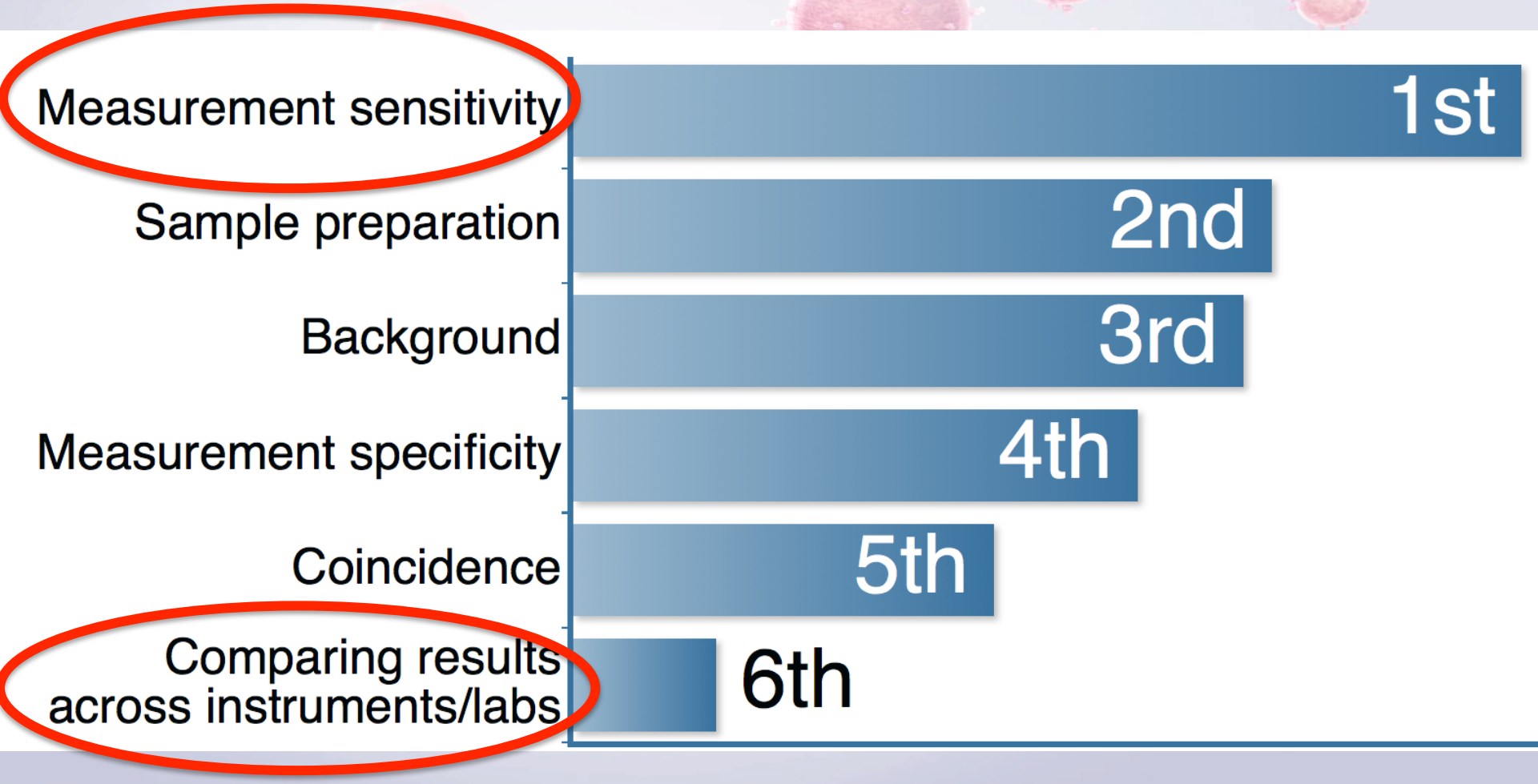
Potential Therapeutic

- Content delivery
- Stem cell development
- Anti-tumorigenesis

The Problem



What is your greatest obstacle?



Multi-organizational Effort

- ISAC-ISEV-ISTH-NIH (Established in April 2015)
  - Flow Cytometry working group with the goal to improve detection and comparison of results
    - ✓Develop standards and reference materials for scatter and fluorescence
    - ✓Develop protocols for objective instrument characterization
    - ✓Develop protocols for reporting results in standardized fluorescence units
    - ✓Develop educational materials

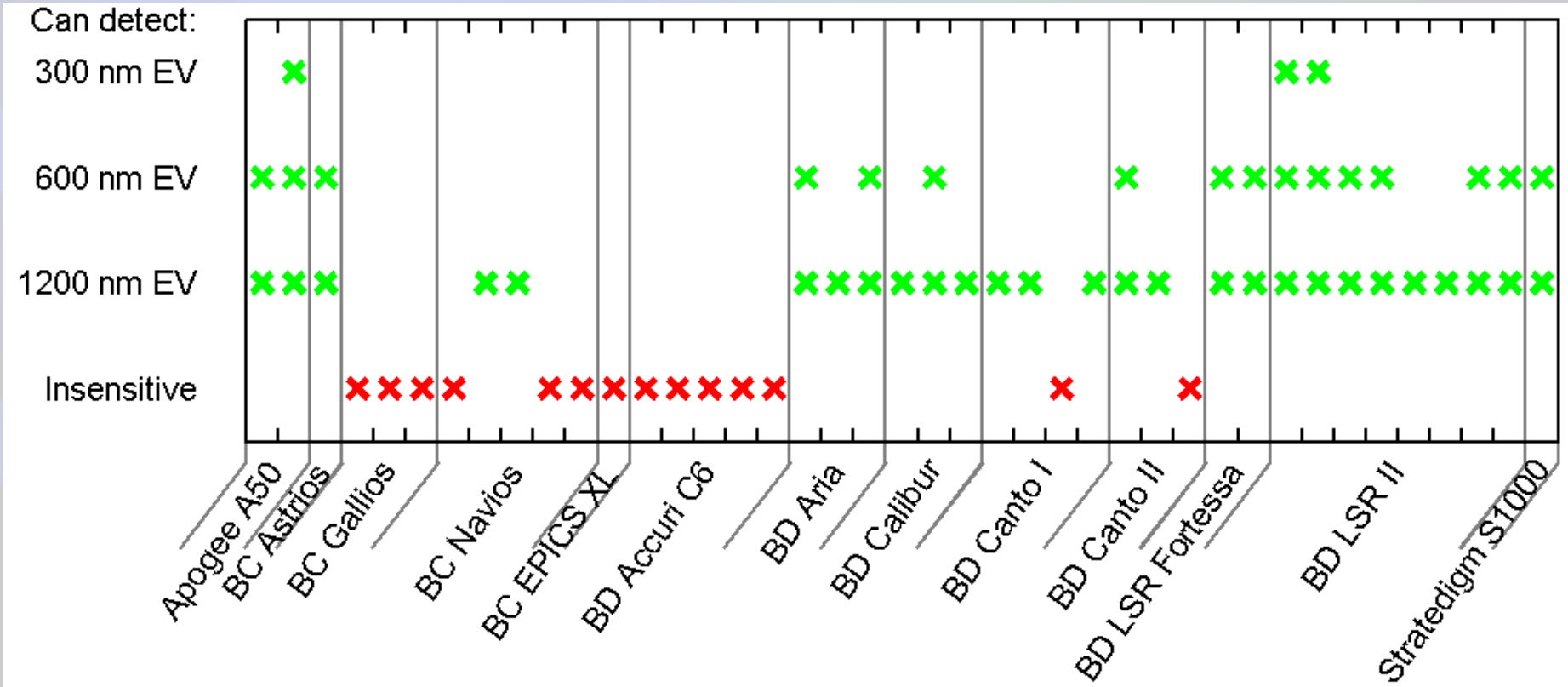
ISAC-ISEV-ISTH Flow Cytometry Working Group

- "Spring Exercise"
  - Multi-site trial of calibration and standardization
- ISTH
  - EMRP Project – METves (Metrological characterization of micro-vesicles from body fluids as non-invasive diagnostic biomarkers http://www.metves.eu/
- ISAC
  - EVIDG http://isac-net.org/Interest-Development-Groups/Microvesicle-Analysis-Interest-Development-Group.aspx
  - workshops http://cytoconference.org/2017/Home.aspx
- ISEV
  - MOOC Coursera http://isev.org/mooc/
  - Annual Conference/Education Day http://isev.org/isev2017/

Why is Instrument Characterization Important?

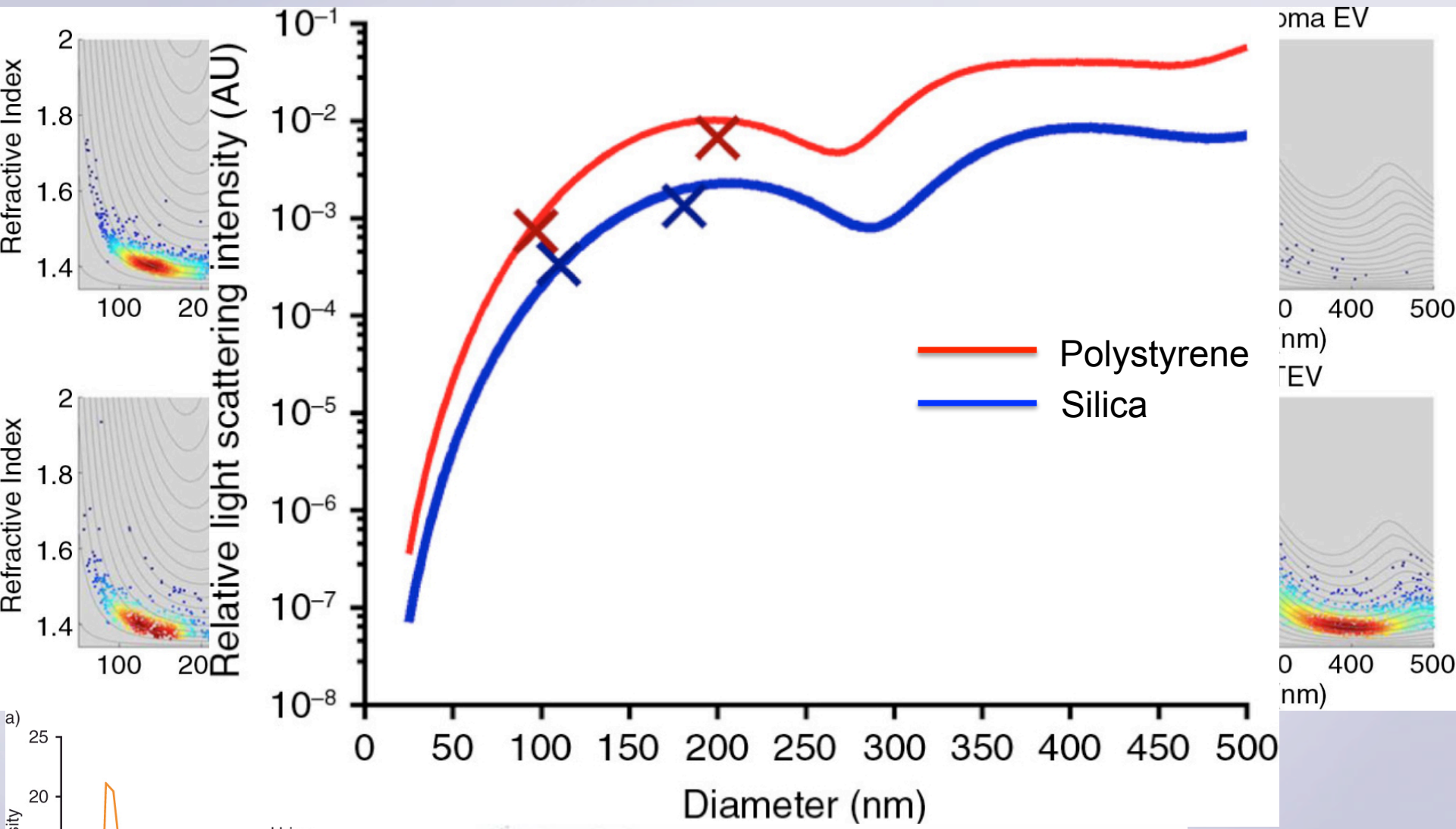
- Need to understand the limits of detection
  - Size
    - Calibrating size using scatter is difficult, complicated and lacks good standards
      - Scatter is impacted by wavelength and power of illumination, RI of medium & particle, and angle of collection
    - However, knowing the limits of size detection is important!
      - With particles whose RI is closely matched to EVs one can approximate limit of size detection
      - Useful in identifying analysis window

