Multi-color Panel Design and Experiment Considerations

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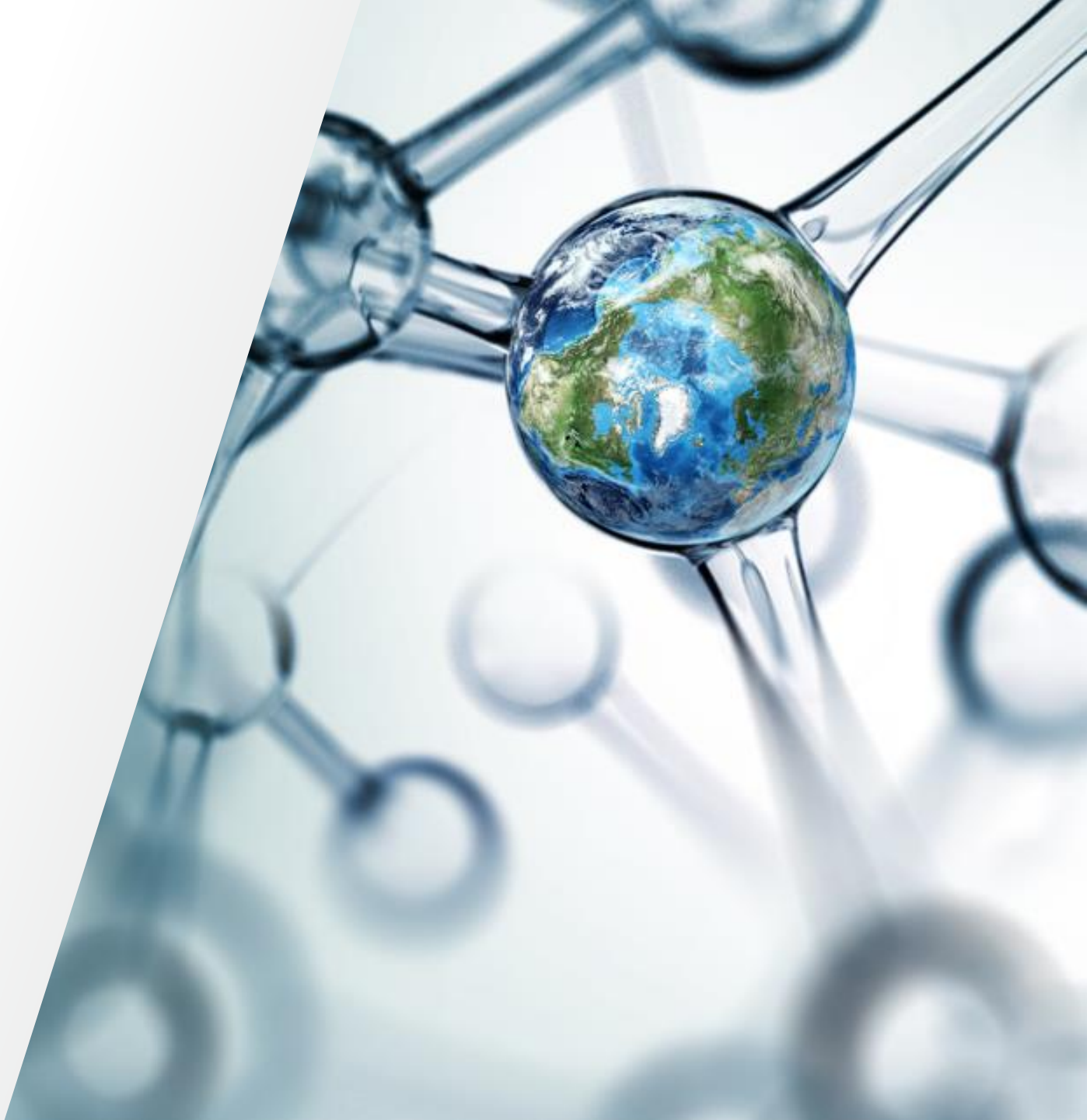
Field Applications Scientist



