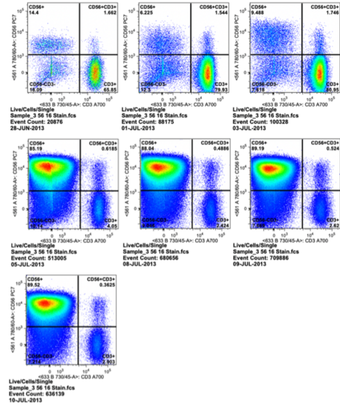
Rigor and Reproducibility in Flow Cytometry



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Rigor and Reproducibility in Flow Cytometry

nature International weekly journal of science

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Archive > Volume 521 > Issue 7552 > News Feature > Article

NATURE | NEWS FEATURE

Reproducibility crisis: Blame it on the antibodies

Antibodies are the workhorses of biological experiments, but they are littering the field with false findings. A few evangelists are pushing for change.

Monya Baker

19 May 2015

https://www.nature.com/news/reproducibility-crisis-blame-it-on-the-antibodies-1.17586

BAD ANTIBODIES The most common problems with antibodies and how to avoid them.

Target protein
Binding site
Non-target protein
Antibody

CROSS-REACTIVITY
Problem: An antibody is supposed to recognize only its target protein, but sometimes binds to others, depending on the proteins present in a sample.
Solution: An antibody should be tested for off-target binding using positive and negative controls.

VARIABILITY
Problem: Separate batches of antibody can perform differently. This happens most often when the antibody is produced from a new set of animals.
Solution: Researchers should confirm lot numbers and characterization data with vendors.

WRONG APPLICATION
Problem: Different experiments and experimental conditions can change a protein's folding and therefore its binding ability.
Solution: Scientists should check supplier's recommended applications.

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Archive > Volume 533 > Issue 7604 > News Feature > Article

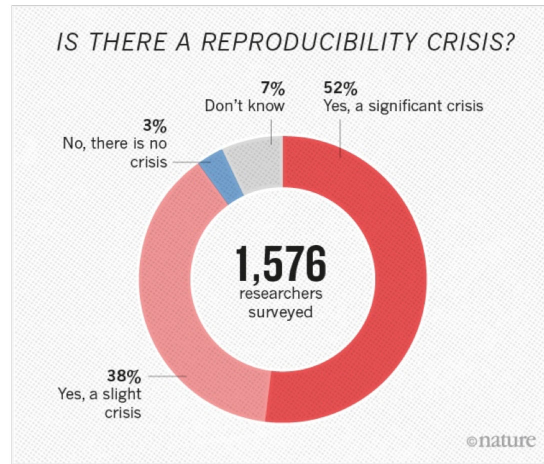
NATURE | NEWS FEATURE

1,500 scientists lift the lid on reproducibility

Survey sheds light on the 'crisis' rocking research.

Monya Baker

25 May 2016 | Corrected: 28 July 2016



https://www.nature.com/news/1-500-scientists-lift-the-lid-on-reproducibility-1.19970

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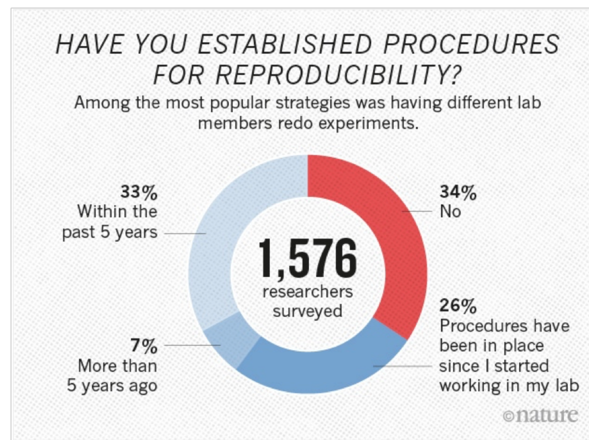

https://www.nature.com/news/1-500-scientists-lift-the-lid-on-reproducibility-1.19970

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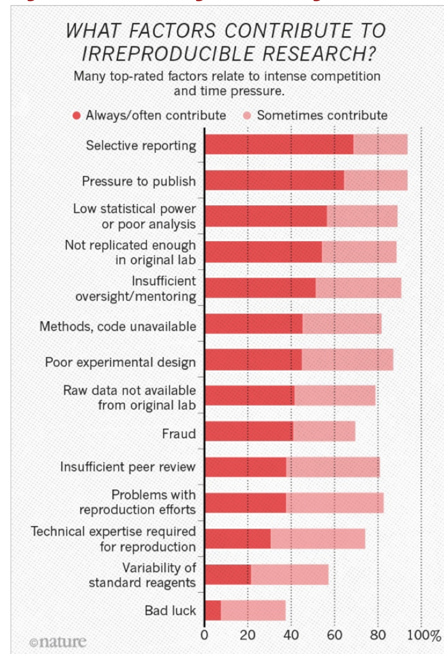
What's Wrong?



https://www.nature.com/news/1-500-scientists-lift-the-lid-on-reproducibility-1.19970

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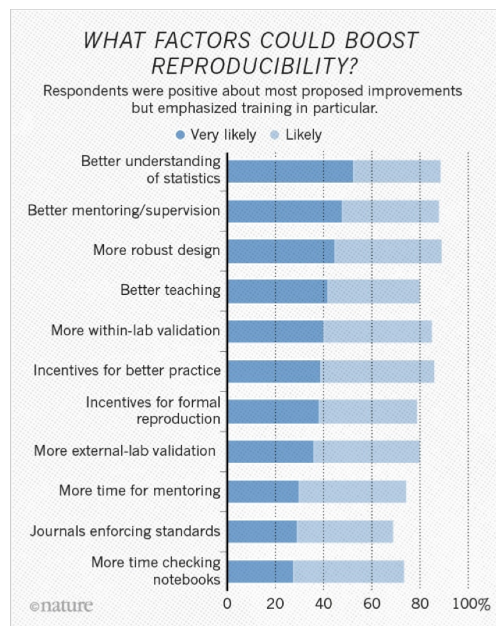
How can we fix it?



https://www.nature.com/news/1-500-scientists-lift-the-lid-on-reproducibility-1.19970

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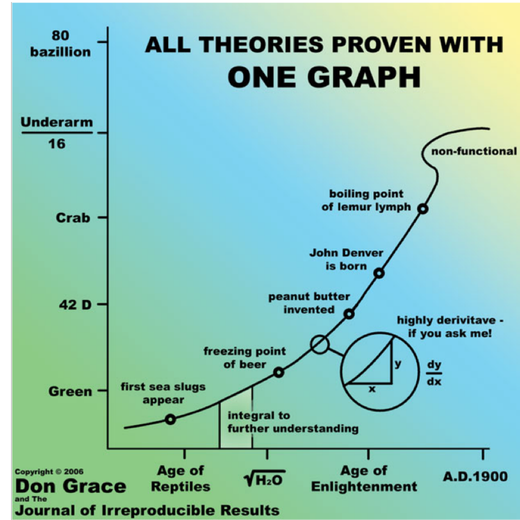
Where do we start?

