SMALL PARTICLE FCM:

Size matters, but it's complicated

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Operations Manager & Adjunct Professor

Flow Cytometry & Virometry Core Facility
University of Ottawa

ISAC SRL Emerging Leader (2018-2022)

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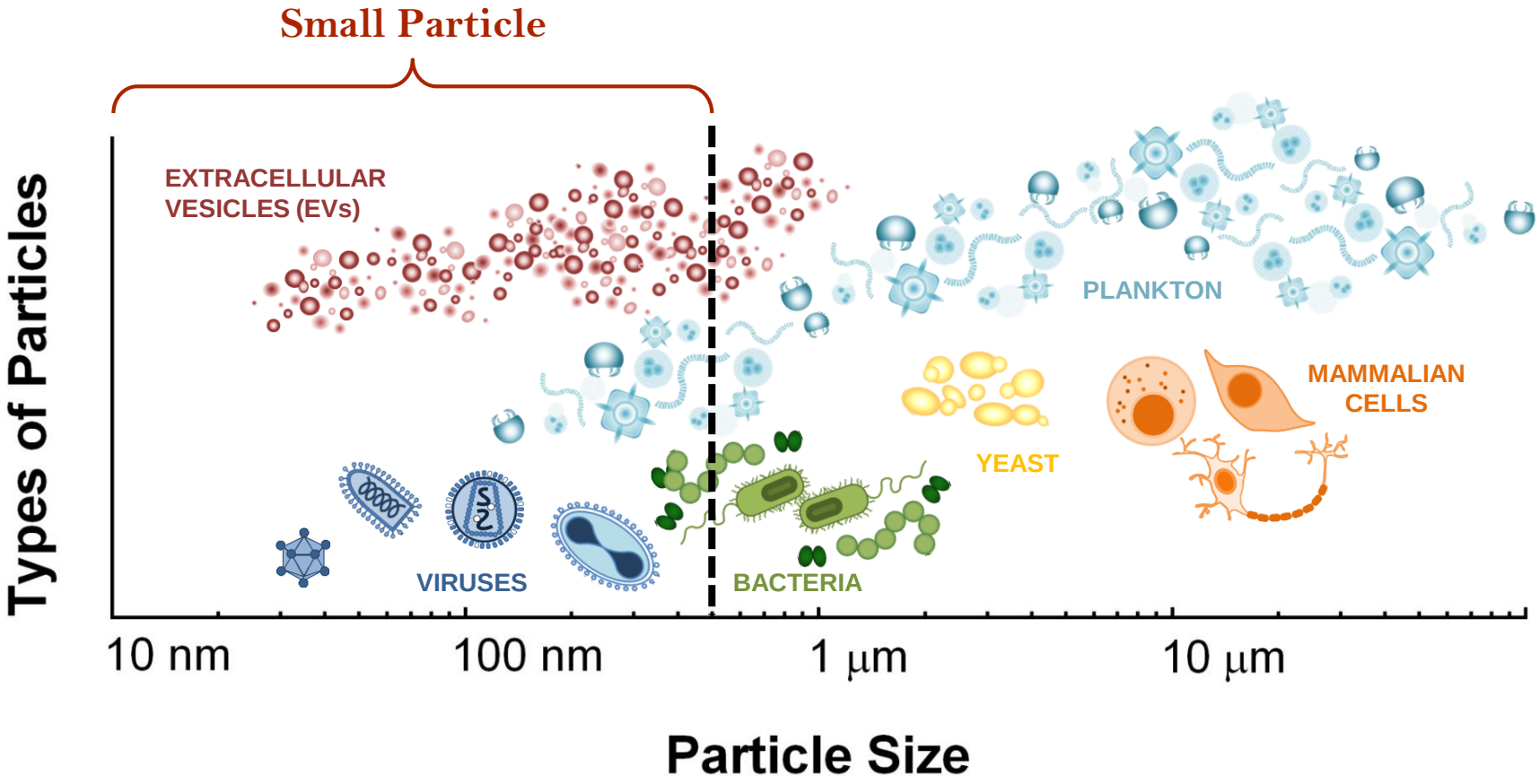
Small Particle Flow Cytometry



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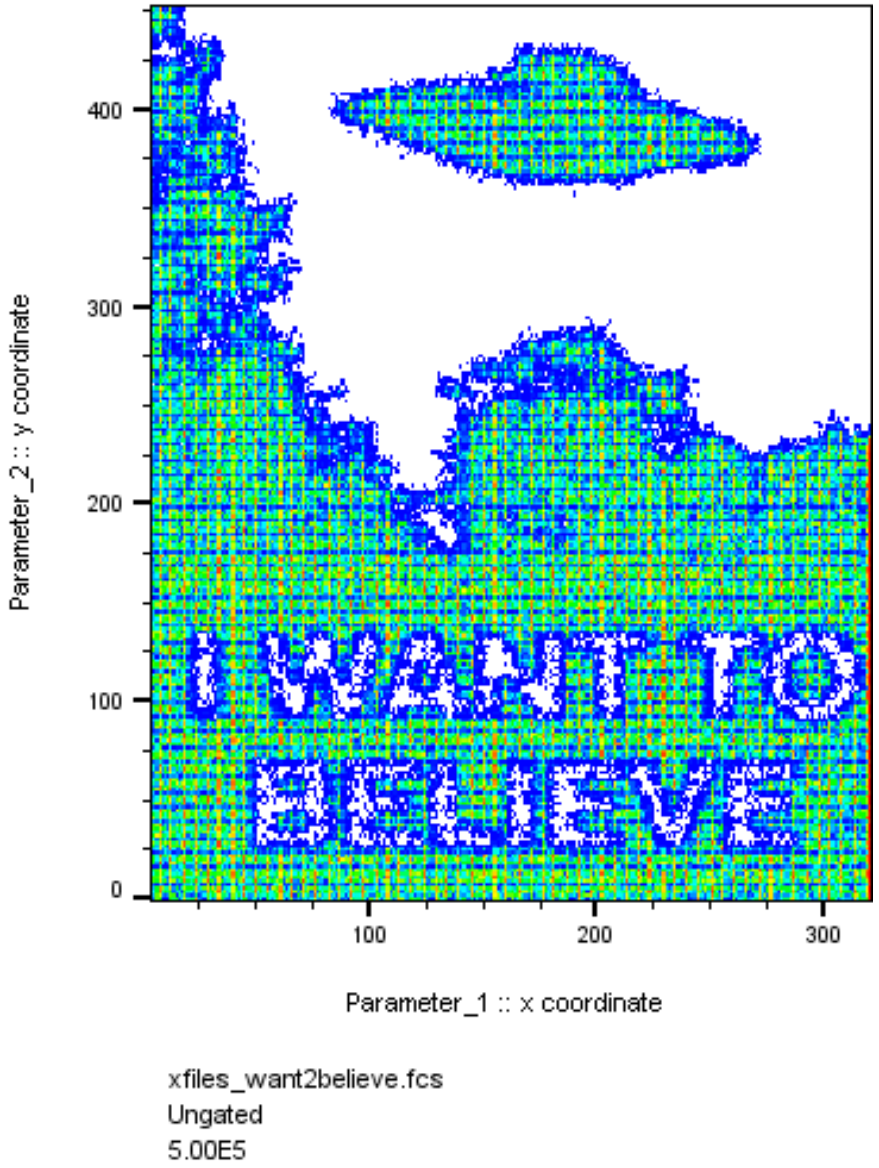
It's Complicated…

- **Size**
  - Amount of light scattered
- **Refractive Index**
  - Amount of light scattered
  - Gold>Polystyrene > Virus > EVs
- **Surface Antigen Density/Quantity**
  - Amount of fluorescence
  - Restricted by small surface area - can be below limit of detection
- **Brightness of fluorophores/dyes**
  - Amount of fluorescence
- **Instrument Configuration & Settings**
  - Optimized for small particle detection

Detected Particle = ∫

Size
Refractive Index
Antigen Quantity
Fluorophore/Dye
Brightness
Instrument Configuration

Are you seeing what you *think* you are seeing?