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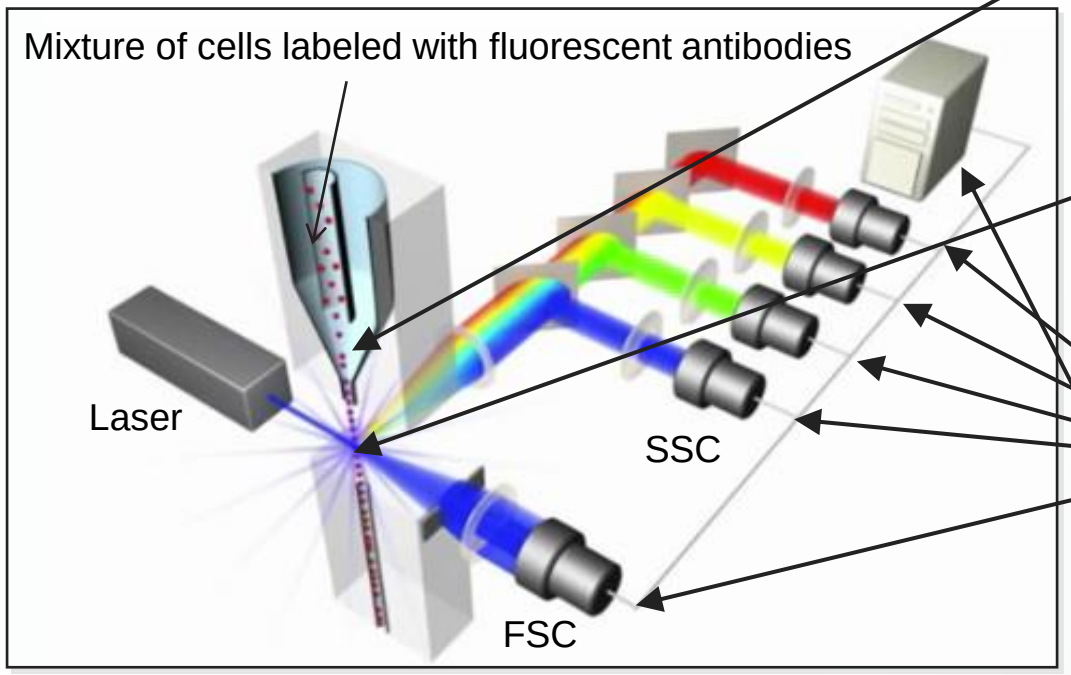
Content

Basic Principle of Flow Cytometery

Principles of Flow Cytometry



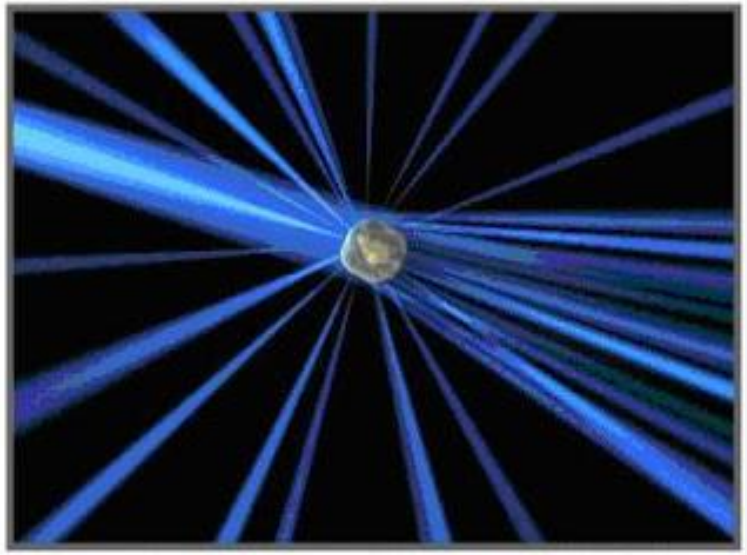
1. Cells in a single profile pass through the narrow tube called flow cell
   - **Cell Focusing**
2. Laser hits individual cell passing through the flow cell
   - **Interrogation Point**
3. Deflected light hits a series of detectors
   - **PMTs**
4. The signals from detectors are interpreted by a computer
   - **Storage and analysis**

Optics System

What Happens to Light When it Hits a Cell?