The Journal of Irreproducible Results®
The Science Humor Magazine

http://www.jir.com



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The NIH

https://grants.nih.gov/policy/reproducibility/index.htm

NIH National Institutes of Health
Office of Extramural Research
Grants & Funding
NIH's Central Resource for Grants and Funding Information

Entire Site | Sea
eRA | eNIH Staff | G

HOME | ABOUT GRANTS | FUNDING | POLICY & COMPLIANCE | NEWS & EVENTS

Home » Policy & Compliance » Rigor and Reproducibility » Enhancing Reproducibility through Rigor and Transparency

Policy & Compliance
- NIH Grants Policy Statement
- Notices of Policy Changes
- Compliance & Oversight
- **Select Policy Topics**
  - Anti-Sexual Harassment
  - Animal Welfare
  - Application Submission Policies
  - Clinical Trial Requirements +
  - Early Stage and Early Established Investigator Policies
  - Financial Conflict of Interest
  - Human Subjects Research
  - Intellectual Property Policy
  - Lobbying Guidance for Grantee Activities
  - NIH Funding Strategies
  - Peer Review Policies and Practices
  - Public Access
  - Research Integrity
  - Rigor and Reproducibility

Enhancing Reproducibility through Rigor and Transparency

The information provided on this website is designed to assist the extramural community in addressing rigor and transparency in NIH grant applications and progress reports. Scientific rigor and transparency in conducting biomedical research is key to the successful application of knowledge toward improving health outcomes.

Definition

Scientific rigor is the strict application of the scientific method to ensure unbiased and well-controlled experimental design, methodology, analysis, interpretation and reporting of results.

Goals

The NIH strives to exemplify and promote the highest level of scientific integrity, public accountability, and social responsibility in the conduct of science. Grant applications instructions and the criteria by which reviewers are asked to evaluate the scientific merit of the application are intended to:

- ensure that NIH is funding the best and most rigorous science,
- highlight the need for applicants to describe details that may have been previously overlooked,
- highlight the need for reviewers to consider such details in their reviews through updated review language, and
- minimize additional burden.

Guidance: Rigor and Reproducibility in Grant Applications

Learn how to address rigor and reproducibility in your grant application and discover what reviewers are looking for as they evaluate the application for scientific merit.

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The NIH

NIH Peer Review

Reviewer Guidance on Rigor and Transparency: Research Project Grant and Mentored Career Development Applications

The goal of this initiative is to enhance reproducibility of research through rigor and transparency. NIH recently updated application instructions and review language for research grant (NOT-OD-16-011) and mentored career development award (NOT-OD-16-012) applications submitted for due dates of January 25, 2016 and beyond. Implementation of rigor and transparency for individual fellowship, institutional career development, and institutional training grant applications will be announced in advance, on a different timeline that allows for training in rigor and transparency to be developed (NOT-OD-16-034).

The four areas of the current rigor and transparency initiative are explained below.

https://grants.nih.gov/grants/peer/guidelines_general/Reviewer_Guidance_on_Rigor_and_Transparency.pdf

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The NIH

The Four Areas of Rigor and Reproducibility

1. **Scientific Premise** – Distinct from hypothesis or justification, refers to the quality and strength of prior research
2. **Scientific Rigor** – Strict application of the scientific method to ensure robust and unbiased experimental design, methodology, analysis interpretation and reporting of results
3. **Consideration of Sex and Other Biological Variables** – includes the critical factors affecting health or diseas in vertebrate animals or human subjects
4. **Authentication of Key Biological and/or Chemical Resources** – Key resources that may vary over time, lab to lab; integral to research; include, but not limited to, cell lines, specialty chemicals, **antibodies**, & other biologicals

https://grants.nih.gov/grants/peer/guidelines_general/Reviewer_Guidance_on_Rigor_and_Transparency.pdf

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Other Resources

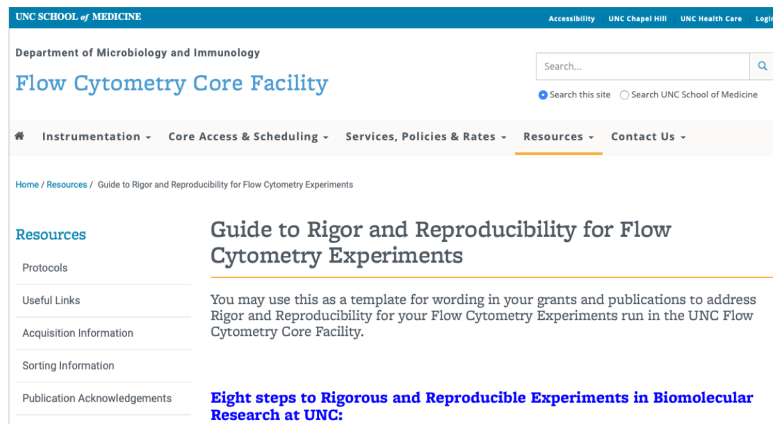
University of Virginia
Flow Cytometry Facility

Association of Biomolecular
Resource Facilities

University of Michigan
Office of Research
Rigor and Reproducibility

Rigor and Reproducibility in Flow Cytometry

University of North Caroline School of Medicine
Flow Cytometry Core Facility



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FASEB report on enhancing research reproducibility identifies three main gaps to research reproducibility:

1. Lack of uniform definitions to describe the problem
2. Insufficient reporting of key experimental details
3. Gaps in scientific training

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Rigor and Reproducibility in Flow Cytometry

Eight steps to Rigorous and Reproducible Experiments in Biomolecular Research

1. If using a core facility, **consult** with the core staff in the planning stage. Consult with a statistician if you need help developing a Power Analysis to assure that your results will be adequately powered.
2. Design your experiment with **sufficient controls** (rigor) **and replicates**(reproducibility).
3. Assure that ALL of your reagents (antibodies, cell lines, mice) are **fully validated**.
4. Have a clear and **detailed protoco**l (SOP) and data analysis plan that can be easily followed. Assure that the protocol is strictly followed or that any deviation is well documented.
5. Assure that the staff or students performing the experiment are **well trained** and understand each step and the importance of performing them precisely (rigor again).
6. Use only **well-maintained instrumentation**, preferably maintained and operated in a core facility with expert staff (see #1 above).
7. **Document all steps**, reagents, equipment and data analysis methods used in the experiment. Assure that the both the documentation and the data itself are properly stored in a safe data management repository.