David Gravano: converting images to fcs https://youtu.be/XC4D1QKKiQA

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Small Particle Framework

J Extracell Vesicles. 2020; 9(1): 1713526.

Minimal Information for a Flow Cytometry experiment on EVs
and other small particles (MIFlowCyt-EV)

Contributing Societies:
International societies for extracellular vesicles, advancement of cytometry and thrombosis and haemostasis (**ISEV-ISAC-ISTH**)

Goal:
To improve the quality of EV and small particle flow cytometry data

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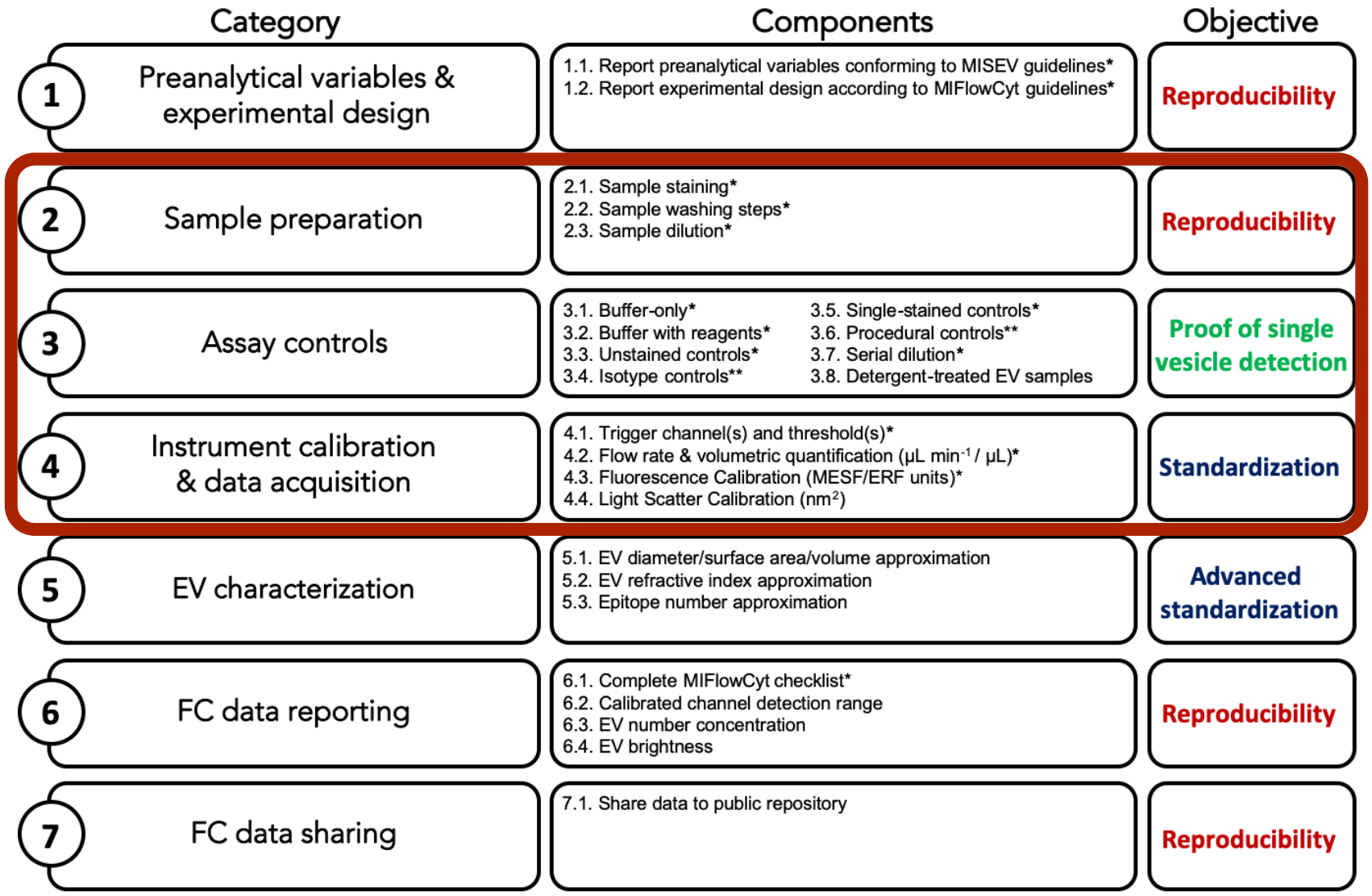

J Extracell Vesicles. 2020; 9(1): 1713526.

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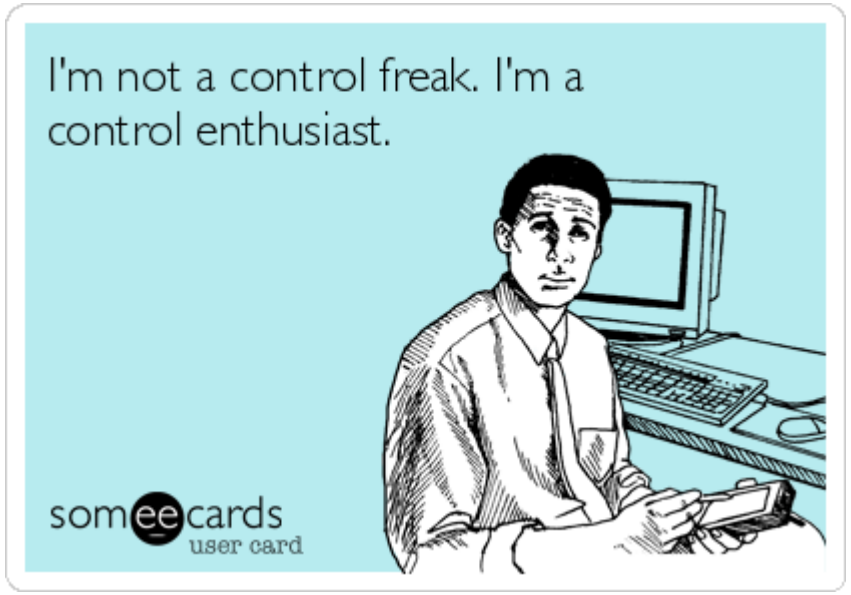
Controls & Calibration

- **Coincidence Controls**
  - Sample dilutions
- **Assay Controls**
  - Buffer only
  - Reagent & buffer only
  - Negative (or Isotype) control
  - Positive control
  - Single-stained controls
  - Unstained control
- **Calibration**
  - Fluorescence – MESF or ERF calibration beads (i.e. Quantibrite PE, Spherotech 8 peak rainbow), Excel
  - Light Scatter – size calibration beads (i.e. NIST-traceable polystyrene & silica beads, **non-fluorescent**), light-scatter calibration software