Laser Light Scatter

- When laser light interacts with a cell, light is scattered in all directions
- The magnitude of the light scatter is dependent on refractive index, size and complexity of the cells or particles passing by the laser
- Differences in Forward Light Scatter and Side Light Scatter can be used to distinguish different types of cells or particles

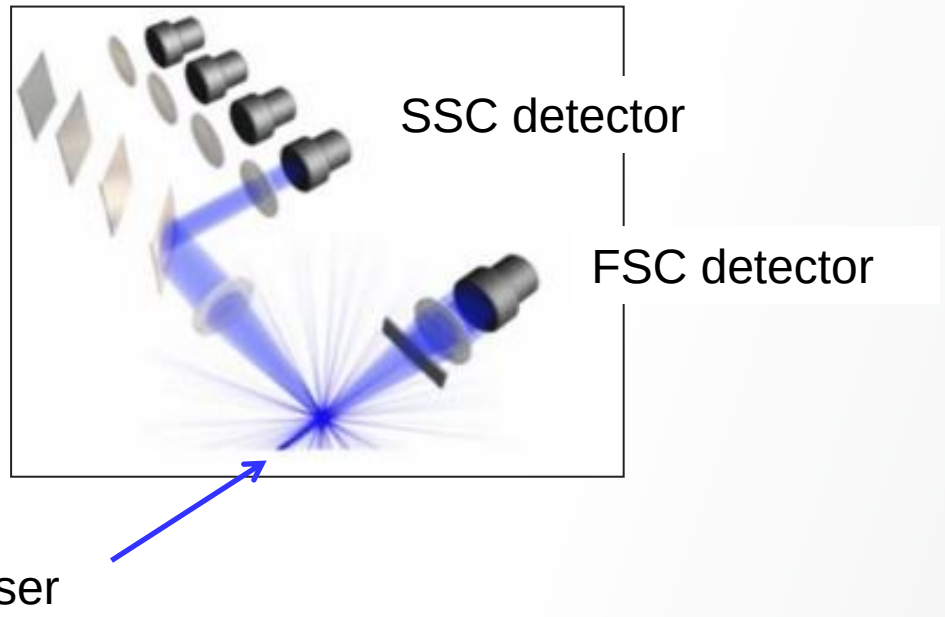


Optics System

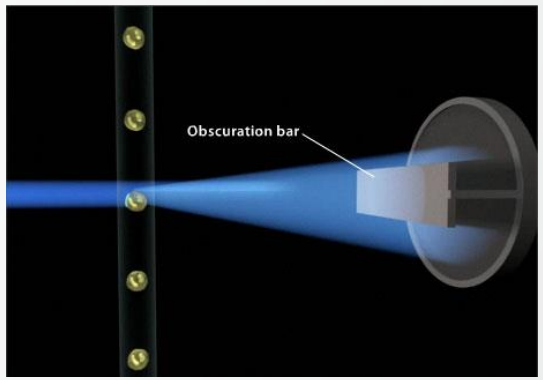
What Happens to Light When it Hits a Cell?