Acknowledge the Cancer Center Support Grant (*P30 CA014520)*, Grant-supported instrumentation and core staff in publications.

https://www.med.unc.edu/flowcytometry/resources-2/guide-to-rigor-and-reproducibility-for-flow-cytometry-experiments/

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Rigor and Reproducibility in Flow Cytometry

"Lack of Statistical Power, Poor Understanding of Statistics"

UWCCC RESEARCH
Resource for Researchers

Search

INNOVATION | RESOURCES | CLINICAL TRIALS | FUNDING OPPORTUNITIES | EDUCATION | RESEARCHERS | CONTACT US

HOME / RESOURCES / BIOSTATISTICS SHARED RESOURCE (BSR)

BIOSTATISTICS SHARED RESOURCE (BSR)

The mission of the Biostatistics Shared Resource (BSR) is to promote excellence in cancer research at UW Carbone Cancer Center (UWCCC) by providing outstanding biostatistical support and collaboration to UWCCC members.

https://cancer.wisc.edu/research/resources/bsr/

MORE ABOUT BSR

- BSR HOME »
- USING THE FACILITY »
- SERVICES »
- SCIENTIFIC ACCOMPLISHMENTS »

BIOSTATISTICS CONSULTING DROP-IN CLINIC

Biostatistics offers a drop-in clinic for UWCCC members Monday and Tuesday afternoons from 2-5pm.

Location: 6153 WIMR
Phone: (608) 265-8477
Mondays: Yanyao Yi
Tuesdays: Colin Longhurst

CONTACT US

Faculty Leader: Menggang Yu, PhD

Associate Leader: Roxana Alexandridis, PhD

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Rigor and Reproducibility in Flow Cytometry

Guide to Rigor and Reproducibility for Flow Cytometry Experiments in the Flow Lab

I. Multicolor Flow Cytometry: all flow cytometry experiments will be run using replicate [animals/cell lines, etc.] according to recommendations based on power calculations.

1. Include single color controls with each experiment for compensation calculations.
2. Include a viability dye to exclude dead cells from the analysis
3. Incorporate Fluorescence Minus One (FMO) as gating controls for data analysis and as controls to validate the expression of rare or low-expressing markers.
4. Run doublet discrimination in your analysis to exclude aggregates
5. Run time as a parameter to assure that the fluidics were running smoothly during acquisition
6. Daily quality control of all instrumentation is performed based on manufacturers' recommendations to ascertain that all of the Flow Lab flow cytometers maintain peak performance. Flow cytometric fluorophore compensation and analysis is completed using FlowJo, or FCS Express.

II. Cell sorting: quality control/laser alignment and drop delay calculation is carried out according to manufacturers' recommendations on all cell sorters prior to sorting. Single color controls are included with each experiment for compensation calculations. Proper controls for gating cells of interest are included. Post-sort analysis are run on each population to verify purity when possible.

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https://www.med.unc.edu/flowcytometry/resources-2/guide-to-rigor-and-reproducibility-for-flow-cytometry-experiments/

Rigor and Reproducibility in Flow Cytometry

III. All antibodies will be validated prior to reporting of results. Recommendations for research using antibodies (page 7-8) 'Although vendor-supplied technical information may help investigators select reagents such as antibodies, this information is insufficient for validation'.
Antibody Validation: Standards, Policies and Practices Workshop Report 2016

Have your antibodies been validated?
Check the Antibody registry.

Resources for antibody validation:

1. Always Titrate
2. Validate Specificity
3. Integrate critical controls like Fluorescence Minus One controls (FMO)
4. See: Uhlen et al., A Proposal for Validation of Antibodies, Nature Methods (2016)
5. More at: https://expertcytometry.com/4-steps-to-validate-flow-cytometry-antibodies-and-improve-reproducibility/
6. Also on the EuroMab network:
7. Guidelines Website: https://www.euromabnet.com/guidelines/index.php Guidelines Manuscript: https://www.ncbi.nlm.nih.gov/pubmed/26418356 Pos/Neg Controls Website: https://www.euromabnet.com/guidelines/positive-negative-controls.php Additional Articles about Ab Validation: https://www.euromabnet.com/guidelines/articles-about-antibody-validation.php

FluoroFinder has partnered with CiteAb to provide validation data for >200,000 reagents.

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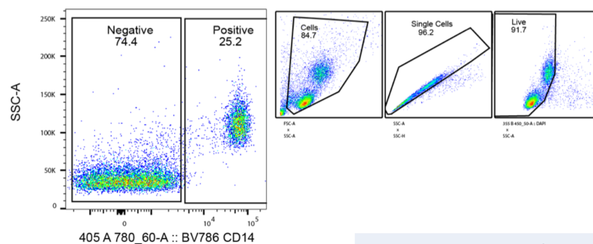
https://www.med.unc.edu/flowcytometry/resources-2/guide-to-rigor-and-reproducibility-for-flow-cytometry-experiments/

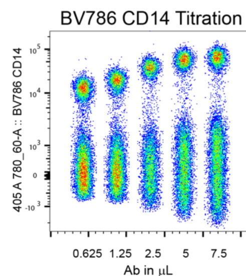
Rigor and Reproducibility in Flow Cytometry

Titration & Separation Index



$$SI = \frac{Med_{pos} - Medneg}{\left[\frac{84\%ileneg - Medneg}{0.995}\right]}$$

Technotes for Performing Titrations & Data Analysis on flowcytometry.wisc.edu



CD14 BV786 Titration

Antibody Amount (µL)	84th Percentile of Negative	Median of Negative	Median of Positive	Separation Index
0.625	713	126	12287	20.6
1.25	752	126	18594	29.4
2.5	904	166	35374	47.5
5	1119	182	53070	56.2
7.5	1285	230	59808	56.2

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Rigor and Reproducibility in Flow Cytometry

Annotate, Annotate, Annotate

"Your closest collaborator is you 6 months from now and you don't answer emails."

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Rigor and Reproducibility in Flow Cytometry

The value of data is only as good as its annotation and accessibility.