Controls & Calibration

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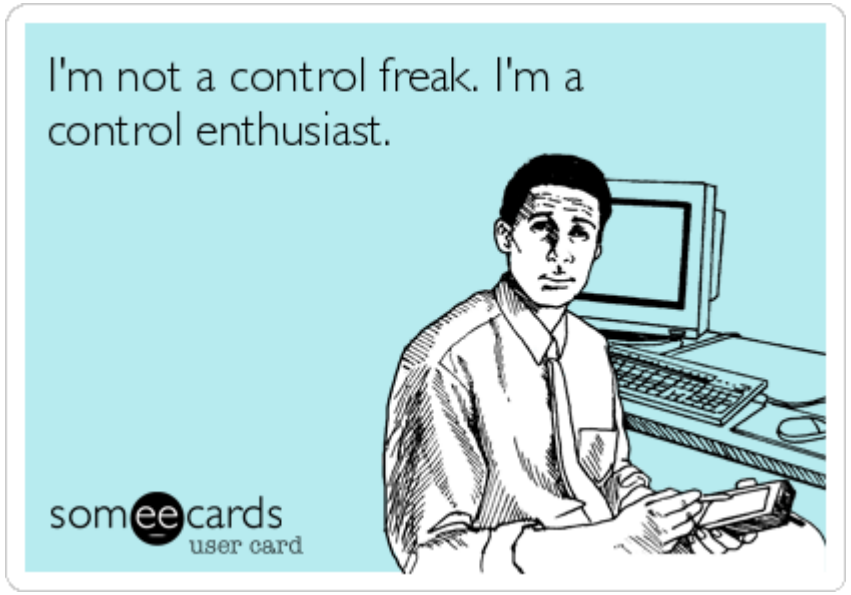
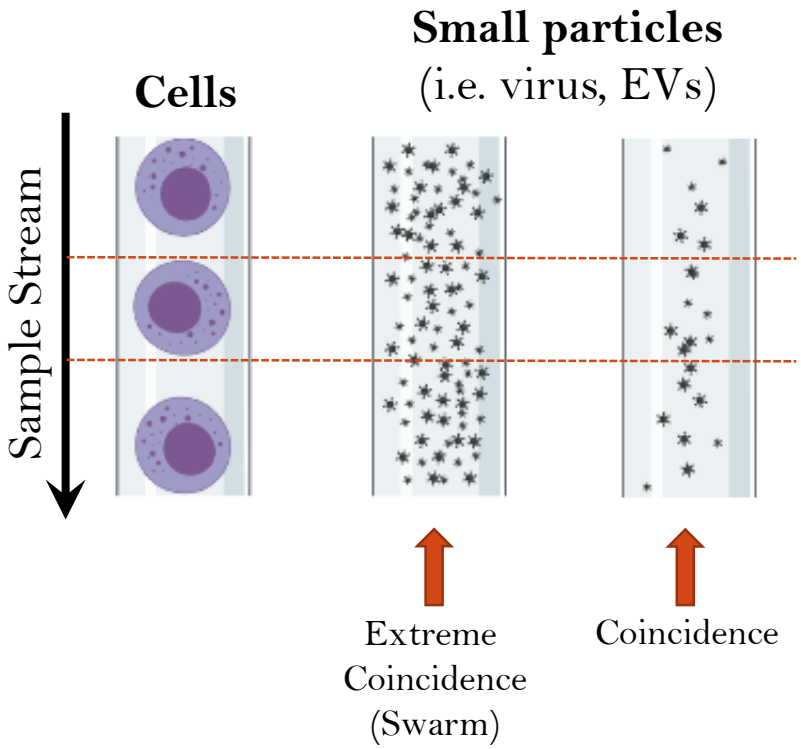
  - Unstained control

Optimize:
- *staining concentration (ab & particles)*
- *concentration to run samples*

Validate multicolor staining



Coincidence



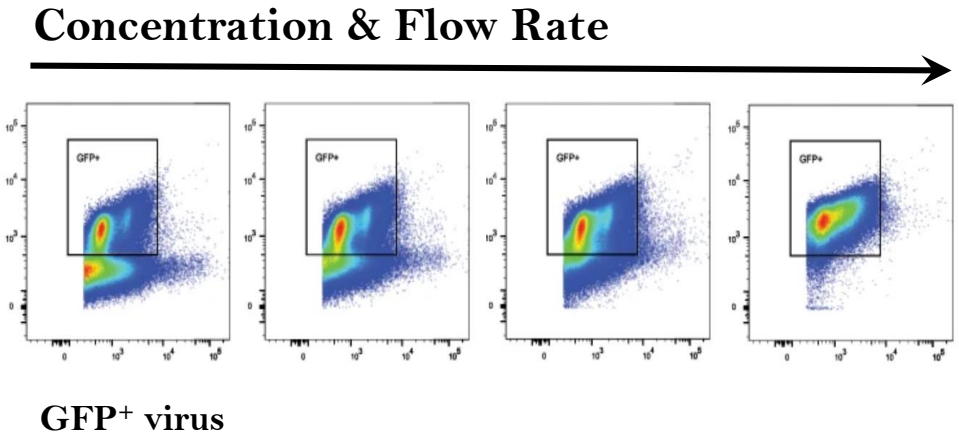
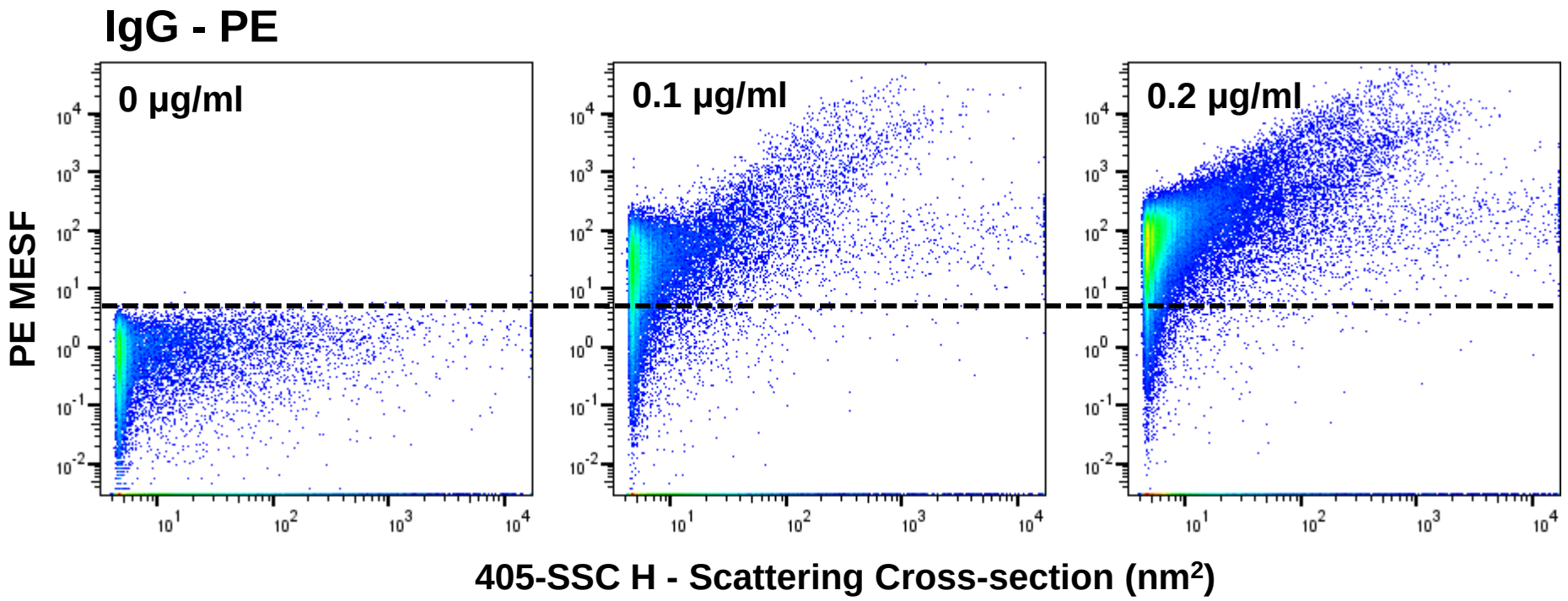


Diagram created with BioRender.com
Data: Scientific Reports **volume 7**, Article number: 17769 (2017)