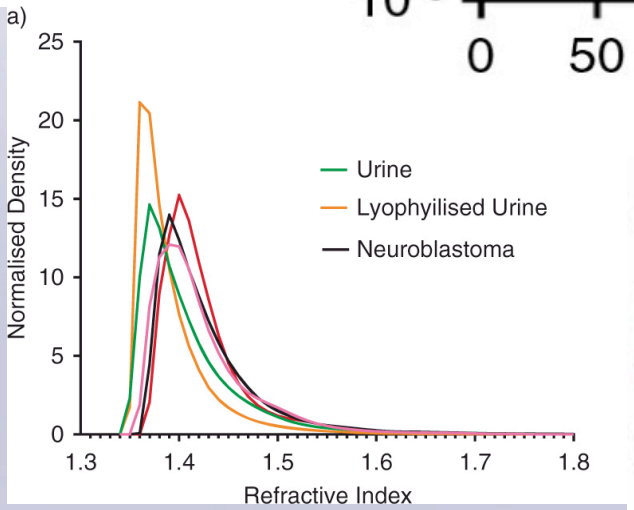
Results – sensitivity of 41 systems



Data provided by Rienk Nieuwland, Ph.D. – Academic Medical Center, Netherlands

Rosetta calibration: Software and reference materials: www.exometry.com





Journal of Extracellular Vesicles

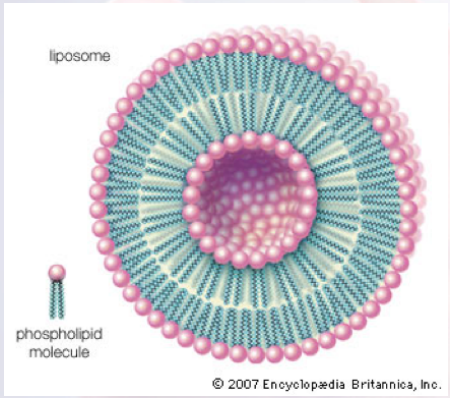
ORIGINAL RESEARCH ARTICLE

Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in extracellular vesicles

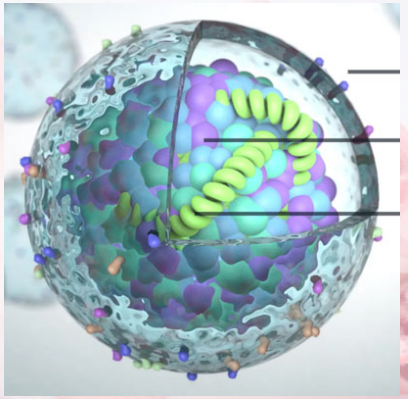
Chris Gardiner[1*†], Michael Shaw[1†], Patrick Hole[2], Jonathan Smith[2], Dionne Tannetta[1], Christopher W. Redman[1] and Ian L. Sargent[1]

Refractive Index and Scatter

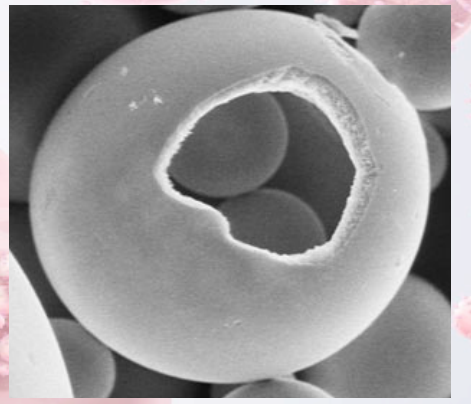
Liposome
RI = ~1.39



EV
RI = ~1.37-1.42



Hollow silica
RI = 1.46



Polystyrene
RI = 1.63

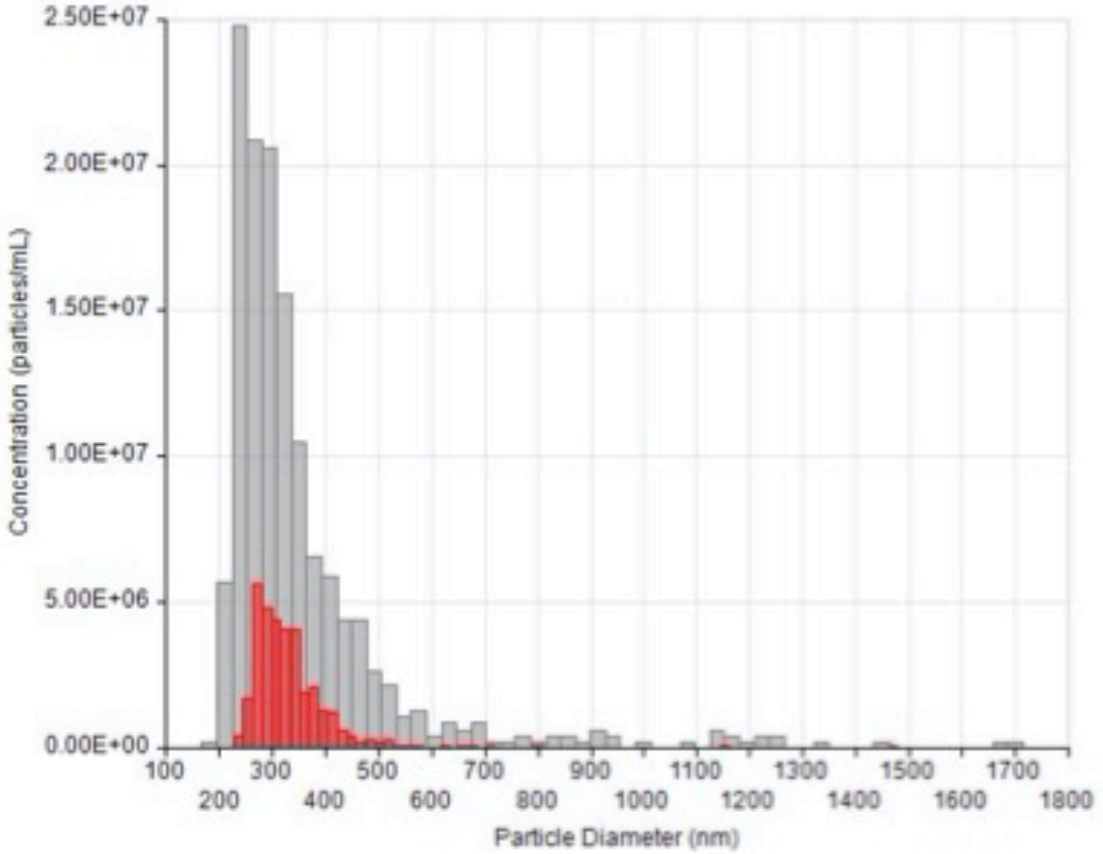
Scatter Intensity

Figure 1

Lannigan and Erdbrugger, METHODS, Oct. 2016

EVs from Human Urine

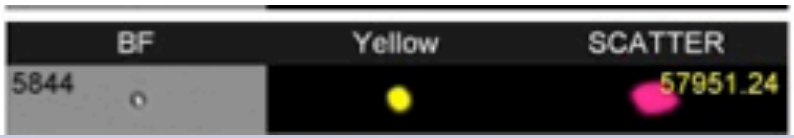
Tunable Resistive Pulse Sensing (TRPS)



no filter (EVs 1.36e08)

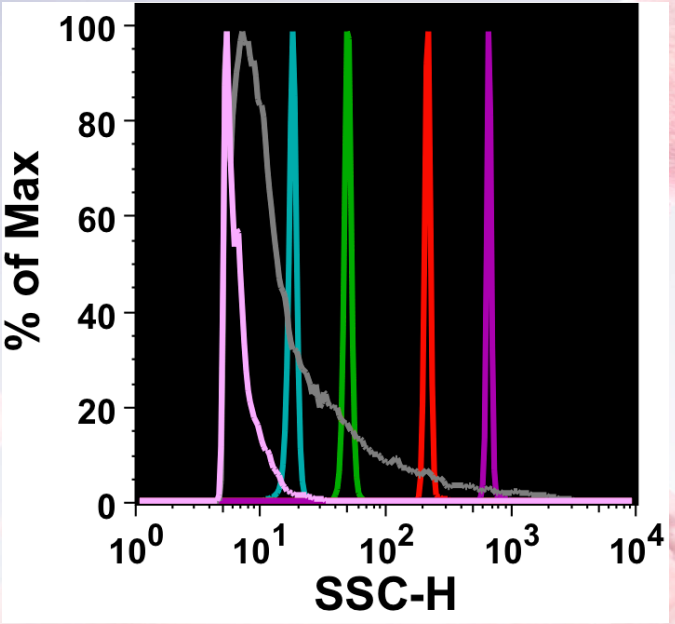
0.8micron filter (EVs 3.43e07)