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

ISAC
International Society for Advancement of Cytometry

Minimum Information About a Flow Cytometry Experiment (MIFlowCyt) Checklist (Numbered in accordance with MIFlowCyt 1.0 document)

Cytometry Part A • 77A: 813, 2010

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Rigor and Reproducibility in Flow Cytometry

MIFlowCyt

1. **Experiment Overview**
   - 1.1. Purpose
   - 1.2. Keywords
   - 1.3. Experiment Variables
   - 1.4. Organization (name and address)
   - 1.5. Primary Contact (name and email address)
   - 1.6. Date (or time period)
   - 1.7. Conclusions (if applicable)
   - 1.8. Quality Control Measures
2. **Flow Sample and Specimen Details**
   - 2.1. Sample/Specimen Material Description (include description, type, source, source treatment, taxonomy, age, gender, phenotype, genotype as applicable for biological samples; description and location for environmental samples)
   - 2.3. Sample Treatment(s) Description
   - 2.4. Fluorescence Reagent(s) Description (include characteristic(s) being measured, analytes, analyte detectors, analyte reporters, clone names/numbers, manufacturer, catalogue numbers as applicable)
3. **Instrument Details**
   - 3.1. Instrument Manufacturer
   - 3.2. Instrument Model
   - 3.3. Instrument Configuration and Settings (provide acquisition settings including detector voltages and describe all custom alterations of the instrument if applicable; include installation dates of optical filters)
4. **Data Analysis Details** (*if data analysis has been performed*)
   - 4.1. List-mode Data File (specify location of original list-mode file, for example supplementary material, URL, website)
   - 4.2. Compensation Details (describe how multicolor compensation was performed by including antibodies, cells, or beads used)
   - 4.3. Data Transformation Details (purpose and description if any transformation of the raw measurement has been performed, including various scales for visualization and gating purposes)
   - 4.4. Gating (Data Filtering) Details (include description of all gates, percentage of events inside, and either mathematical descriptions of each gate boundary or appropriate gate images; mathematical description of gate boundaries can be provided in using Gating-ML or an appropriate project or workspace file); description of the algorithm by which gates were created (for example, subjective, based on FMO (how?), same gate for all analyses, etc.)
5. **Data Presentation Requirements**
   - 5.1. Axes legends (antibody and dye; linear- or logarithmic-scaled axes)
   - 5.2. Graphical example for full gating strategy
   - 5.3. Positive/negative control or FMO

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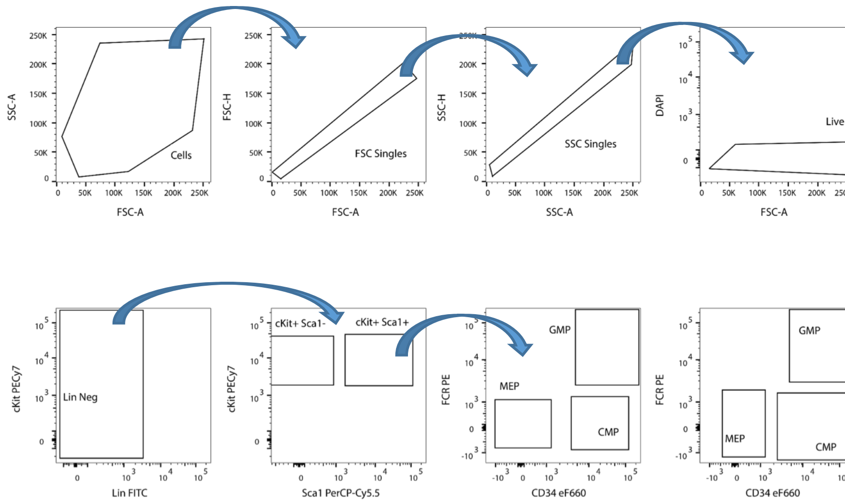

UWCCC Flow Lab for Kirby Johnson, PhD, Bresnick Lab

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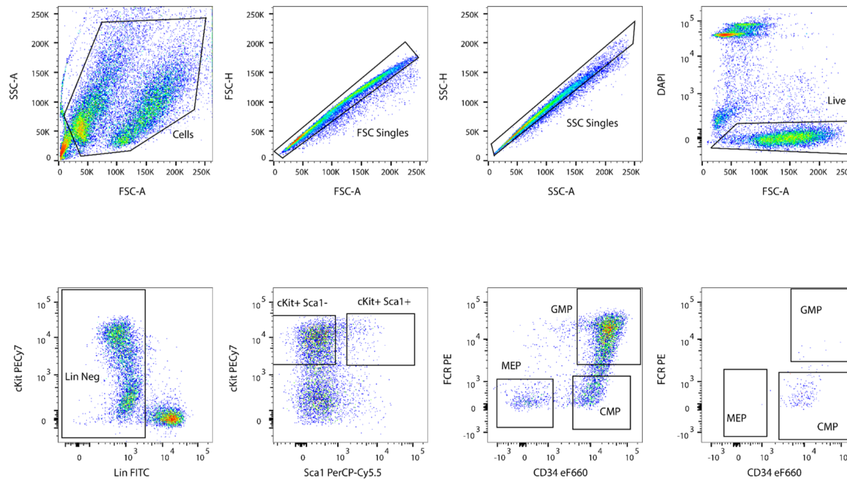

UWCCC Flow Lab for Kirby Johnson, PhD, Bresnick Lab

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Flow Cytometry Controls

Experimental Controls

- Time zero
- Untreated
- Positive control
- Mock transfected
- Wild Type
- Known Standard
- Anything else that will help you prove your hypothesis

Cytometer Controls

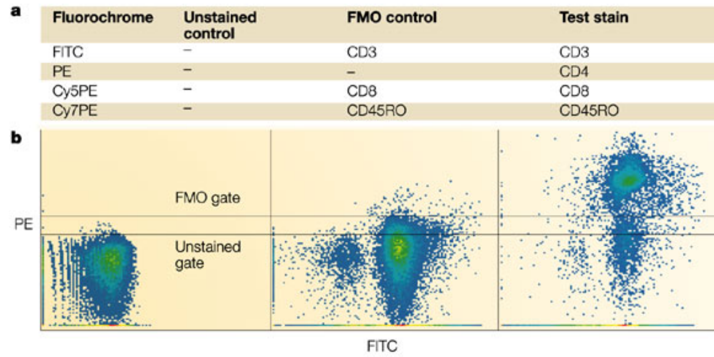
- Single Color Controls
- Bead Standard

Data Analysis Controls

- Fluorescence Minus One (FMO)
- Reference Standard

Rigor and Reproducibility in Flow Cytometry

FMO: Fluorescence Minus One



a

Fluorochrome	Unstained control	FMO control	Test stain
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO

Gating control that takes into account all spread in the data

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Nature Reviews Immunology **4**, 648-655 (August 2004)