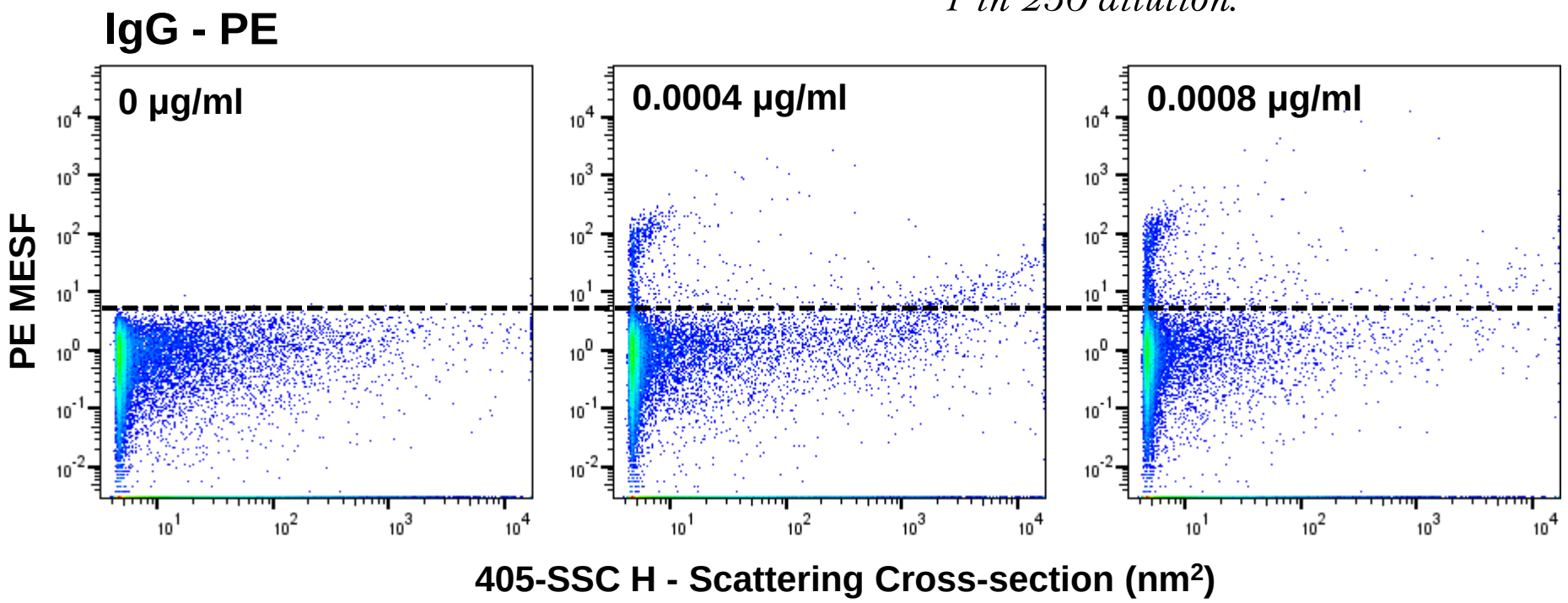
Coincidence: Reagent Alone



Antibody dilution samples from Jonathan Burnie & Dr. Christina Guzzo (Guzzo Lab), University of Toronto

Coincidence: Reagent Alone

1 in 250 dilution:



Antibody dilution samples from Jonathan Burnie & Dr. Christina Guzzo (Guzzo Lab), University of Toronto

Coincidence

Common protocol:

- Label with reagents (antibodies or dyes) without removing excess
- Dilute sample with buffer prior to analysis

Majority of particles in sample = reagent

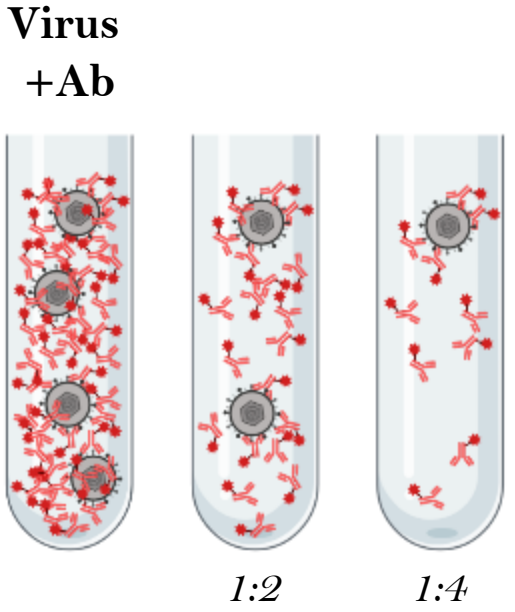
Example:

Antibody labeling of virus particles:

1µg/ml Ab + 10^9 virus particles/ml

4000 Ab: 1 virus

IgG molecular weight = 150,000g/mol



Serial Dilutions

Diagram created with BioRender.com

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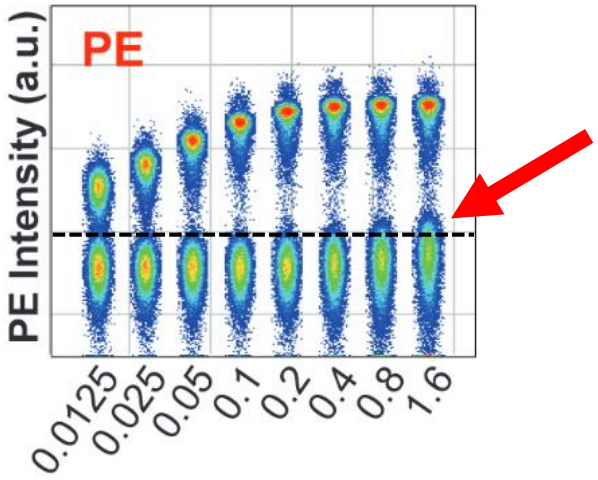
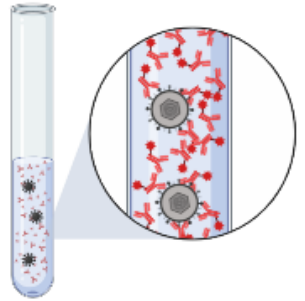
Coincidence: Negative Ctrl + Reagents

Anti-GFP-PE:
MLV-GFP + MLV



Non-specific labeling or coincidence?



Antibody labeling of virus particles:
1.6µg/ml Ab + 10^9 virus particles/ml

6400 Ab: 1 virus

IgG molecular weight = 150,000g/mol

MLV = murine leukemia virus

Antibody titration using MLV and MLVsfGFP: bioRxiv pre-print location https://doi.org/10.1101/614461
Diagram created with BioRender.com