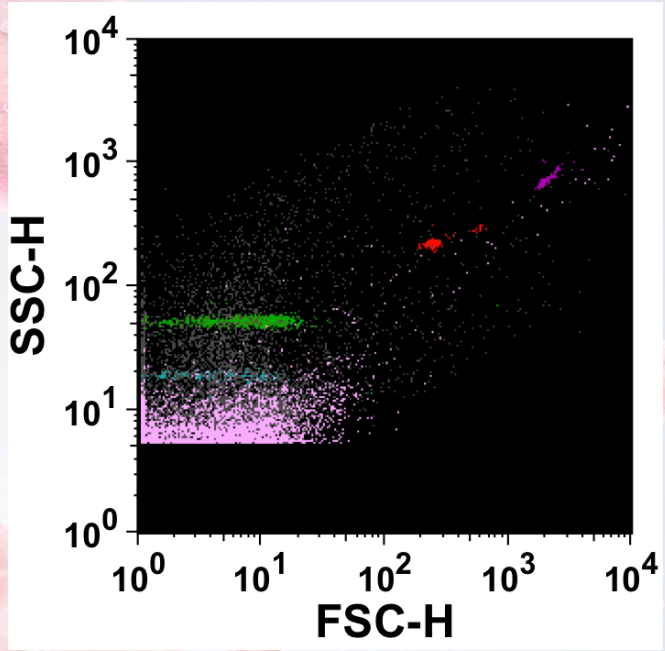
220-500nm



Erdbrugger, Lannigan et.al. Cytometry Part A 85A: 756-770, 2014

FCM

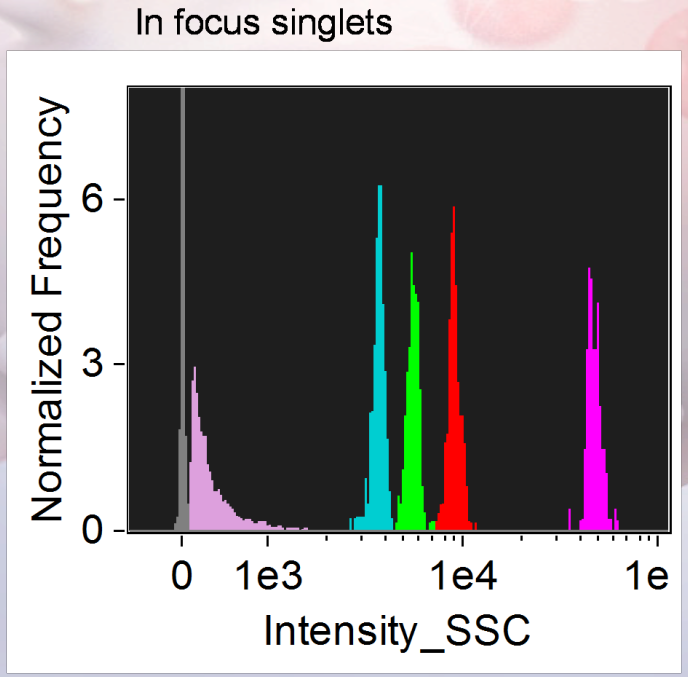


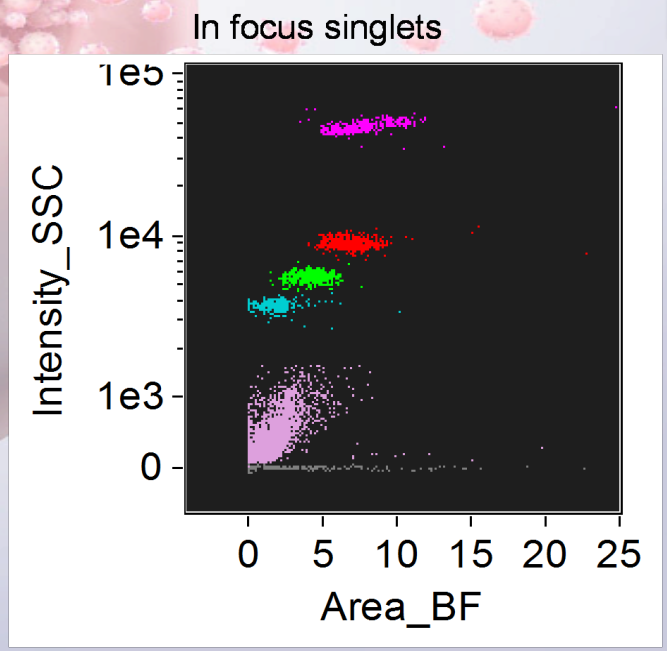


Legend

- 0.20u Lipos
- 0.22u PSB
- 0.45u PSB
- 0.88u PSB
- 1.33u PSB
- Buffer

ISX



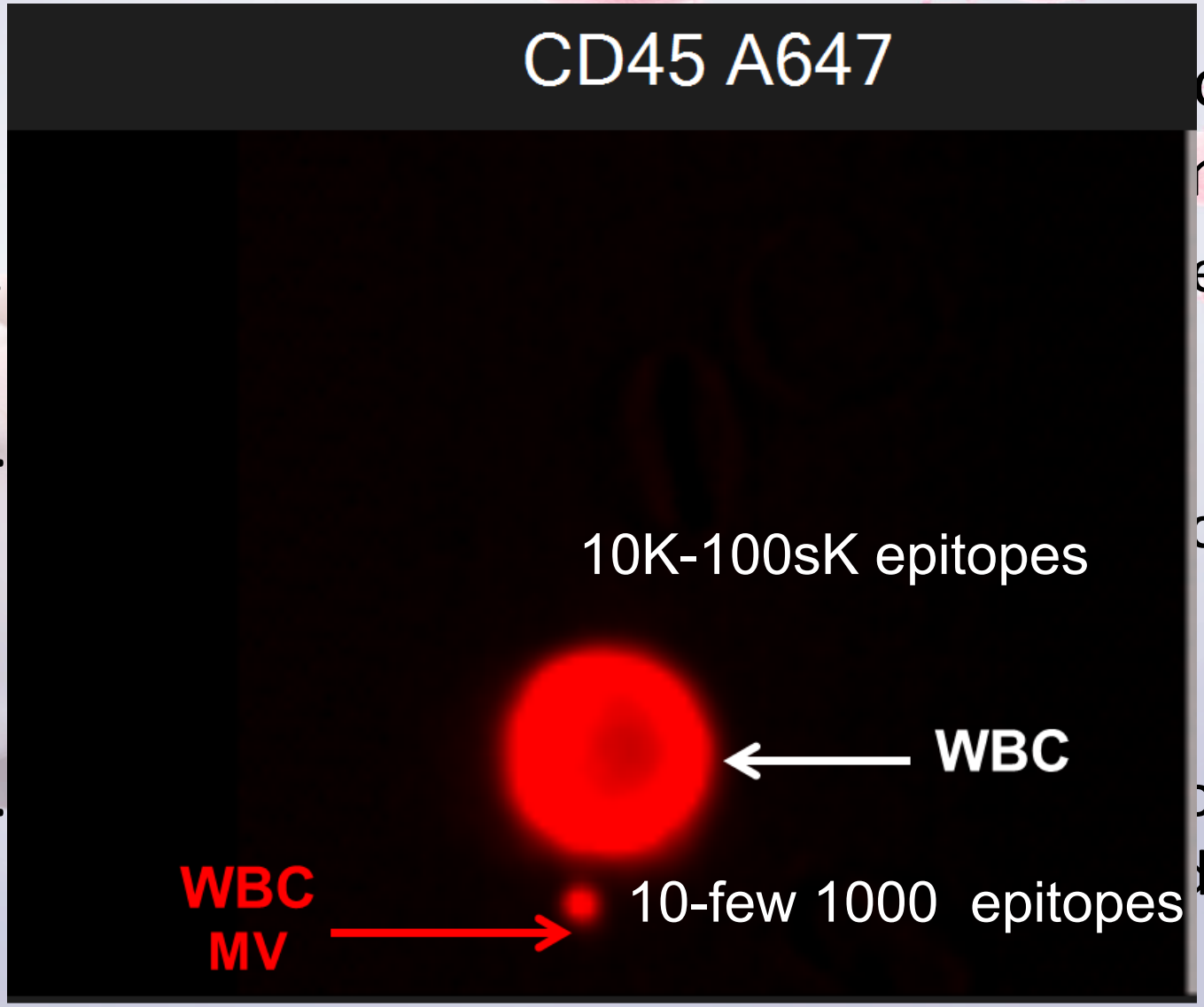


Light Scatter is complicated! But….

- Using particles with RI close to EVs
  - Allows better characterization of instrument scatter sensitivity
  - Can serve as a daily calibrator for instrument set up and performance
  - Can be useful in determining gates for window of analysis