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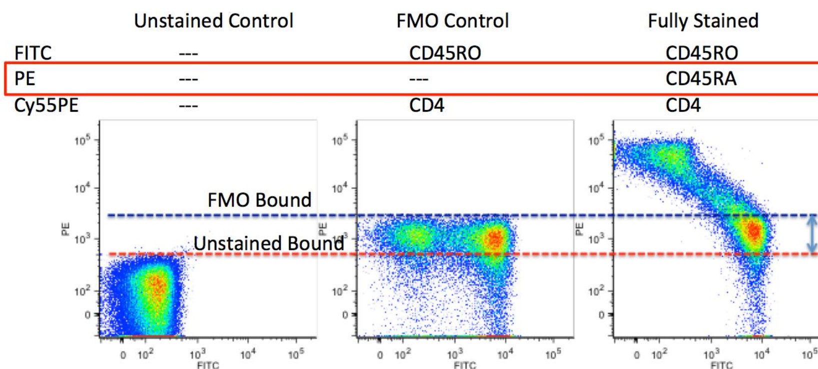
Another FMO Example



	Unstained Control	FMO Control	Fully Stained
FITC	---	CD45RO	CD45RO
PE	---	---	CD45RA
Cy55PE	---	CD4	CD4

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Tim Bushnell, Excyte Cytometry

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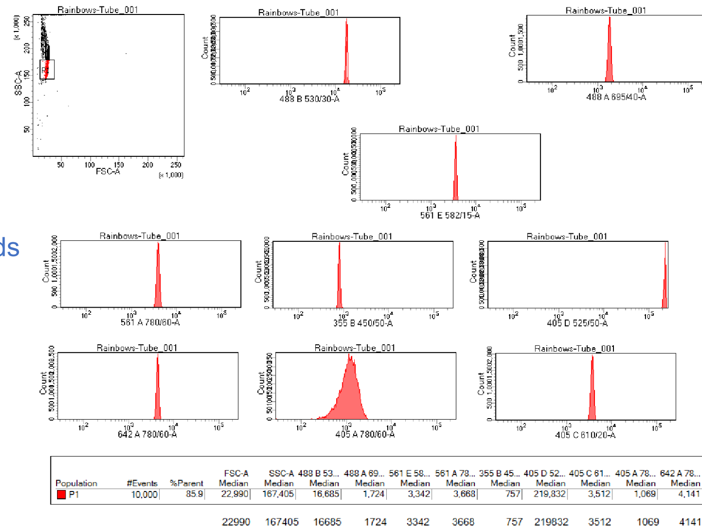
Assay Standardization

Spherotech Midrange Rainbow Beads

- Run after optimizing voltages
- Median Fluorescence Intensity Values = Target Values for Successive Runs



Population	#Events	%Parent	FSC-A Median	SSC-A Median	488 B 53... Median	488 A 69... Median	561 E 58... Median	561 A 78... Median	355 B 45... Median	405 D 52... Median	405 C 61... Median	405 A 78... Median	642 A 78... Median
P1	10,000	85.9	22,990	167,405	16,685	1,724	3,342	3,668	757	219,832	3,512	1,069	4,141
			22990	167405	16685	1724	3342	3668	757	219832	3512	1069	4141

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Rainbow Bead Standardization

UWCCC Flow Cytometry Laboratory
https://cancer.wisc.edu/research/resources/flow/

1111 Highland Ave
7016 WIMR
Madison, WI 53705
608.263.0313

Why Standardize My Flow Assay?

Assay standardization in flow cytometry improves reproducibility and reduces cytometer set-up time. For assays evaluating protein expression levels using MFI (median fluorescence intensity), this standardization is critical for reducing user set-up variation among replicates. For all flow assays, standardization expedites cytometer set-up and data analysis.

How Does Rainbow Bead Standardization Work?

Do I Still Have to Compensate?

Yes! Standardization tells you where to set voltages (instrument sensitivity) using multicolor beads, but compensation uses single stained controls to account for fluorescence spillover. Compensation is still critical for obtaining good multicolor flow cytometry data. For the most

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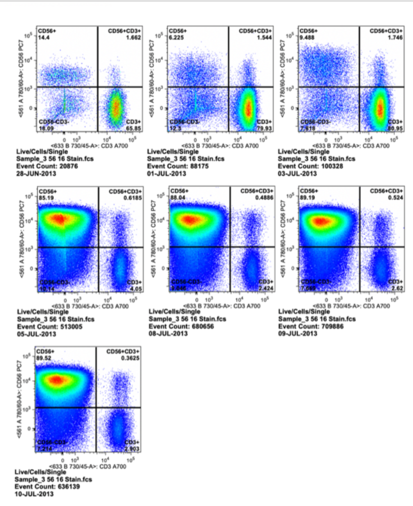
Rigor and Reproducibility in Flow Cytometry

- Experimental Design
- Antibody Validation
- Antibody Titration
- Proper Controls
- Instrument Optimization
- Annotation
  - Standardized Naming Conventions
  - Data Collection
  - Reagent Lot #'s & Exp. Dates
  - Deviations
  - Instrument QC
- Rainbow Standard
- Data Acquisition Template
- Data Analysis Template
- Data Reporting Template
- Peer Review
- Cross-Training
- Detailed SOP & SOP Template to record annotations above **per run**



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Rigor and Reproducibility in Flow Cytometry

Instrument Characterization

Determination of operational voltages on an instrument-by-instrument basis as a means to facilitate high-dimensional panel design in a shared resource setting

Derek D. Jones*, Richard D. Schretzenmair*, and Jonni S. Moore
University of Pennsylvania Abramson Cancer Center, Flow Cytometry and Cell Sorting Resource Laboratory, Philadelphia, PA
*Equal contribution

Jones, DD, et. al., U Penn Flow Cytometry & Cell Sorting Resource Laboratory

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Rigor and Reproducibility in Flow Cytometry

Instrument Characterization

Figure 2. Sample type does not affect the SI-PMTV curve. Human PBMC or lyophilized PBMC were stained with anti-CD4 BV605, and acquired over a range of voltages. Depicted are stain indices as a function of PMT voltage; EC_{90} are indicated. The operational voltage was nearly identical for each sample type, and is indicated by a dashed line.

Figure 3. The SI-PMTV curve is independent of fluorochrome brightness. Human PBMC were stained with anti-CD4 BB700 and PerCP-Cy5.5 (both in the 695/40 Blue detector), and acquired over a range of voltages. Depicted are stain indices as a function of PMT voltage (left). Stain indices reported as a % of maximum (right) allow for a meaningful comparison of different fluorochromes. EC_{90} were identical for each fluorochrome.