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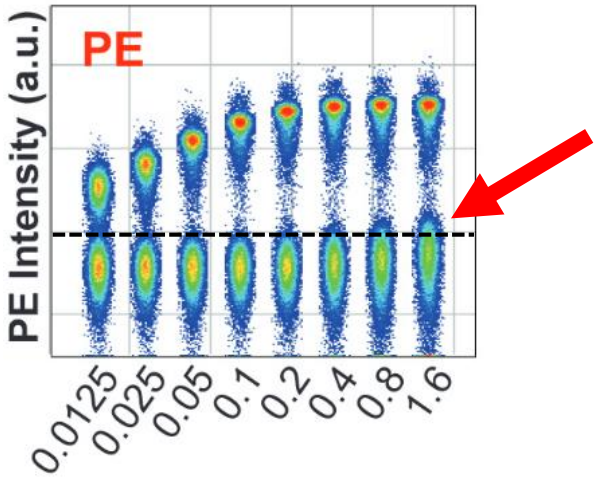
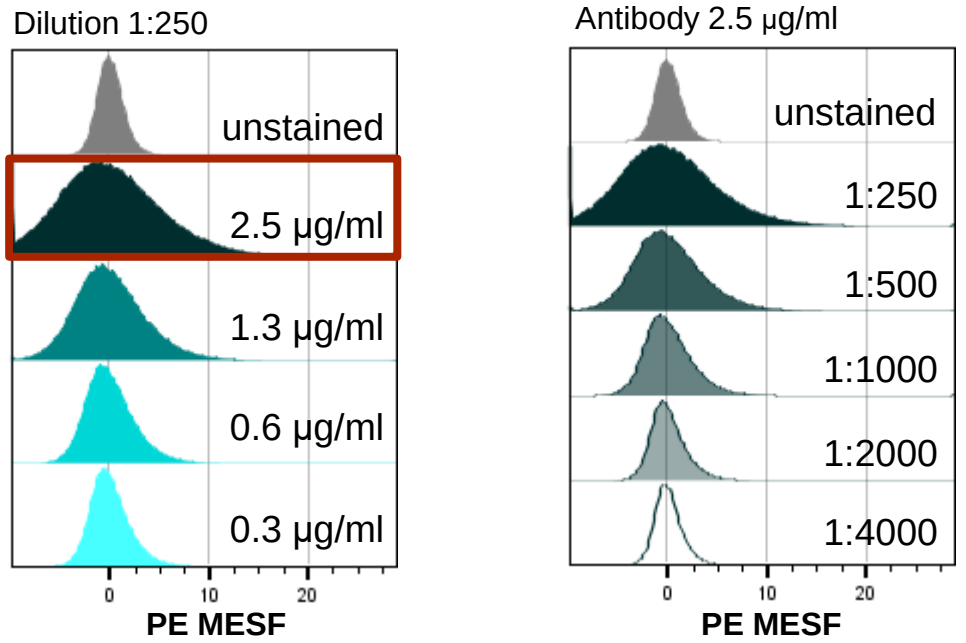
Coincidence: Negative Ctrl + Reagents

Anti-GFP-PE:

MLV-GFP + MLV



Negative Virus + Antibody:



Dilution 1:250 = ~2 x 10^6 virus particles/ml

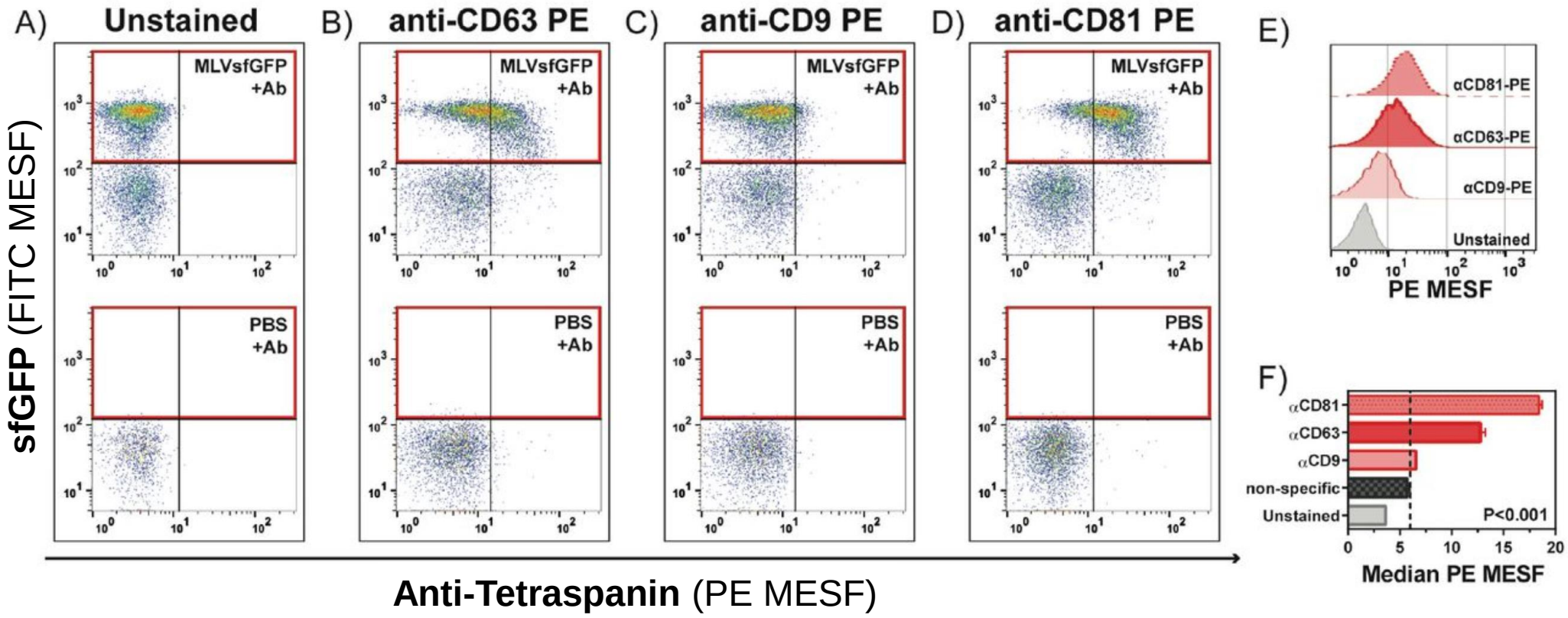
MLV = murine leukemia virus

Antibody titration using MLV and MLVsfGFP: bioRxiv pre-print location https://doi.org/10.1101/614461
& Negative virus and antibody dilution samples from Jonathan Burnie & Dr. Christina Guzzo (Guzzo Lab), University of Toronto.
Data acquired at uOttawaw FCV core facility.

METROFLOW 2020

Phenotyping viruses:

Retrovirus expression of host cell tetraspanins



MLV = murine leukemia virus

Data: bioRxiv pre-print location https://doi.org/10.1101/614461

Controls & Calibration

- Coincidence Controls
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- Assay Controls
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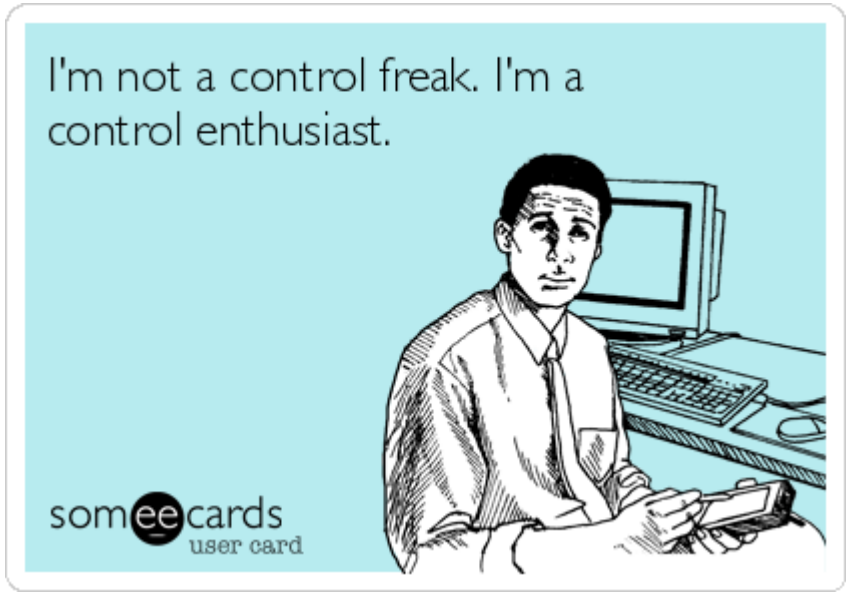