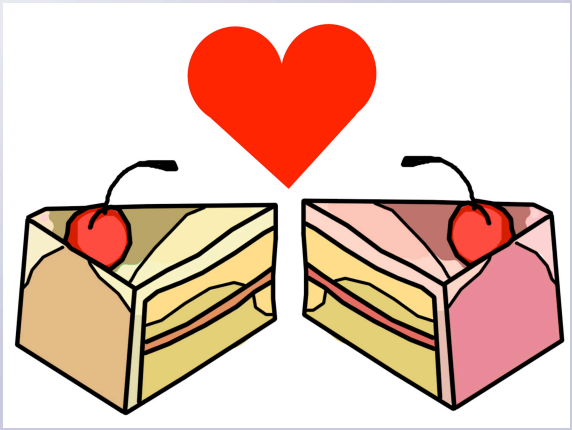
Characterizing Fluorescence Sensitivity

- La ction
  se nents
  a. es of
  b. ciency
  c.
  d. ore

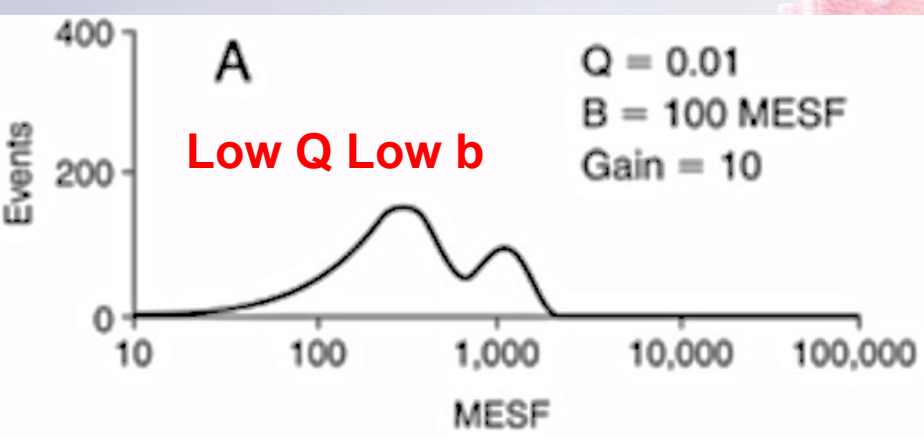


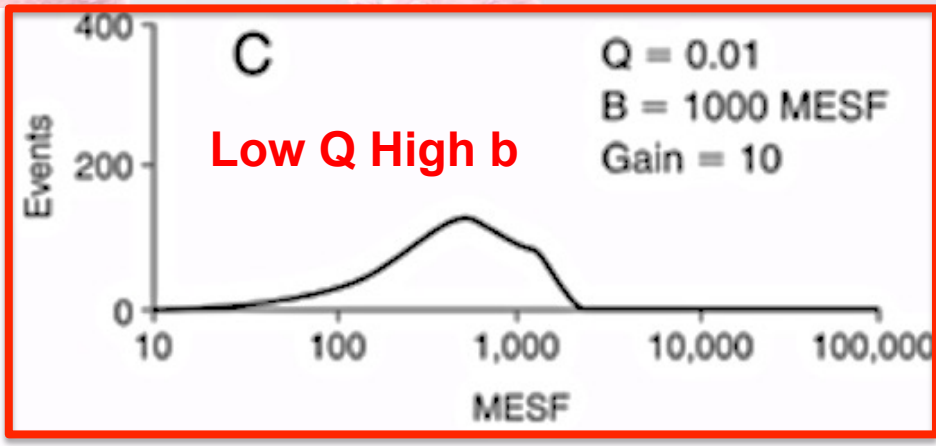
Things that affect Q, b, & RL

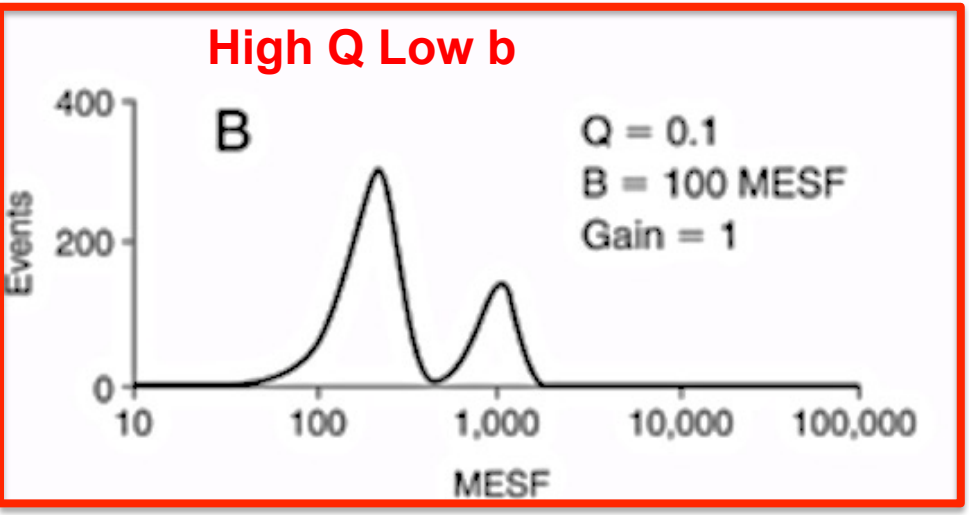
- **L**aser power and alignment
- **O**ptical Filters/mirrors
- **V**elocity of fluidics (laser dwell time)
- **E**lectronic noise
- **P**MT sensitivity (voltage)
- **I**nstrument design



Impact of Q & b on Resolution







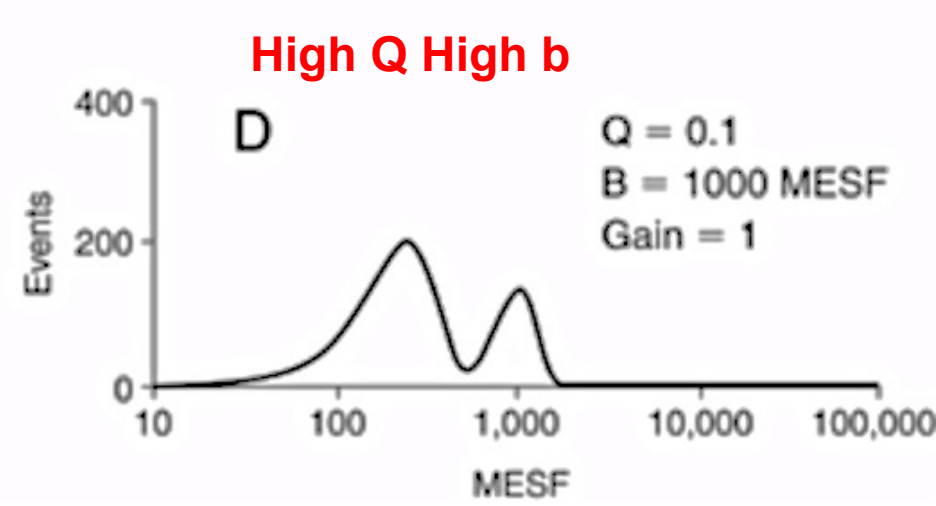


Figure 1.20.3. Theoretical fluorescence histograms demonstrating the effect of detection efficiency and background. Q is low (0.01) for panels A and C and high (0.1) for panels B and D. Background is low (100 MESF) in panels A and B, and high (1000 MESF) in panels C and D. **Characterization of flow cytometer instrument sensitivity** Hoffman, R. A.; Wood, J. C.; *Current protocols in Cytometry*

Approaches

- Multipeak bead method

Chase, ES, Hoffman, RA.. Cytometry. 1998; 33:267-279.

  - Requires bead set with
    - 3 or more beads: blank, moderately bright (photon limited- CV predicts # photons) and very bright (intrinsic CV) beads
    - MESF values for specific excitation and emission wavelengths
    - SD blank and CVs of moderately bright beads
    - $Q=1/(CV^2 * MESF)$; $b=(SD_{blank}/SD_{dim})^2 * MESF_{dim}$
    - $RL=4*((\sqrt{Q}*B) +1)/Q$

Approaches

- LED Method
  - Eliminates the bead intrinsic CV from observed CV
  - Primarily a measure at the detector level
    - Does not take into account impact of fluidics and laser power/alignment
  - Requires specialized equipment

Predicting the best resolution and sensitivity in panel development and reducing inter-instrument variability in Flow Cytometry
Steve Perfetto (NIH) and Jim Wood (Wake Forest)

Pros Cons – Bead Method

- Cons
  - Contribution of intrinsic CV of beads (may not be the same for all bead intensities)
  - Lack of MESF assignments for all lasers and filter combinations
  - Beads are too bright to run at same settings as EVs
  - Recurring bead expense
- Pros
  - Relatively easy to perform
  - Doesn't require any specialize equipment

Pros Cons – LED Method

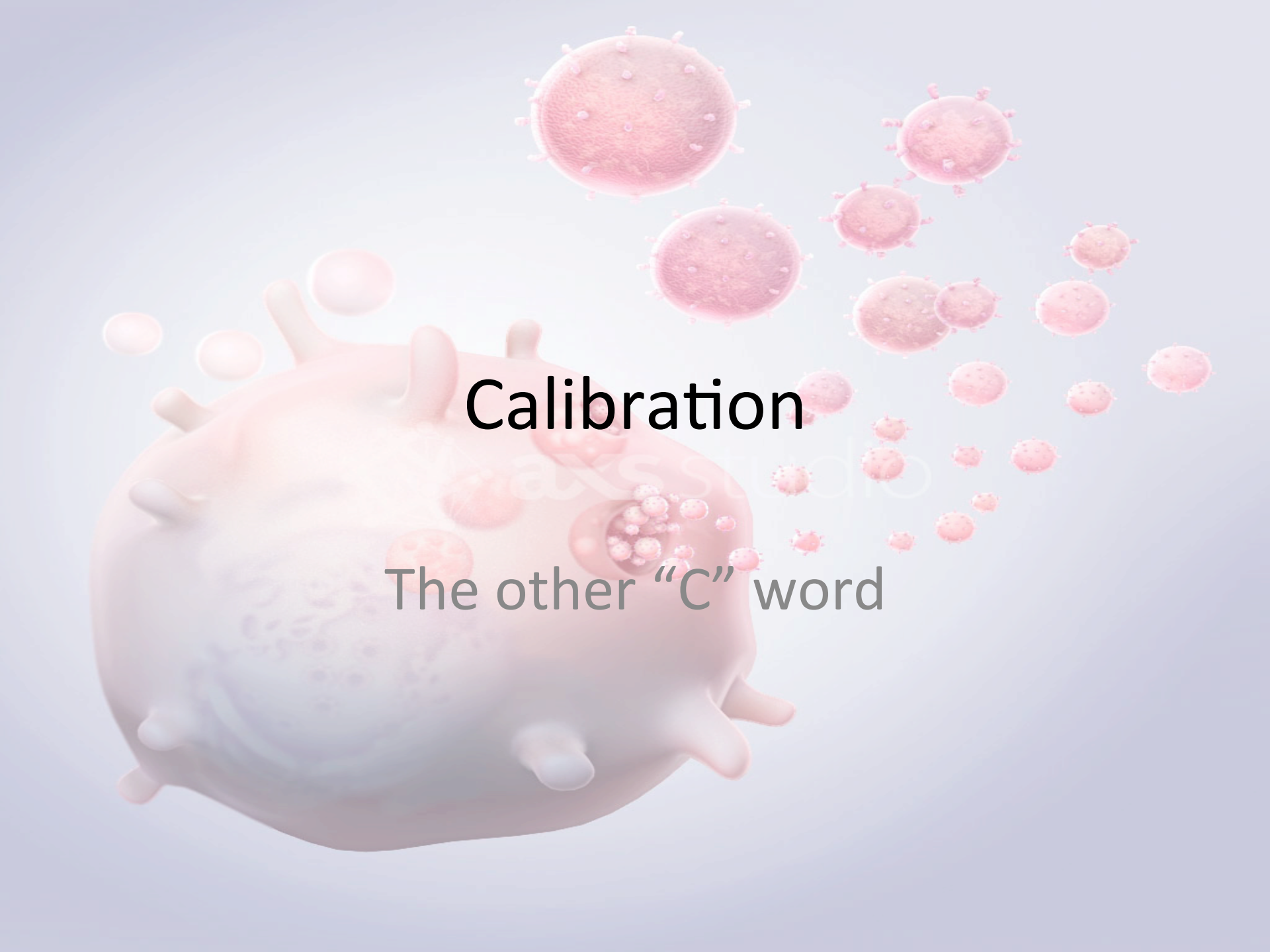
- Cons
  - Requires specialized equipment
  - Doesn't take into account laser power/ alignment and fluidics
  - Requires some level of skill to set up
- Pros
  - More accurate at the detector (very low intrinsic CVs)
  - Measurable across more levels of photons
  - Allows calibration of intensity scales to standardized photonics scale (spE/channel)

Implications of High RL in EV measurements

- Under estimation of EVs
  - EVs below the detection sensitivity of the instrument
- Data biased to larger EVs
  - Subpopulation representation biased to larger EVs
- Some phenotypes with low density epitopes may be missed entirely