Figure 5. Operational voltages reduce spillover spread and facilitate panel design. Human PBMC were stained with anti-CD4 antibodies conjugated to the indicated fluorochromes. Samples were acquired on a BD LSR II at CST voltages (left) and Operational voltages (right), as determined by CD4 voltage titration. Spillover spread matrices were computed using FlowJo, and pseudocolored according to intensity of spillover spread.

Jones, DD, et. al., U Penn Flow Cytometry & Cell Sorting Resource Laboratory

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Rigor and Reproducibility in Flow Cytometry

Instrument Characterization

Conclusion/Summary

- Providing SIs and SSMs to users will support panel design and enhance users' understanding of cytometry
- Comparison of stain indices between instruments can help in the identification of poorly performing cytometers or individual PMTs
- Facilitate the construction of higher-dimensional panels (20+ colors)

Future Directions:

- CD4 voltage titration and SSM calculation for every cytometer (>20) in the UPenn SRL
- Designate a target MFI for each detector, and use application settings to update cytometer changes daily
- Provide a means for eventual cross-instrument standardization
- This approach will ultimately be used to guide panel design for a 30+-parameter Symphony A5.
  - *Will facilitate several pre-clinical studies focusing on T cells, NK cells, and myeloid subsets*

Jones, DD, et. al., U Penn Flow Cytometry & Cell Sorting Resource Laboratory

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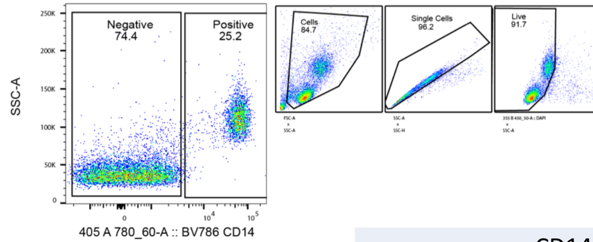
Rigor and Reproducibility in Flow Cytometry

Titration & Separation Index



$$SI = \frac{Med_{pos} - Medneg}{\left[\frac{84\%ileneg - Medneg}{0.995}\right]}$$

BV786 CD14 Titration

405 A 780_60-A :: BV786 CD14

0.625 1.25 2.5 5 7.5

Ab in µL

CD14 BV786 Titration				
Antibody Amount (µL)	84th Percentile of Negative	Median of Negative	Median of Positive	Separation Index
0.625	713	126	12287	20.6
1.25	752	126	18594	29.4
2.5	904	166	35374	47.5
5	1119	182	53070	56.2
7.5	1285	230	59808	56.2

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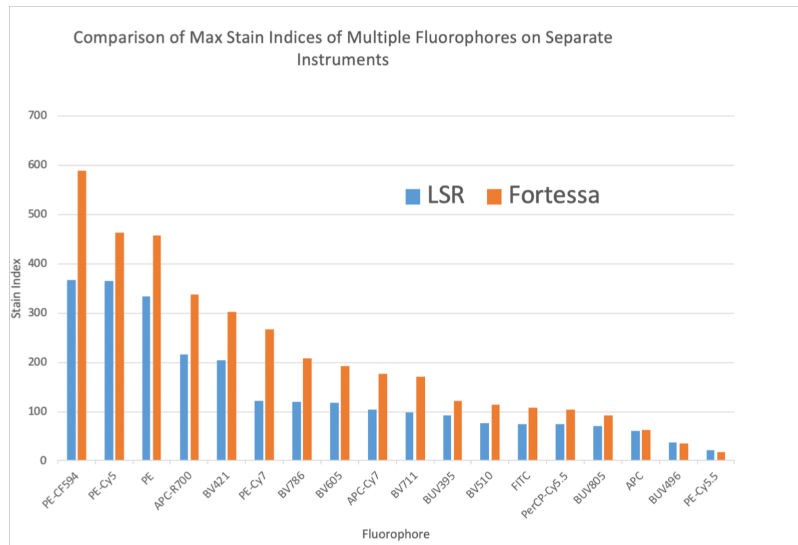
Instrument Characterization

CD4 Staining Index

Comparison of LSR to Fortessa



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Rigor and Reproducibility in Flow Cytometry

Instrument Characterization – LSR

LSR II - Spillover Spreading Matrix - CST Voltages

	355 A	355 B	355 C	405 A	405 B	405 C	405 D	405 E	488 A	488 B	561 A	561 B	561 C	561 D	561 E	640 A	640 B	640 C
	820_60	530_30	820_60	780_60	710_20	610_20	525_50	450_50	695_40	530_30	780_60	710_50	670_30	610_20	582_15	780_60	730_45	675_20
BUV805		0.00	0.09	0.07	0.00	-0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.13	0.00	0.00
BUV496	0.01		0.31	0.01	0.01	0.98	0.64	0.15	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUV395	0.00	0.01		0.00	0.00	-0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV786	0.04	0.00	0.00		0.01	0.01	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00
BV711	0.02	0.00	0.00	0.51		0.00	0.00	0.03	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.05	0.18	0.00
BV605	0.00	0.00	0.00	0.03	0.08		0.00	0.01	0.00	0.00	0.00	0.02	0.02	0.03	0.01	0.00	0.00	0.00
BV510	0.00	0.06	0.00	0.06	0.13	5.20		0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV421	0.00	0.00	0.00	0.00	0.00	0.04	0.06		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PerCP-Cy5.5	0.01	0.00	0.00	0.44	0.95	-0.01	0.00	0.00		0.00	0.04	0.33	0.11	0.00	0.00	0.08	0.23	0.07
FITC	0.00	0.01	0.00	0.01	0.00	0.09	0.05	-0.01	0.03		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PE-Cy7	0.03	0.00	0.00	0.27	0.00	0.00	0.00	0.00	0.00	0.00		0.03	0.00	0.01	0.01	0.16	0.01	0.00
PE-Cy5.5	0.00	0.00	0.00	0.03	0.07	0.01	0.00	0.00	0.47	0.00	0.10		0.14	0.01	0.03	0.04	0.13	0.02
PE-Cy5.5	0.00	0.00	0.00	0.02	0.04	0.00	0.00	0.00	0.41	0.00	0.07	0.83		0.00	0.01	0.06	0.22	0.40
PE-CF594	0.00	0.00	0.00	0.01	0.03	1.23	0.00	0.00	0.29	0.00	0.04	0.50	0.52		0.10	0.00	0.00	0.00
PE	0.00	0.00	0.00	0.00	0.01	0.74	0.00	0.00	0.10	0.00	0.01	0.14	0.16	0.50		0.00	0.00	0.00
APC-Cy7	0.02	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.01	0.01	0.00	0.00		0.08	0.05
APC-R700	0.00	0.00	0.00	0.02	0.06	0.00	0.00	0.00	0.01	0.00	0.03	0.25	0.02	0.00	0.00	0.23		0.05
APC	0.00	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.16	0.27	0.00	0.00	0.12	0.49	
Voltage Setting	847	822	616	879	819	944	525	576	603	453	620	604	533	561	561	597	673	558