Laser Light Scatter

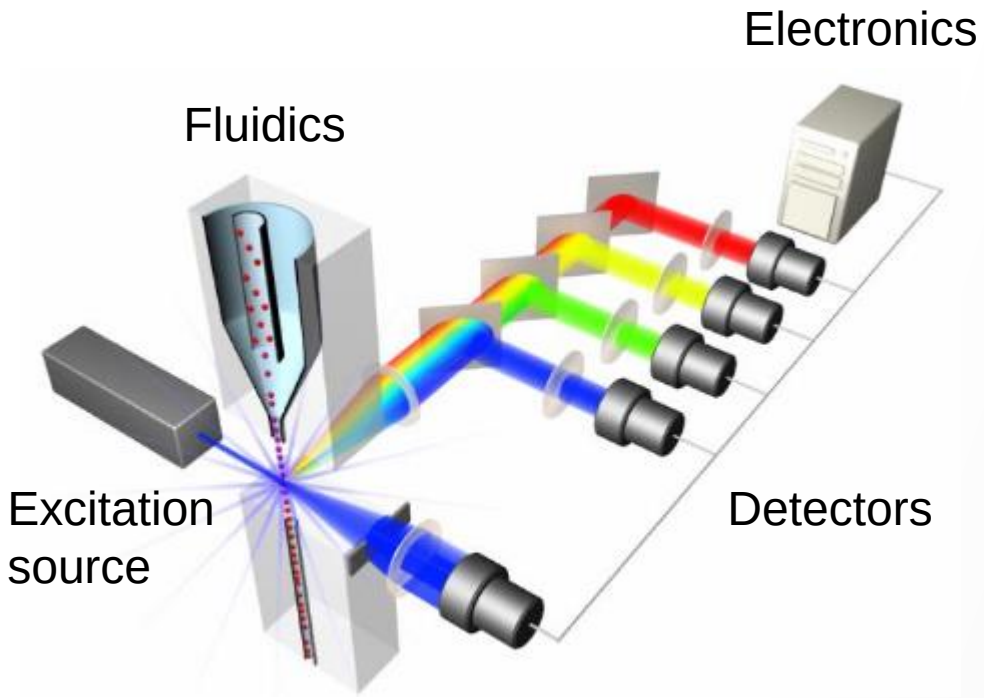
- **Forward Scattered light (FSC)** is impacted by both refractive index and can sometimes be used as a measure of relative cell **size**.
- **Side-scattered light (SSC)** is a measure of cellular **complexity**, both surface and internal. SSC is usually collected at 90 degrees to the laser beam.