Characterization Summary

- Characterization of instrumentation
  - determines the detection sensitivity
    - Size
    - Fluorescence
  - Identifies baseline performance
    - Allows the monitoring of performance over time
  - Identifies which instruments (or detectors) are best for measuring EVs (maximum sensitivity with minimal noise)



Calibration

The other “C” word

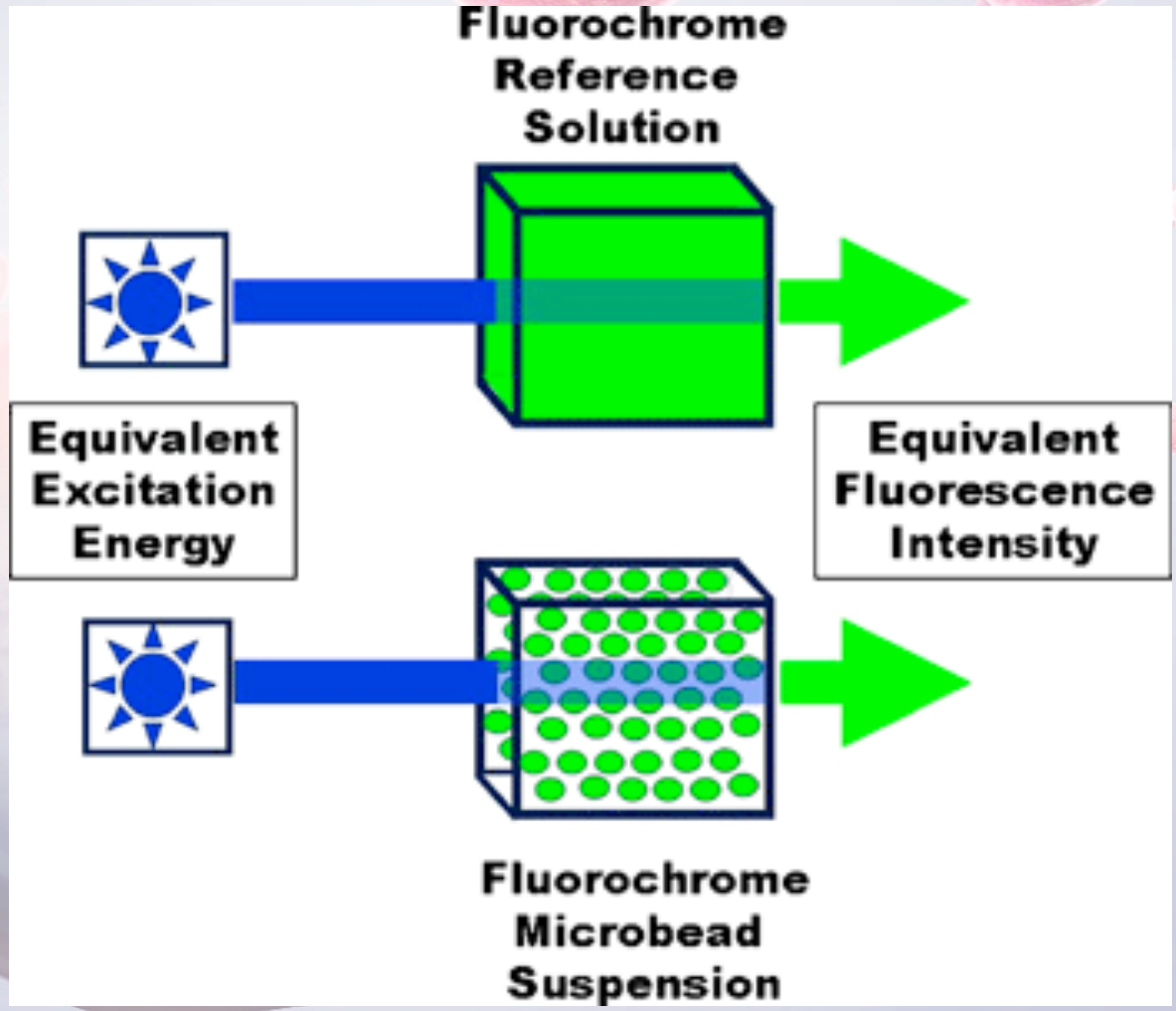
Why Calibration?

- Different cytometers have different optical configurations and sensitivity
- Data are presented in arbitrary units
  - Comparison of data across labs and cytometers is difficult without standardization
  - Comparison of results is required for a complete understanding of EVs and their role as biomarkers

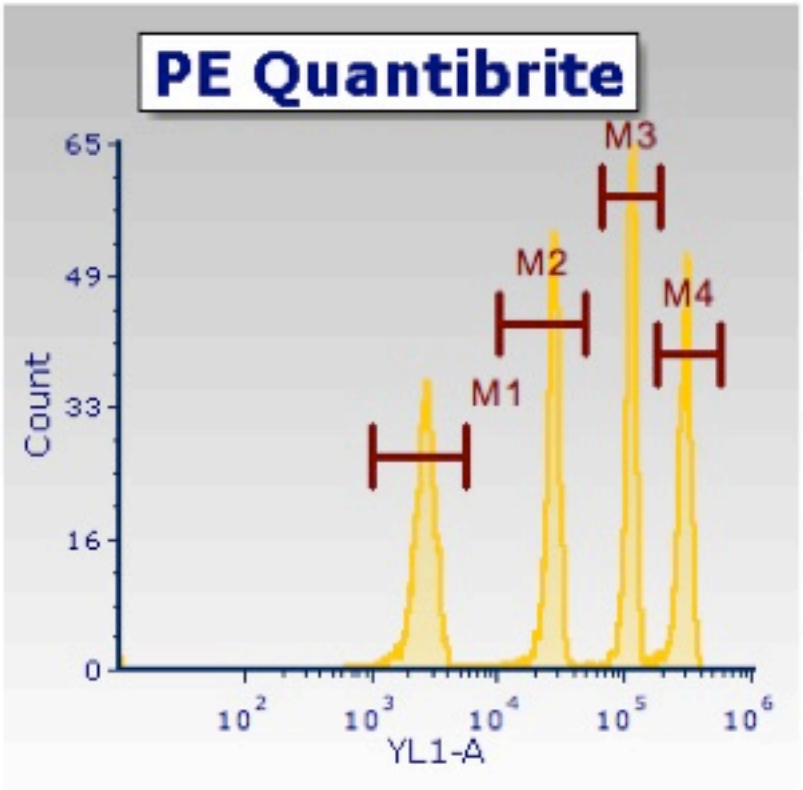
Calibration

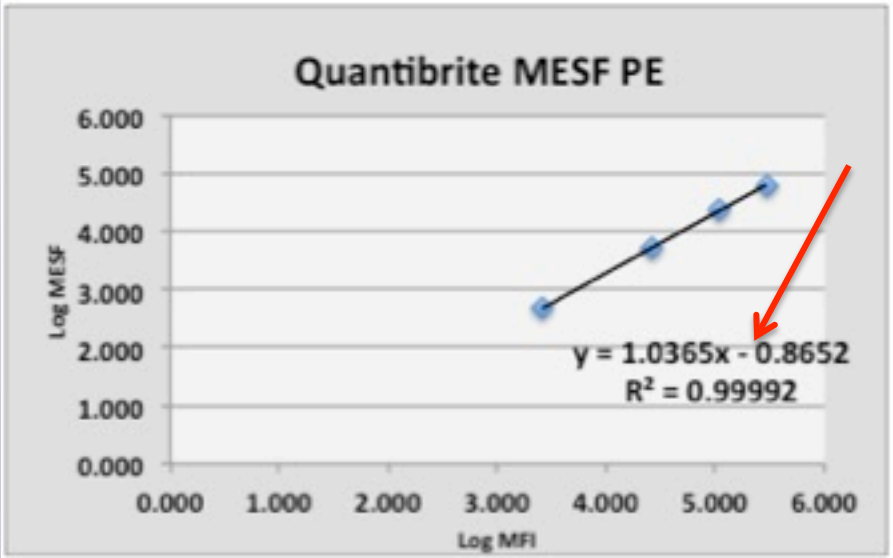
- No generally accepted standards available for size (Yet!)
- Fluorescence calibration standards are available for common fluorophores
  - MESF beads
  - Hard dyed beads
  - antibody capture beads
  - biological standard

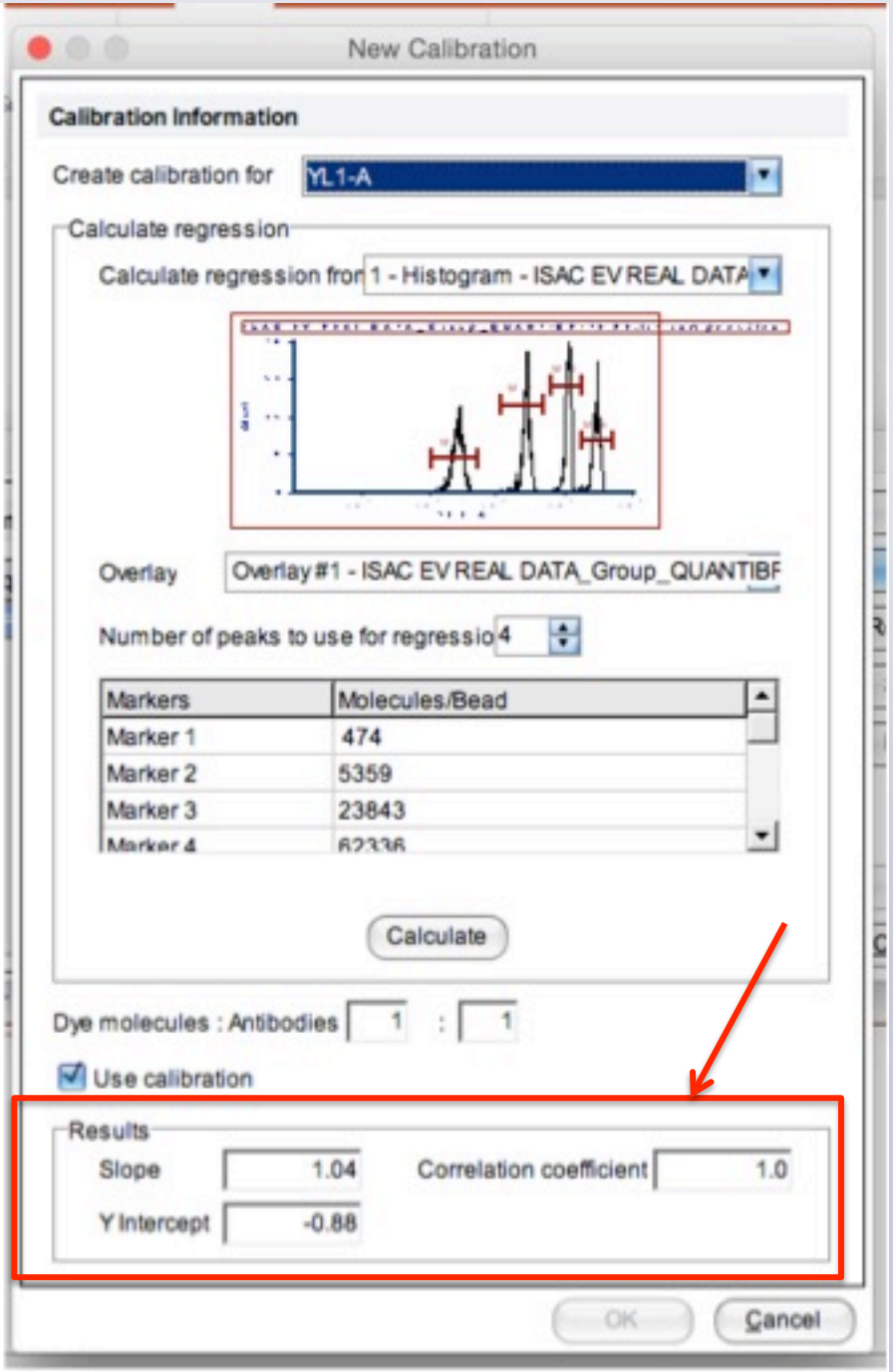
Molecules of Equivalent Soluble Fluorochrome (MESF)