LSR II - Spillover Spreading Matrix - Optimized Voltages

	355 A	355 B	355 C	405 A	405 B	405 C	405 D	405 E	488 A	488 B	561 A	561 B	561 C	561 D	561 E	640 A	640 B	640 C
	820_60	530_30	820_60	780_60	710_20	610_20	525_50	450_50	695_40	530_30	780_60	710_50	670_30	610_20	582_15	780_60	730_45	675_20
BUV805		0.01	0.32	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.64	0.03	0.00
BUV496	0.00		0.27	0.01	0.01	0.10	0.56	0.12	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUV395	0.00	0.02		0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV786	0.02	0.00	0.00		0.01	0.00	0.00	0.05	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.07	0.02	0.00
BV711	0.01	0.00	0.00	0.57		0.00	0.00	0.05	0.02	0.00	0.01	0.03	0.00	0.00	0.00	0.13	1.29	0.01
BV605	0.00	0.00	0.00	0.07	0.14		0.00	0.03	0.00	0.00	0.01	0.05	0.06	0.06	0.03	0.00	0.00	0.00
BV510	0.00	0.07	0.00	0.04	0.08	0.61		0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV421	0.00	0.00	0.00	0.00	0.00	0.00	0.05		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PerCP-Cy5.5	0.00	0.00	0.00	0.46	0.88	0.00	0.00	0.00		0.00	0.10	0.42	0.14	0.00	0.00	0.18	1.59	0.12
FITC	0.00	0.01	0.00	0.00	0.00	0.01	0.03	0.00	0.01		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PE-Cy7	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00		0.01	0.00	0.00	0.01	0.13	0.02	0.00
PE-Cy5.5	0.00	0.00	0.00	0.03	0.05	0.00	0.00	0.00	0.37	0.00	0.23		0.14	0.01	0.04	0.08	0.71	0.02
PE-Cy5.5	0.00	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.32	0.00	0.16	0.82		0.00	0.01	0.11	1.16	0.56
PE-CF594	0.00	0.00	0.00	0.02	0.03	0.26	0.00	0.00	0.33	0.00	0.13	0.74	0.76		0.19	0.00	0.01	0.00
PE	0.00	0.00	0.00	0.00	0.01	0.08	0.00	0.00	0.06	0.01	0.01	0.10	0.12	0.26		0.00	0.00	0.00
APC-Cy7	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.01	0.01	0.00	0.00		0.25	0.04
APC-R700	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.05	0.00	0.00	0.00	0.08		0.01
APC	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.11	0.19	0.00	0.00	0.16	1.87	
Voltage Setting	652	575	561	754	690	637	477	521	520	445	609	535	473	471	516	570	745	517

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Rigor and Reproducibility in Flow Cytometry

Instrument Characterization - Fortessa

LSR Fortessa - Spillover Spreading Matrix - CST Voltages

	355 A	355 B	355 C	405 A	405 B	405 C	405 D	405 E	488 A	488 B	561 A	561 B	561 C	561 D	561 E	640 A	640 B	640 C
	820_60	530_30	379_28	780_60	710_20	610_20	525_50	450_50	695_40	530_30	780_60	710_50	670_30	610_20	582_15	780_60	730_45	670_14
BUV 805		0.00	0.06	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.02	0.00	0.00
BUV 496	0.01		0.29	0.01	0.01	0.87	1.34	0.07	0.01	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUV 395	0.00	0.03		0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV 786	0.12	0.00	0.00		0.01	0.03	0.02	0.05	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00
BV 711	0.04	0.00	0.00	0.34		0.01	0.01	0.03	0.12	0.00	0.01	0.01	0.00	0.00	0.00	0.02	0.06	0.01
BV 605	0.00	0.00	0.00	0.01	0.02		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.02	0.00	0.00	0.00
BV 510	0.00	0.05	0.00	0.01	0.04	2.00		0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV 421	0.00	0.00	0.00	0.00	0.00	0.07	0.23		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PerCP-Cy5.5	0.03	0.00	0.00	0.19	0.52	0.00	0.00	0.00		0.00	0.08	0.22	0.17	0.00	0.00	0.02	0.06	0.14
FITC	0.00	0.01	0.00	0.00	0.00	0.10	0.11	0.00	0.02		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PE-Cy7	0.04	0.00	0.00	0.11	0.00	0.01	0.00	0.00	0.01	0.00		0.01	0.00	0.01	0.03	0.02	0.00	0.00
PE-Cy5.5	0.01	0.00	0.00	0.05	0.13	0.04	0.00	0.00	1.51	0.00	0.38		0.35	0.06	0.17	0.02	0.06	0.08
PE-Cy5	0.00	0.00	0.00	0.01	0.03	0.01	0.00	0.00	0.49	0.00	0.10	0.32		0.01	0.02	0.01	0.03	0.28
PE-CF594	0.00	0.00	0.00	0.01	0.02	0.78	0.00	0.00	0.24	0.00	0.04	0.14	0.41		0.20	0.00	0.00	0.00
PE	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.04	0.00	0.01	0.02	0.08	0.35		0.00	0.00	0.00
APC-Cy7	0.21	0.00	0.00	0.39	0.01	0.01	0.00	0.00	0.00	0.00	0.75	0.03	0.09	0.00	0.00		0.15	0.24
APC-R700	0.03	0.00	0.00	0.07	0.29	0.00	0.00	0.00	0.13	0.00	0.20	0.57	0.09	0.00	0.00	0.22		0.38
APC	0.01	0.00	0.00	0.01	0.04	0.01	0.00	0.00	0.01	0.00	0.03	0.09	0.43	0.00	0.00	0.03	0.14	
Voltage Setting	636	408	394	517	535	636	465	409	494	357	515	472	488	488	503	431	411	525