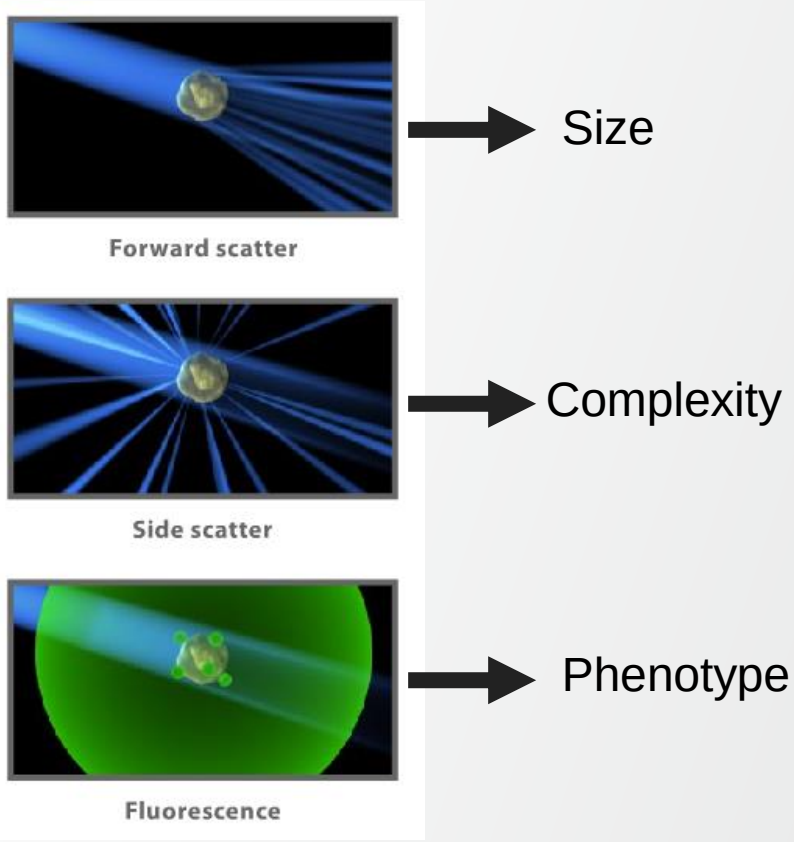


Obscuration Bar

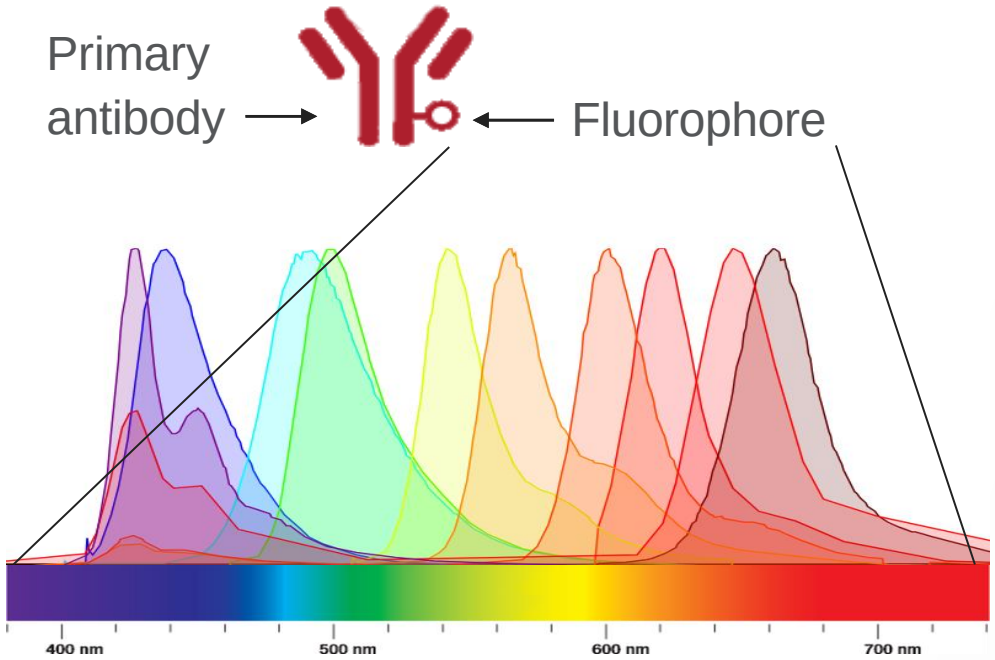


Principles of Flow Cytometry





What is a Flow Cytometry Antibody?



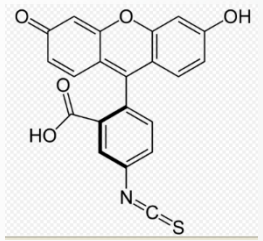
Conjugated antibodies allow for less experimental steps like washes and centrifugations

Multiple antibodies and reagents are often required for an experiment to detect the cell population of interest

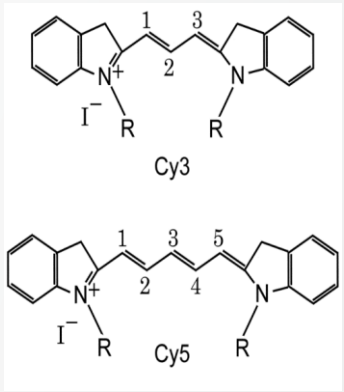
Fluorophore is attached to the primary antibody

Different Fluorophores

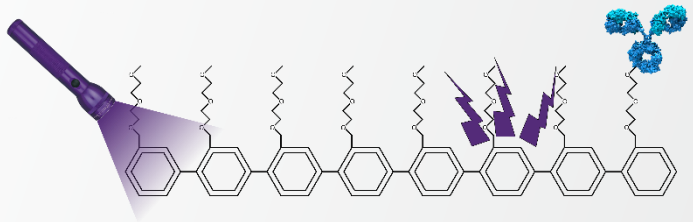
- **ORGANIC**:
  - Contain several aromatic groups
  - FITC, PE, Per-CP, APC, eFluors, Alexa Fluor®, Pacific Blue, Cy5, Cy5.5, Cy7