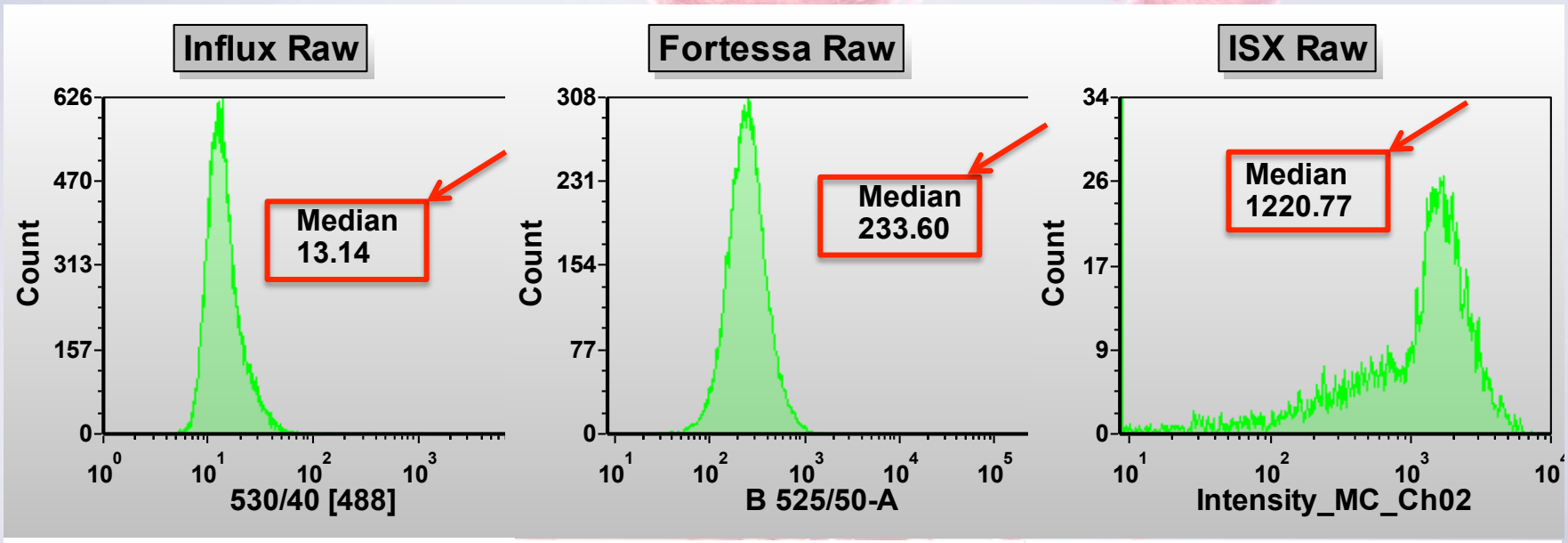
Quantitative Fluorescence Calibration: a Tool for Assessing the Quality of Data Obtained by Fluorescence Measurements 18 April 2008 Robert F. Vogt Jr. , Gerald E. Marti, Vincent Zenger


PE Quantibrite
M1
M2
M3
M4
Count
65
49
33
16
0
10^2
10^3
10^4
10^5
10^6
YL1-A

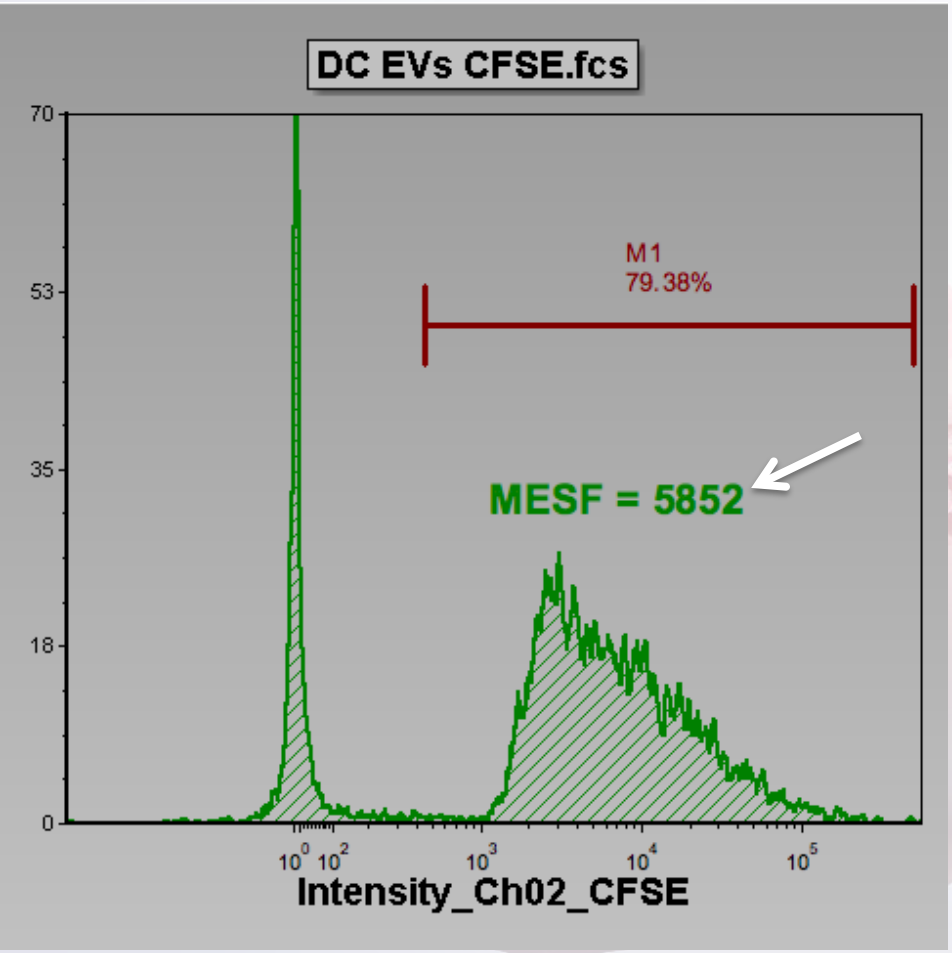

Quantibrite MESF PE
Log MESF
6.000
5.000
4.000
3.000
2.000
1.000
0.000
0.000
1.000
2.000
3.000
4.000
5.000
6.000
Log MFI
y = 1.0365x - 0.8652
R^2 = 0.99992

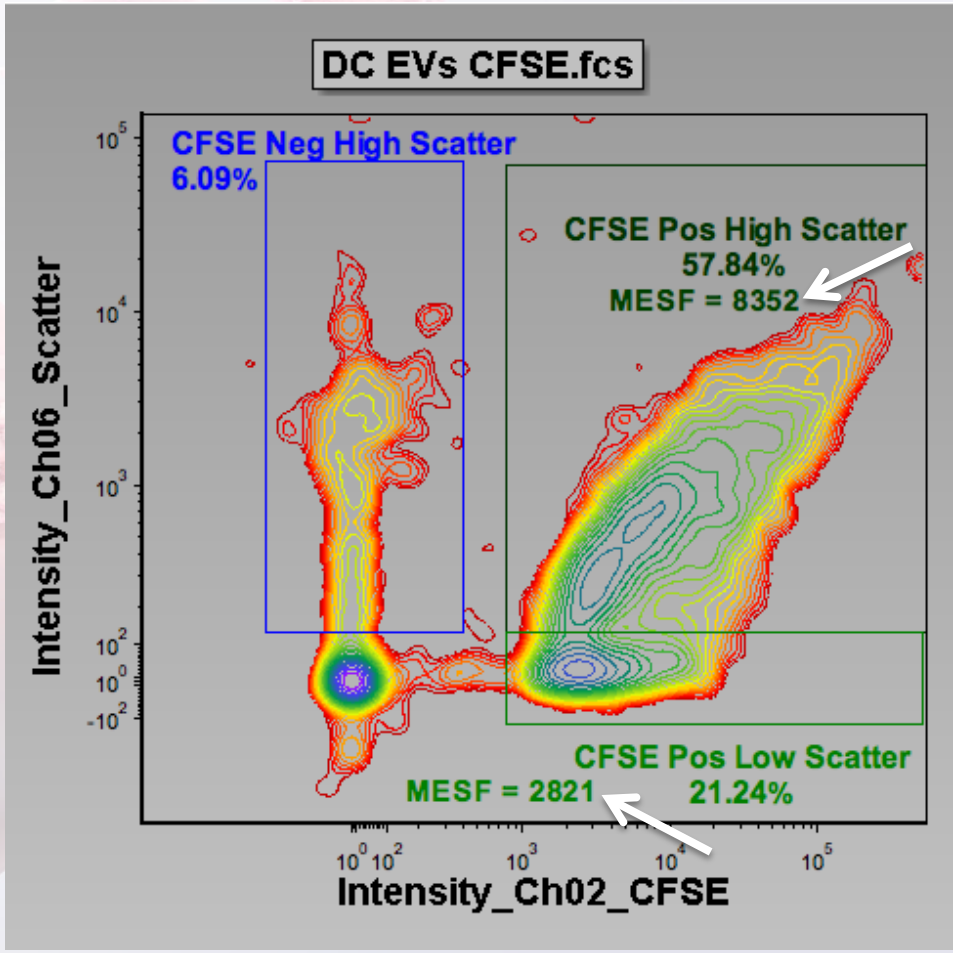

New Calibration
Calibration Information
Create calibration for
YL1-A
Calculate regression
Calculate regression from
1 - Histogram - ISAC EV REAL DATA
Overlay
Overlay #1 - ISAC EV REAL DATA_Group_QUANTIBF
Number of peaks to use for regressio
4
Markers
Molecules/Bead
Marker 1
474
Marker 2
5359
Marker 3
23843
Calculate
Dye molecules : Antibodies
1
:
1
Use calibration
Results
Slope
1.04
Correlation coefficient
1.0
Y Intercept
-0.88
OK
Cancel