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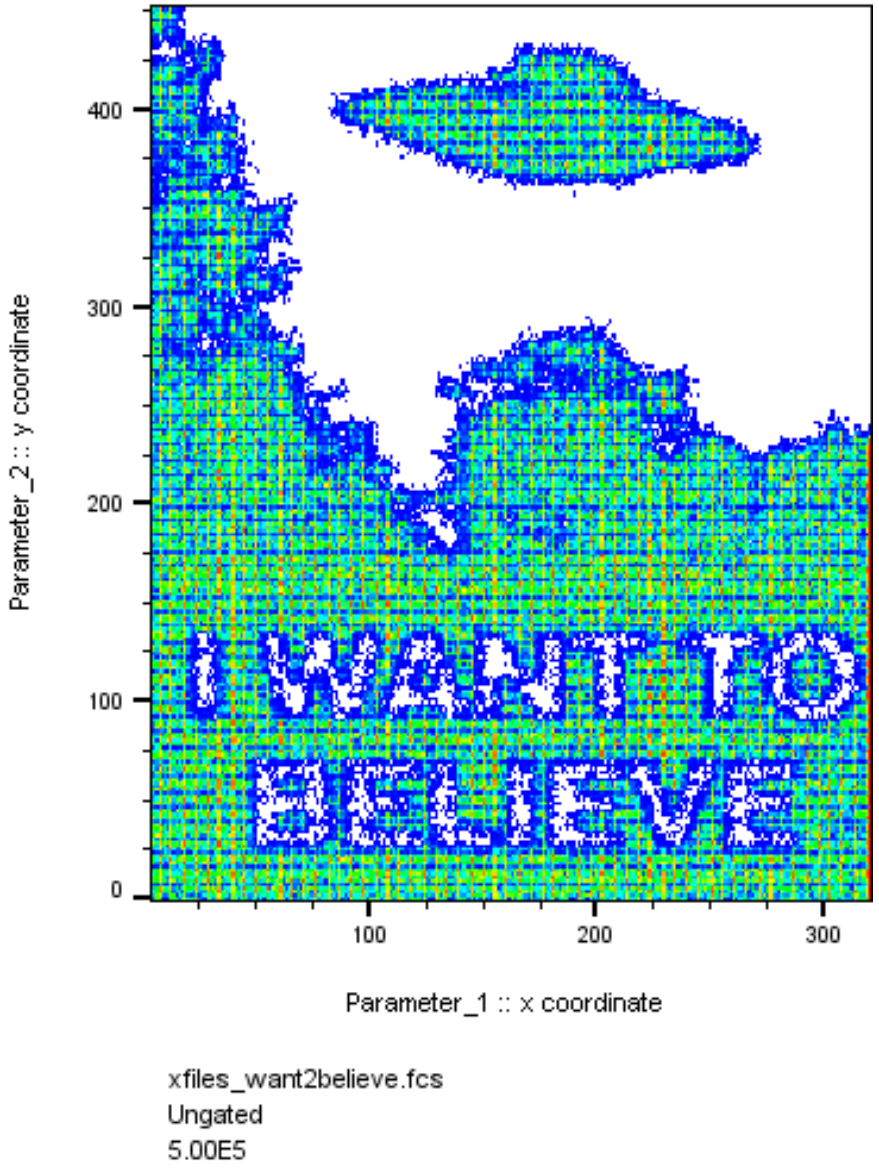

David Gravano: converting images to fcs https://youtu.be/XC4D1QKKiQA

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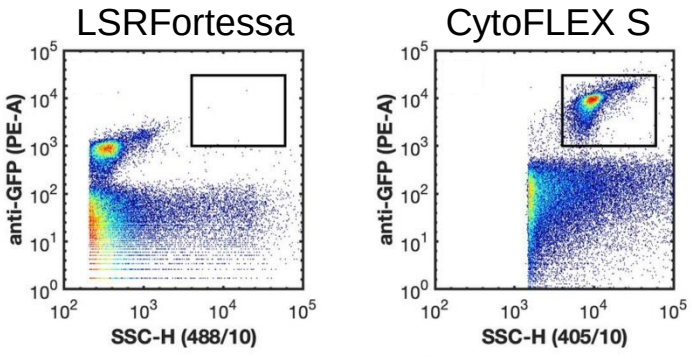
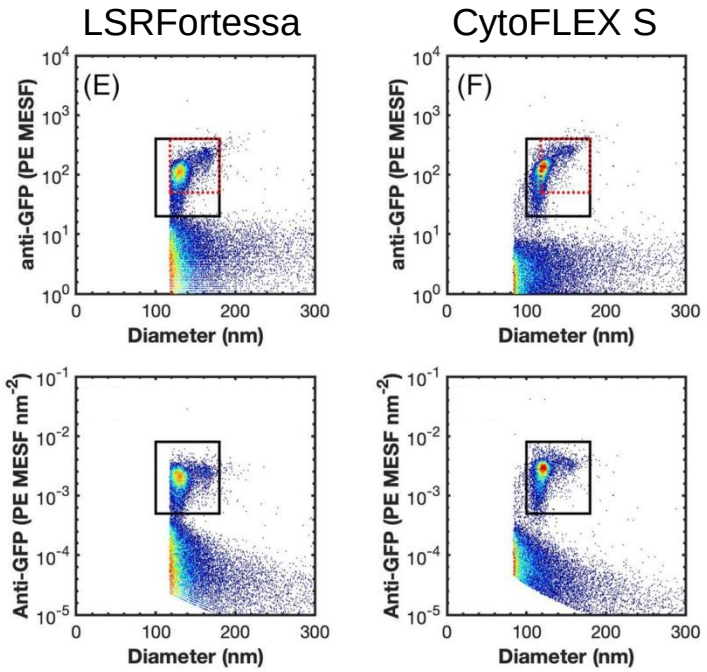
Calibration



calibrate



FL (a.u.) & SSC (a.u.)

Different instruments

- settings & configuration
- 488 vs 405 nm
- Sample – MLVsfGFP + anti-GFP-PE

Antigen Density (MESF/nm²)

J Welsh & VA Tang CYTO2019 Workshop, https://doi.org/10.1002/cyto.a.24029

HOW?

Software for Calibration

- **Scatter Calibration**
- **Fluorescence Calibration**
- Refractive Index Conversion

Download: https://nano.ccr.cancer.gov/fcmpass/ Free for academic use
Protocol: https://currentprotocols.onlinelibrary.wiley.com/doi/10.1002/cpcy.79

Materials required: **MESF or ERF beads for FL calibration, NIST-traceable polystyrene and silica beads (non-fluorescent)***