LSR Fortessa - Spillover Spreading Matrix - Optimized Voltages

	355 A	355 B	355 C	405 A	405 B	405 C	405 D	405 E	488 A	488 B	561 A	561 B	561 C	561 D	561 E	640 A	640 B	640 C
	820_60	530_30	379_28	780_60	710_20	610_20	525_50	450_50	695_40	530_30	780_60	710_50	670_30	610_20	582_15	780_60	730_45	670_14
BUV 805		0.00	0.20	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00
BUV 496	0.00		0.62	0.00	0.00	0.09	0.71	0.05	0.01	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUV 395	0.00	0.01		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV 786	0.10	0.00	0.00		0.01	0.00	0.01	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.01	0.00
BV 711	0.08	0.00	0.00	0.94		0.00	0.02	0.10	0.63	0.00	0.01	0.00	0.00	0.00	0.00	0.21	0.46	0.01
BV 605	0.01	0.00	0.00	0.05	0.06		0.00	0.02	0.04	0.00	0.01	0.00	0.04	0.08	0.05	0.00	0.00	0.00
BV 510	0.00	0.10	0.00	0.02	0.02	0.39		0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV 421	0.00	0.01	0.00	0.00	0.00	0.01	0.16		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PerCP-Cy5.5	0.01	0.00	0.00	0.10	0.10	0.00	0.00	0.00		0.00	0.02	0.00	0.03	0.00	0.00	0.05	0.09	0.02
FITC	0.00	0.01	0.00	0.00	0.00	0.01	0.04	0.00	0.03		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PE-Cy7	0.07	0.00	0.00	0.23	0.00	0.00	0.00	0.00	0.05	0.00		0.00	0.00	0.01	0.03	0.19	0.01	0.00
PE-Cy5.5	0.32	0.01	0.00	1.39	1.48	0.25	0.01	0.00	86.07	0.07	5.27		3.11	0.83	3.05	2.64	4.93	0.66
PE-Cy5	0.01	0.00	0.00	0.03	0.04	0.00	0.00	0.00	3.07	0.00	0.15	0.04		0.01	0.04	0.12	0.31	0.27
PE-CF594	0.00	0.00	0.00	0.02	0.02	0.34	0.00	0.00	1.43	0.00	0.06	0.02	0.41		0.26	0.00	0.00	0.00
PE	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.20	0.01	0.01	0.00	0.06	0.27		0.00	0.00	0.00
APC-Cy7	0.04	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.01	0.00	0.00		0.10	0.02
APC-R700	0.01	0.00	0.00	0.02	0.04	0.00	0.00	0.00	0.09	0.00	0.03	0.01	0.01	0.00	0.00	0.35		0.04
APC	0.01	0.00	0.00	0.04	0.04	0.00	0.00	0.00	0.07	0.00	0.05	0.01	0.44	0.00	0.00	0.47	1.35	
Voltage Setting	621	426	454	516	469	495	445	410	537	385	466	301	419	420	448	531	475	449

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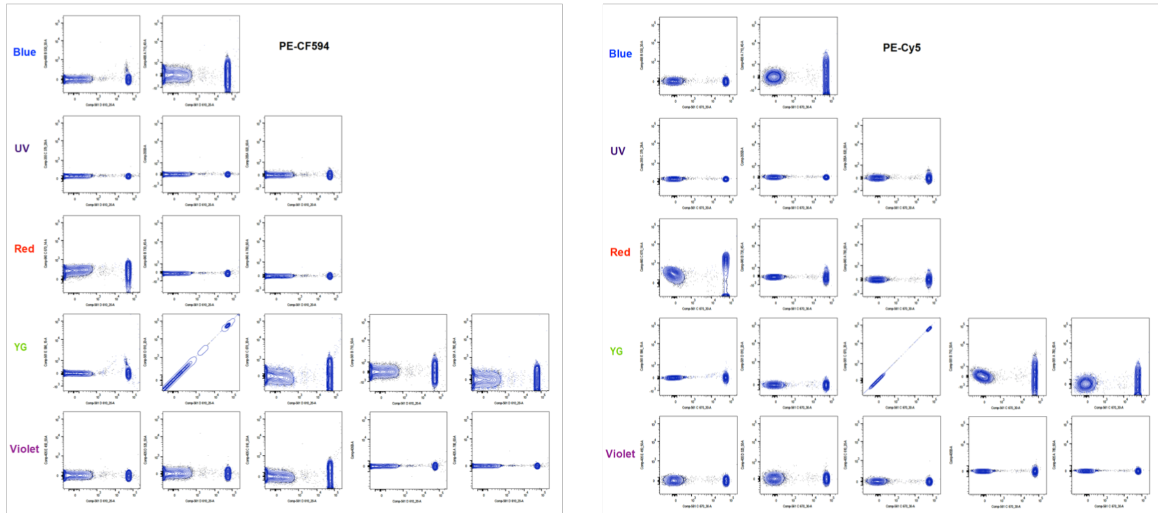
Rigor and Reproducibility in Flow Cytometry

Instrument Characterization



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Rigor and Reproducibility in Flow Cytometry

Instrument Characterization

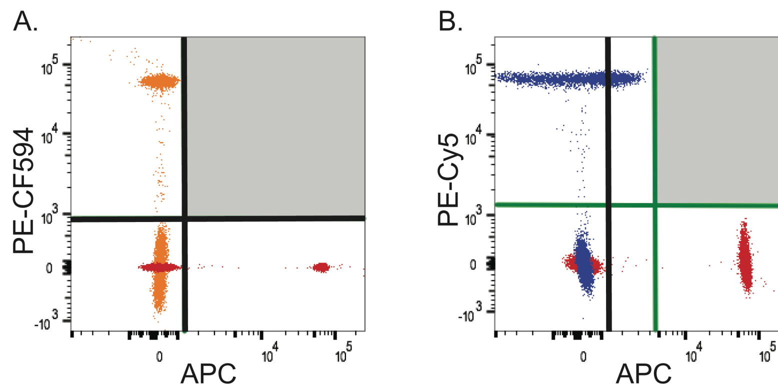

Figure 5. Loss of resolution due to Spectral Spreading. Single stained compensation beads are used to illustrate loss of resolution and sensitivity due to spectral spillover. Grey boxes represent area of double positivity over background due to spectral spillover. A. Spectral spillover of PE-CF594 fluorescence (orange) into APC detector (red) is 0.27%. B. Spectral spillover of PE-Cy5 (blue) fluorescence into the APC (red) detector is 51.53%. Note smaller area of grey box in B. compared to A. Black line represents background for PE-CF954 spectral spillover into APC channel for comparison.

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Mark your calendars for upcoming UWCCC Flow Lab Seminars!

Overview of Computational Data Analysis Platforms for Flow Cytometry
Friday, January 11, 2019
10am, WIMR 7001A

Flow Cytometry – Compensation with Confidence
Friday February 1, 2019
10am, WIMR 7001A

Flow Cytometry Current Best Practices for PIs
Thursday, February 14, 2019
7:30am, WIMR 7170

Multicolor Panel Design for Flow Cytometry
Tuesday, March 5, 2019
2pm, WIMR 7001A

Data Analysis with Alex II
Tuesday, March 7, 2019
10am, WIMR 7170

Data Analysis with Alex III
Wednesday, May 16, 2019
10am, WIMR 7170