EVs Calibrated in MESF Across Platforms



EV Data expressed in MESF





DC EVs stained with CFSE

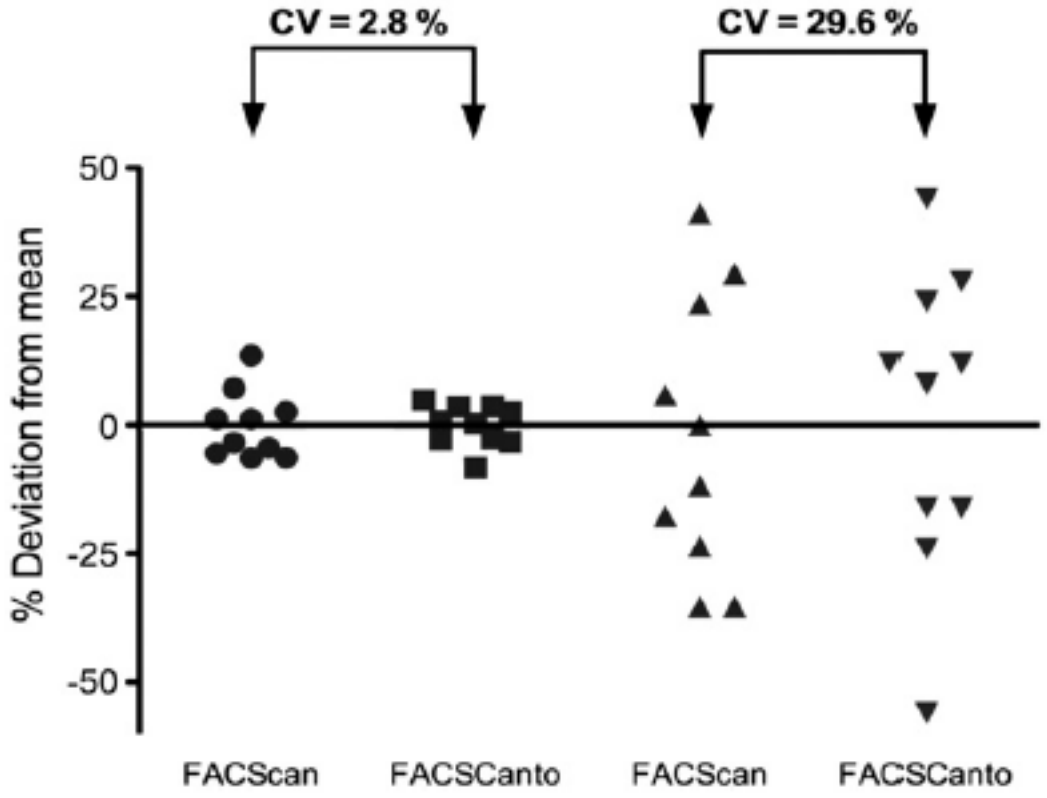
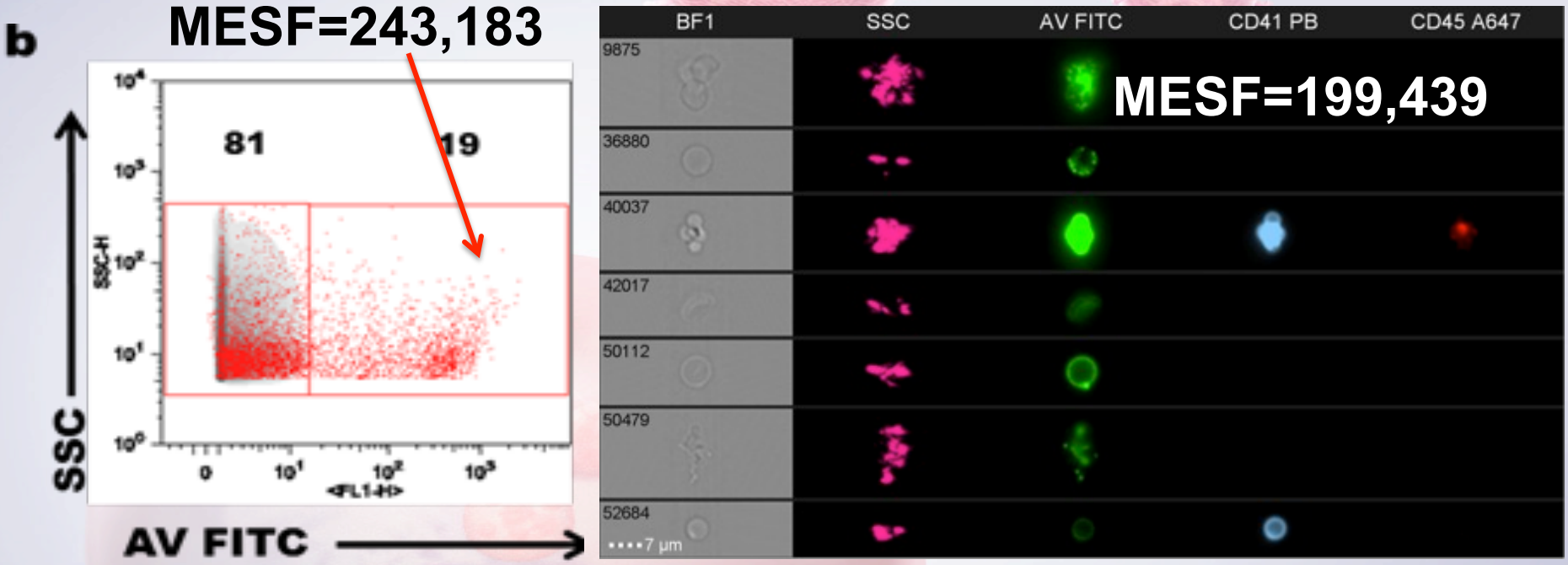


Fig. 4. PDMPs expressing CD42a and CD62p in a plasma sample from a healthy control subject measured on two different flow cytometers (BD FACSscan and BD FACSCanto). Results are shown as MESF values and number of positive events.

	Intra-assay	Interassay
MESF TF	7.1%	6.4%
Events TF	18.2%	19.9%
MESF CD62p	6.2%	7.2%
Events CD62p	14.3%	16.5%

Its More than a Number!



BF1	AV FITC	CD31 PE	CD42a AF647	Scatter	3 Color Composite
658					
BF1	AV FITC	CD31 PE	CD42a AF647	Scatter	3 Color Composite
356					
BF1	AV FITC	CD31 PE	CD42a AF647	Scatter	3 Color Composite
672					
BF1	AV FITC	CD31 PE	CD42a AF647	Scatter	3 Color Composite
727					
BF1	AV FITC	CD31 PE	CD42a AF647	Scatter	3 Color Composite

Limitations of MESF Beads

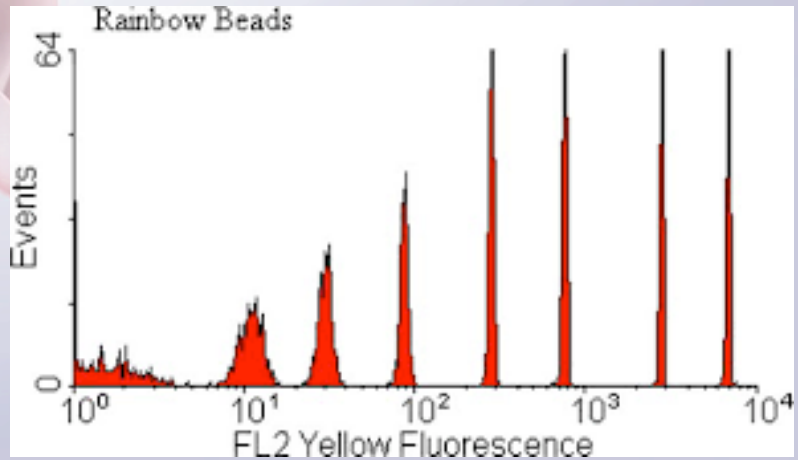
- Phototypically unstable (requires refrigeration, short shelf life)
- Expensive
- Limited fluors:
  - AF488 - CY5 -mCherry
  - FITC - AF647
  - PE - APC
  - PE-CY5 - GFP

Hard Dyes Beads

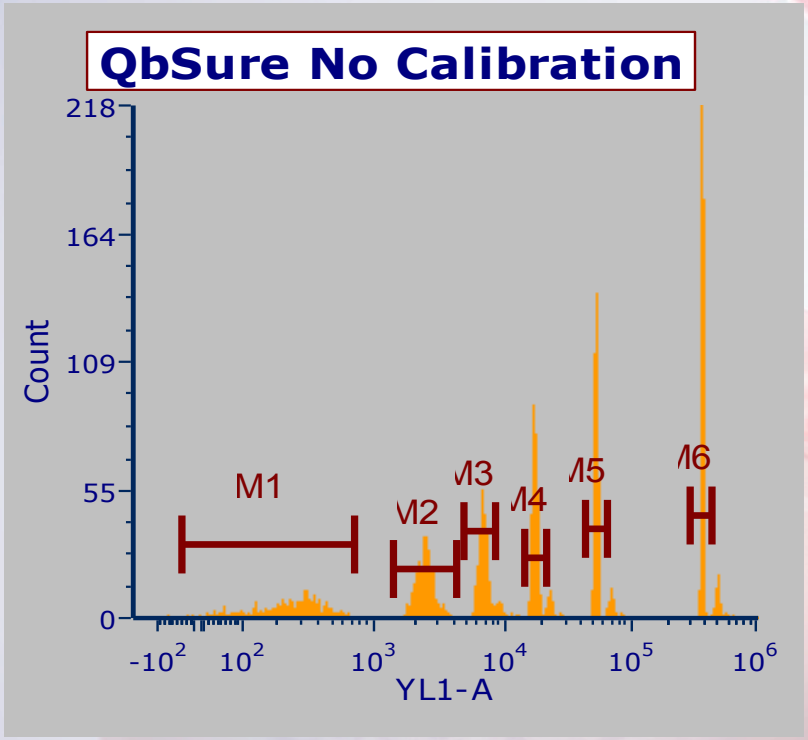
- Not spectrally matched to fluorochromes, broad excitation/emission
  - can be read in most channels
  - No real MESF values
- Fluorescence not impacted by environment
- More photostable, long shelf life
- Less expensive to use