**FCM$_{PASS}$ recommended beads are listed in protocol reference*

EXOMETRY

Rosetta Calibration

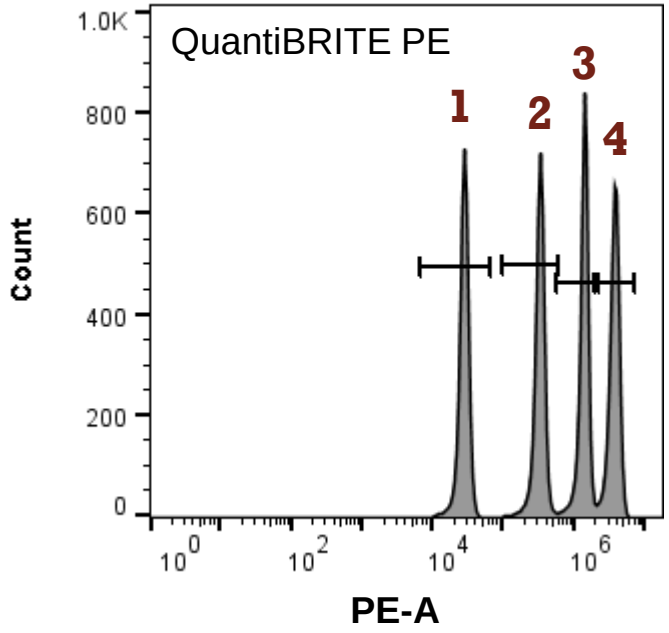
- **Scatter Calibration**

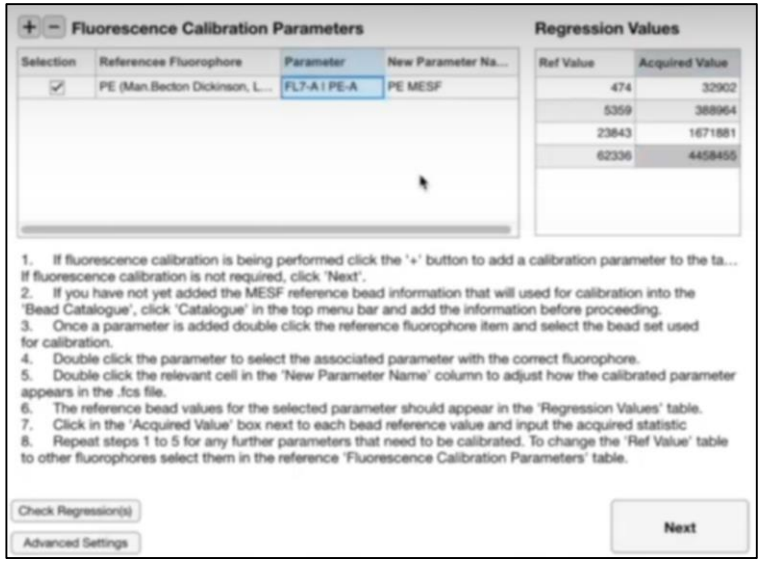
Website for purchase: https://www.exometry.com/products/rosetta-calibration

Materials required: **Rosetta Calibration Beads**

FL & SSC Calibration: FCM$_{PASS}$

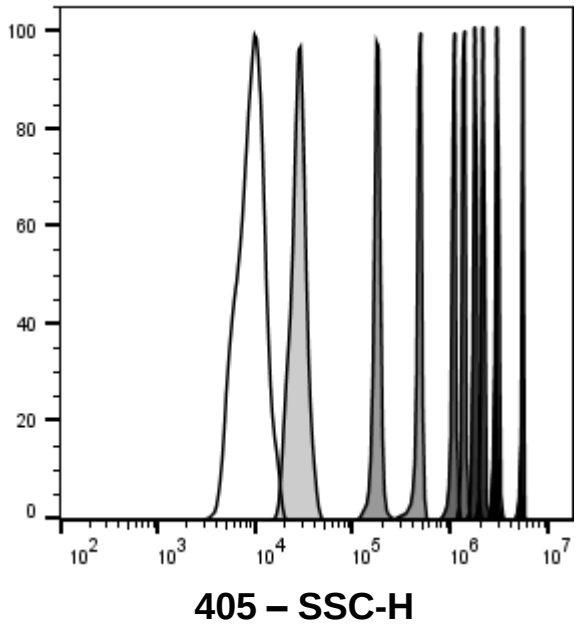
MESF Beads = Molecules of Equivalent Soluble Fluorophore beads





FL & SSC Calibration: FCM$_{PASS}$

NIST-traceable Polystyrene Beads: 80 nm – 600nm
NIST-traceable Silica Beads: 480 nm, 730 nm



405 – SSC-H

FCM PASS

Menu Windows Catalogue Help

Datasets | 1. File Import | 2. Fluorescence Calibration | 3. Scatter Calibration | 4. Perform Calibration | Log

Side Scatter Calibration

Selection	Scatter Parameter	Scatter Wavelength (nm)	Scatter Threshold	Bead Set	Sheath RI
☑	FL5-H | VSSC-H	405	1400	Example Set	1.3431

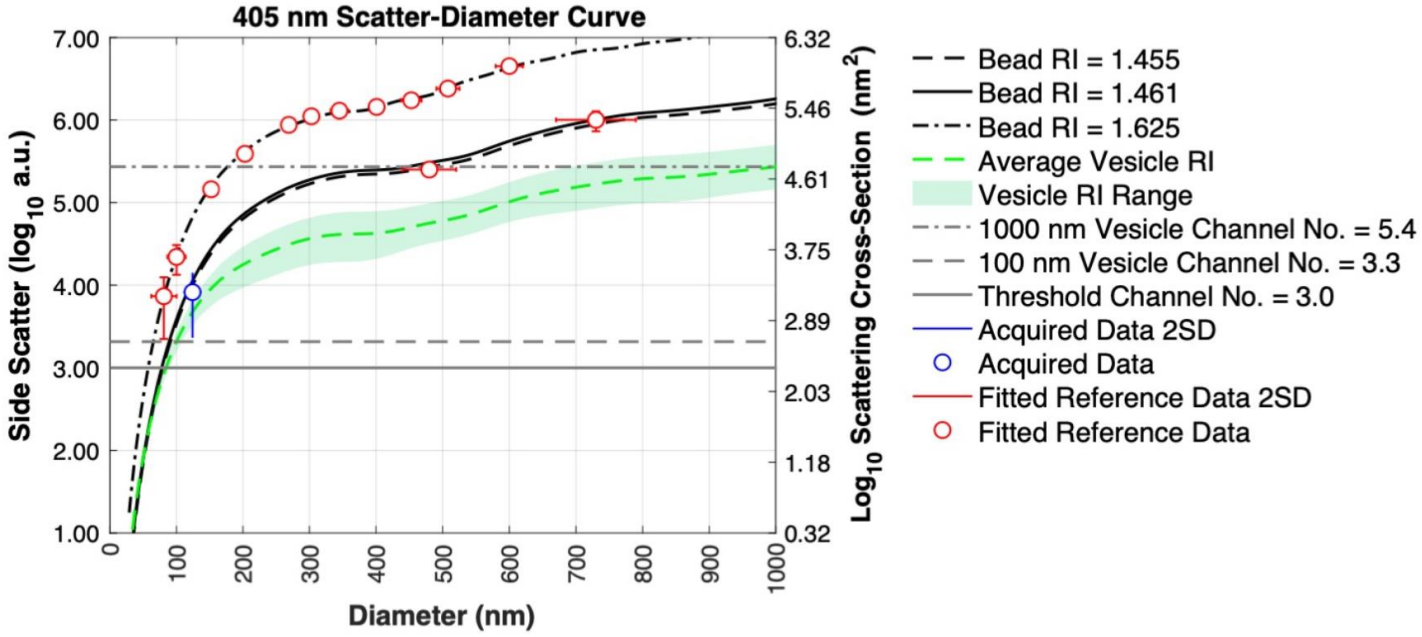
Diameter (nm)	Composition	Acquired Stat (au)	Acquired CV (%)
81	Polystyrene	8485	33.7000
100	Polystyrene	26134	29.3000
152	Polystyrene	170220	9.8300
203	Polystyrene	458000	8.8200
269	Polystyrene	1030000	5.5300
303	Polystyrene	1300000	4.3400
345	Polystyrene	1530000	4.3200
401	Polystyrene	1690000	4.6900
453	Polystyrene	2040000	4.7300
480	Silica	293082	7.8100
508	Polystyrene	2830000	6.2300
600	Polystyrene	5170000	4.7200
730	Silica	1180000	10.1000