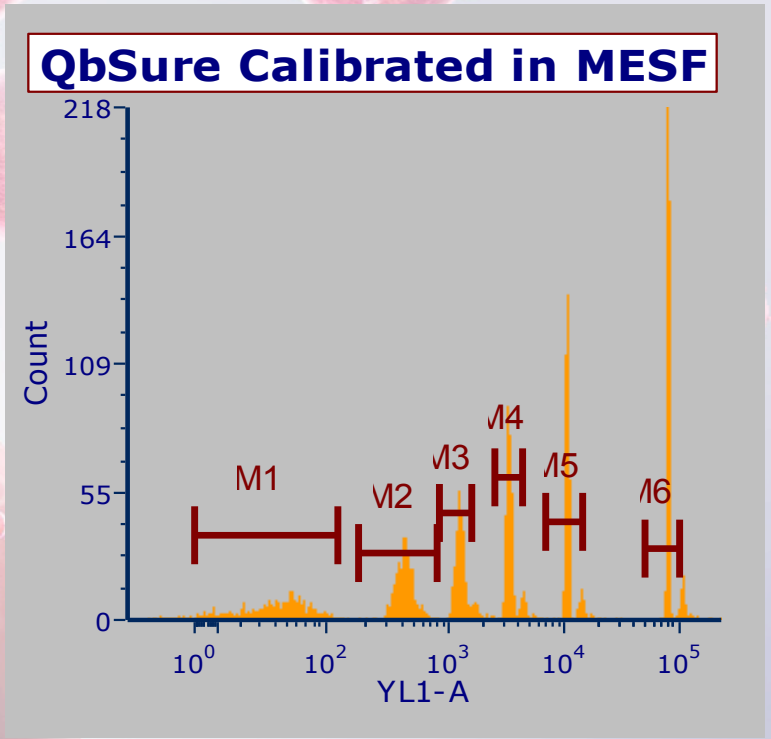
Hard Dyed

Fluorochrome surface dyed



Cross Calibration



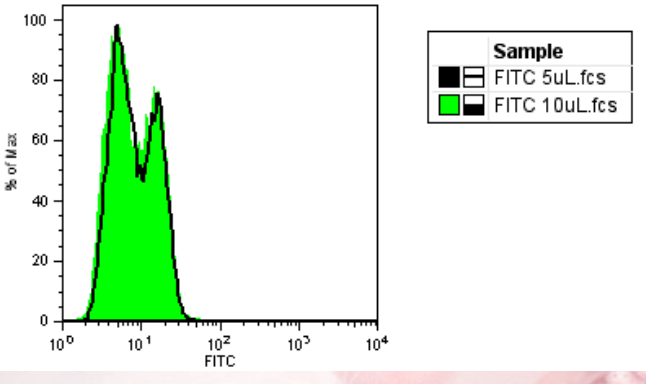


Parameter	Marker	Median	Geometric Mean
YL1-A	M1	258.50	n/a
YL1-A	M2	2384.50	2392.15
YL1-A	M3	6615.50	6638.71
YL1-A	M4	16945.50	16972.37
YL1-A	M5	52453.50	52625.87
YL1-A	M6	365186.50	365474.24

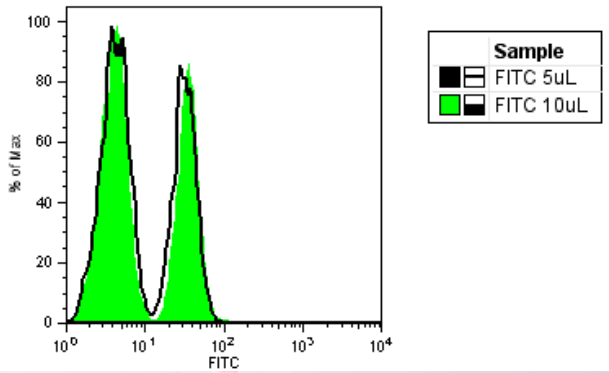
Parameter	Marker	Median	Geometric Mean
YL1-A	M1	43.73	36.12
YL1-A	M2	423.06	424.22
YL1-A	M3	1219.77	1224.21
YL1-A	M4	3243.08	3262.71
YL1-A	M5	10480.87	10707.82
YL1-A	M6	78293.19	78690.98

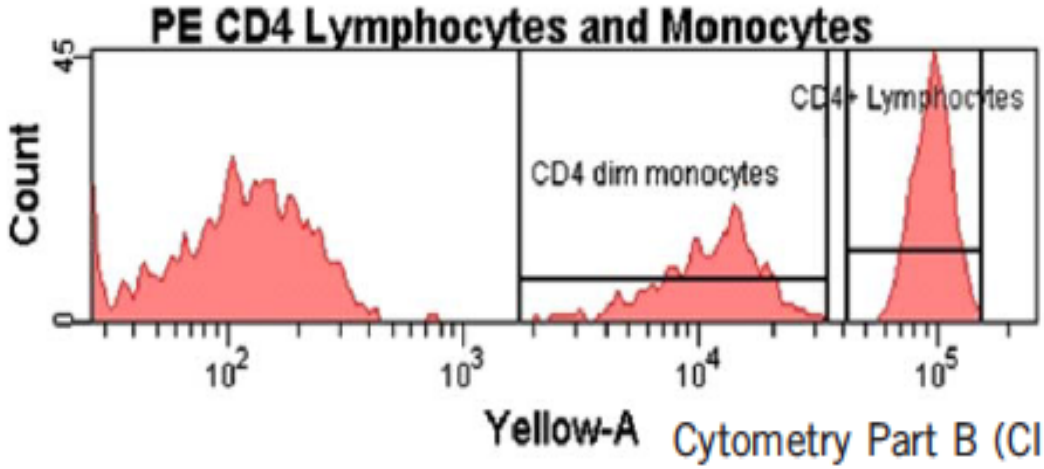
Other Options

Vendor A
CD4 (RPA-T4, Ms IgG1, k)



Vendor B
CD3 (UCHT1, Ms IgG1)





Biological standard

antibody molecules

Cytometry Part B (Clinical Cytometry) 84B:291–308 (2013)

Calibrating for Particle Concentration

- Two methods
  - Bead based (beads with known concentration)
    - Prone to pipetting errors
    - Need to have beads of similar size
    - Expensive
  - Volumetric
    - Generally more accurate
    - Inexpensive

Calibrating for Volumetric Concentrations

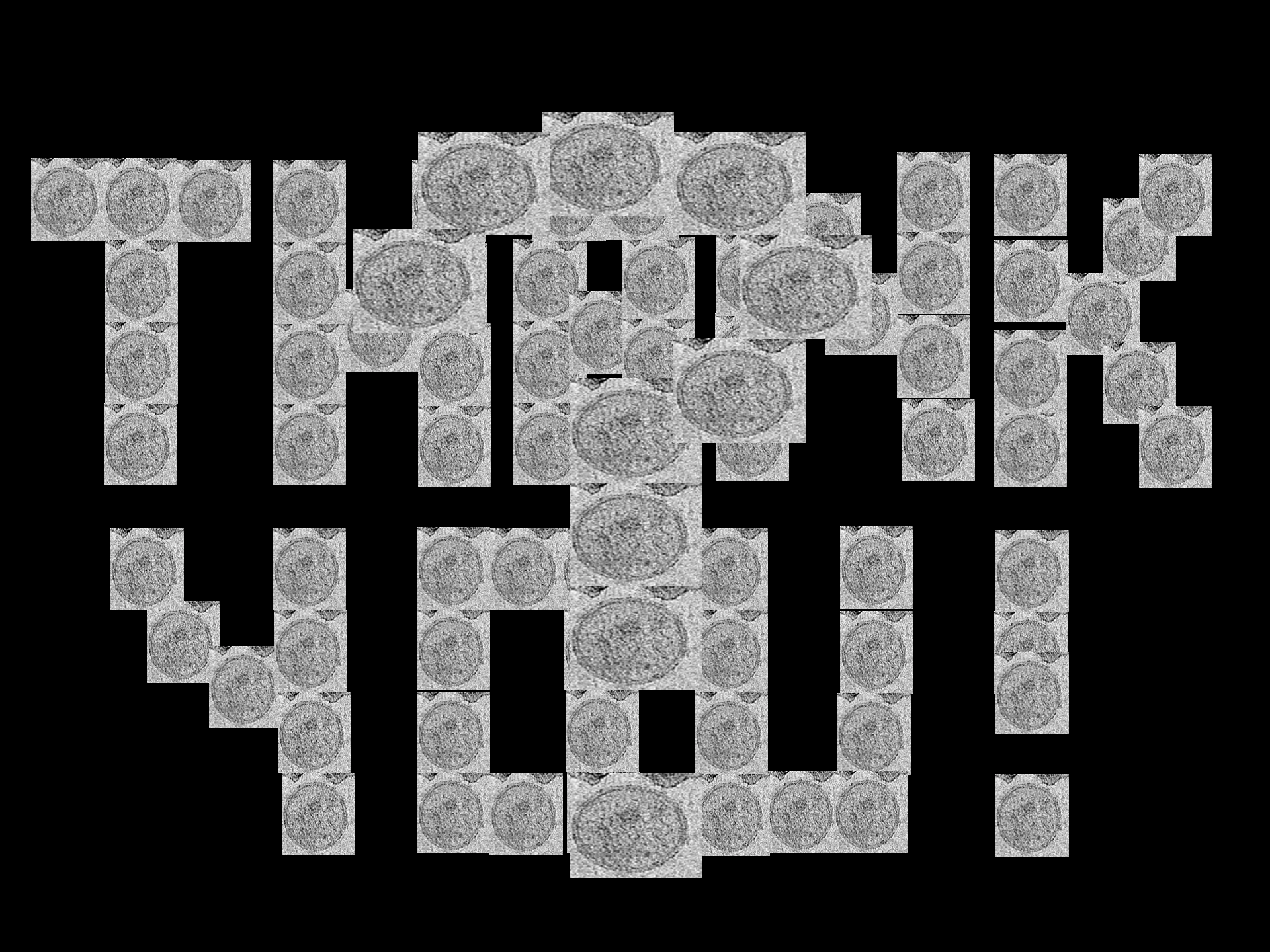
- For instruments with syringe based volume determination
  - Verify with known bead/particle concentrations
- For instruments w/o built in volume determination
  - Calibrate sample flow rates/unit time

Standardization of Result Reporting

- Source of EVs
- Concentration
  - absolute number/mL (Total and dilution)
  - validated by additional method
  - evidence of single particle detection
- Size information
  - independent of flow cytometer
- Standardized measure of fluorescence intensity
- Detection and gating strategies
- % expressed as % of detectable EVs

Still Have Far To Go!

- Better standards/calibrators
  - size standards better matched to composition of EVs
  - fluorescence calibrators and standards which more closely reflect Fluorescence Intensity range of EVs
- Education
  - Protocols
  - Training
- Easy to use software
- Reagents
  - EV validated antibodies

Acknowledgements

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