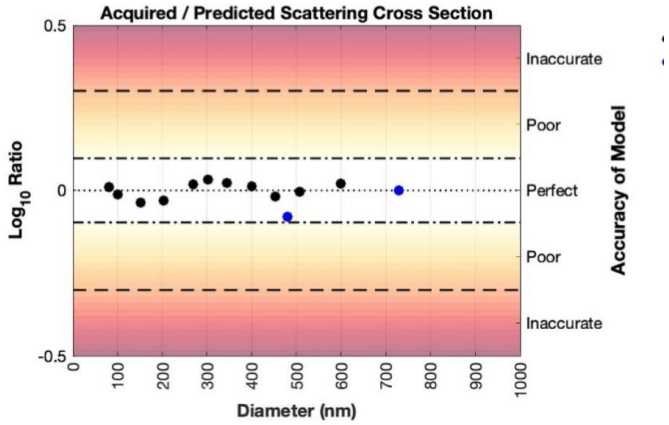
Advanced Settings

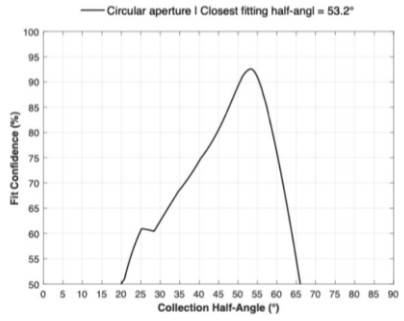
Next

FCM$_{PASS}$ recommended beads are listed in protocol reference

FL & SSC Calibration: FCM$_{PASS}$







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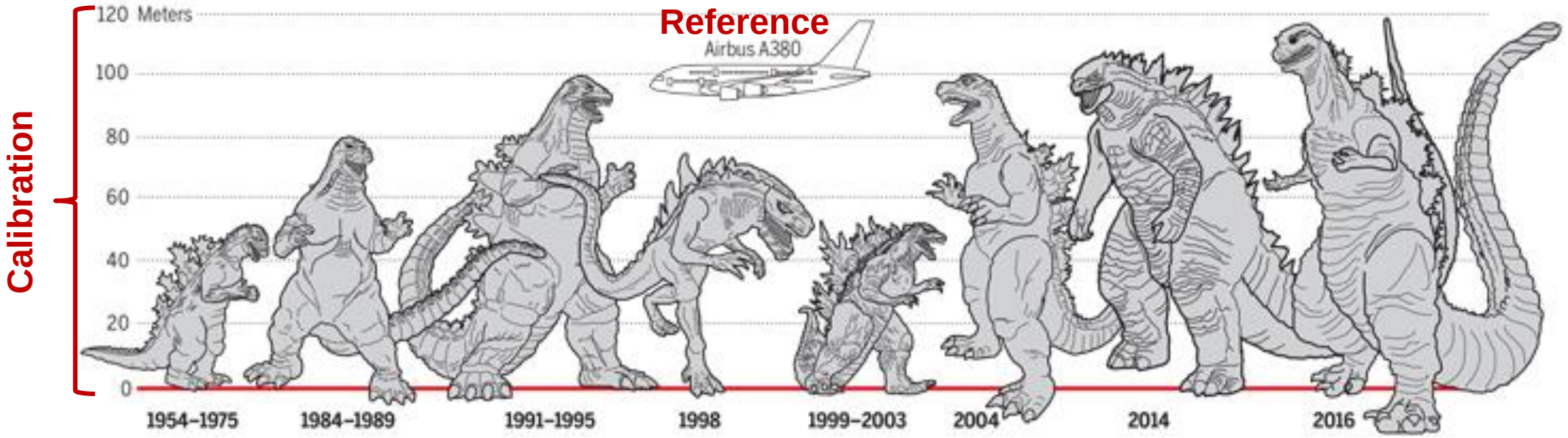
Reference & Calibration Materials



Fluorescence Calibration Beads: MESF Beads (ex. QuantibritePE), ERF Beads (Spherotech 8 peak Rainbow) NIST
Scatter Calibration Beads: non-fluorescent, NIST-traceable, reported diameter, material with reported RI @specific wavelength

Reference Materials: fluorescent EVs, fluorescent virus, fluorescent bead mixes

Godzilla image: Science 31 May 2019: Vol. 364, Issue 6443, pp. 840-841 DOI: 10.1126/science.aax5394

SUMMARY

- **MIFlowCyt-EV Framework** outlines workflow, controls & methods for small particle FCM
- **Coincidence** – both samples & reagents contribute to the total # of events in a sample
- **Assay Controls & Dilutions** – controls for coincidence, but also helps assay optimization
  - Sample concentration – optimize to minimize coincidence
  - Titration of reagents = optimize labeling + minimize coincidence, less is more!
- **Data Calibration** – allows for conversion of arbitrary units fluorescence and light scatter
  - Important to choose well-characterized calibration materials
  - Show data in calibrated units, don't just run the beads!
  - Standardization, reproducibility, validation

METROFLOW 2020

Acknowledgements

uOttawa Flow Cytometry & Virometry Core Facility

- Anna Fritzche, MSc (2017-2019 Technician & Research Assistant)
- Lionel Filion, PhD (1985-2014 Facility Director)
- Marc-Andre Langlois, PhD (2014-2019 Facility Director)
- Kristin Baetz, PhD (2019-present Interim Facility Director)

Collaborators & Colleagues

- Jones Lab (NIH/NCI)
  - Joshua Welsh, PhD
- Langlois Lab (University of Ottawa)
  - Anna Fritzche, MSc
  - Tyler Renner, PhD
- Guzzo Lab (University of Toronto)
  - Christina Guzzo, PhD
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- Des Pink, PhD
- ISAC SRL Emerging Leaders
- Canadian Cytometry & Microscopy Association

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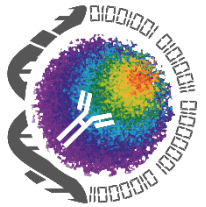

ISAC
INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

CANADIAN CYTOMETRY AND MICROSCOPY ASSOCIATION
ASSOCIATION CANADIENNE DE CYTOMÉTRIE ET DE MICROSCOPIE
CCMA ACCM
CYTOMETRY.CA CYTOMETRIE.CA

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Online Resources

- **How to perform fluorescence and scatter calibration using FCM_{PASS}**
  - Software Download: https://nano.ccr.cancer.gov/fcmpass/
  - Protocol: https://currentprotocols.onlinelibrary.wiley.com/doi/10.1002/cpcy.79
  - Video from CYTO2020: https://www.youtube.com/watch?v=41n4-im-Okg
- **MIFlowCyt-EV & Introduction to Calibration Tools**
  - Video from CYTO2020: https://www.youtube.com/watch?v=mKA9dB_g19M
  - Video from EV Flow Series – MIFlowCyt-EV: https://www.youtube.com/watch?v=4a5mVqbGR9E
- **Slack channel: EVFlowCytometry**
  - Forum for EV Flow Cytometry (& other small particles)– ask the experts directly
  - EV Flow Series ~monthly webinars on EVs & other small particle flow cytometry research
  - http://bit.ly/EVflowslack

Publications Referenced

- JA Welsh *et al.*, **MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments.** J Extracell Vesicles. 2020; 9(1): 1713526. doi: 10.1080/20013078.2020.1713526
- JA Welsh, JC Jones, VA Tang. Fluorescence and **Light Scatter Calibration Allow Comparisons of Small Particle Data in Standard Units across Different Flow Cytometry Platforms and Detector Settings.** Cytometry A. 2020 Jun;97(6):592-601. doi: 10.1002/cyto.a.24029.
- J Burnie, VA Tang, JA Welsh, AT Persaud, L Thaya, JC Jones, C Guzzo. **Flow Virometry Quantification of Host Proteins on the Surface of HIV-1 Pseudovirus Particles.** Viruses 2020, 12(11), 1296. doi:10.3390/v12111296
- VA Tang *et al.*, **Engineered Retroviruses as Fluorescent Biological Reference Particles for Small Particle Flow Cytometry.** June 2019 bioRxiv pre-print doi:10.1101/614461