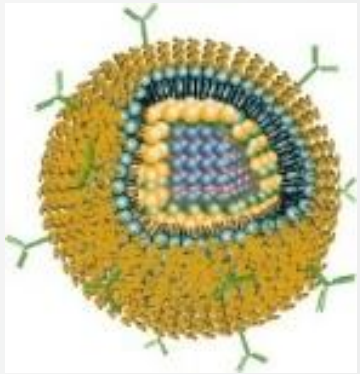
FITC



- **Polymer-dyes**
  - Super Bright Dyes, Brilliant Violet Dyes™

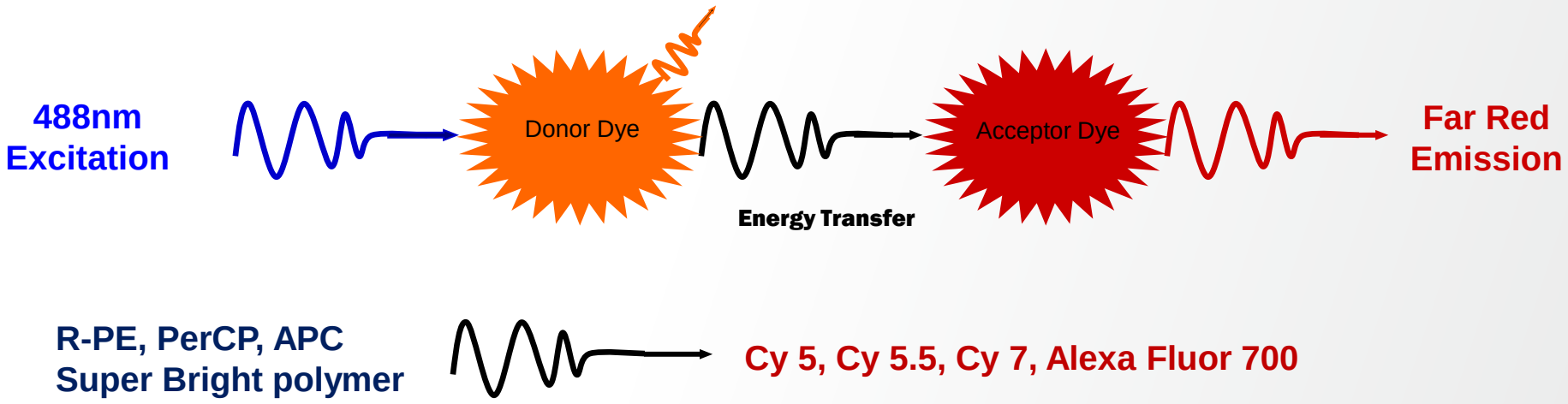


- **Non-ORGANIC**:
  - Semiconductor particles
  - Qdots



Tandem Dyes (Bi-Molecular Dyes)

Förster Resonance Energy Transfer (FRET)



Tandem dyes shift emission spectra to higher wavelengths.

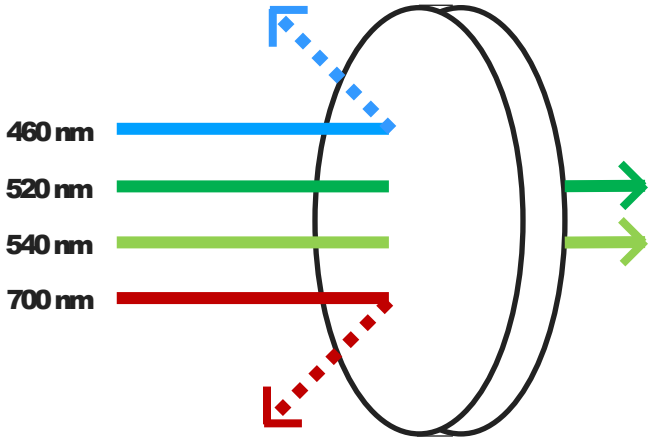
Examples:

PE-Cy7, PE-Alexa Fluor® 700, PerCP-Cy5.5, PerCP-eFluor710

APC-Cy7, APC-eFluor 780, Super Bright 600, 645, 702 and 780

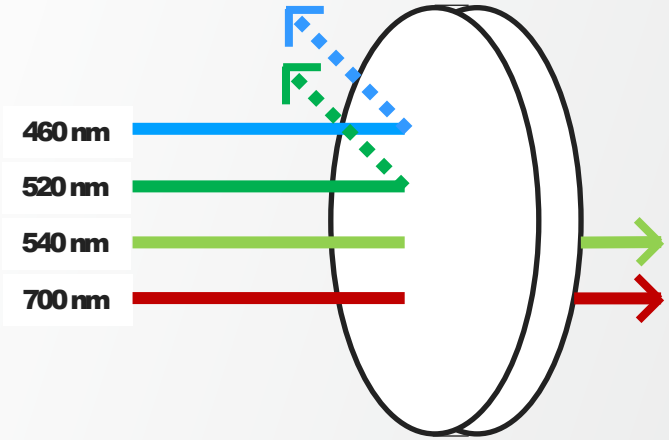
Collect Precise Range of the Emitted Light Wavelengths

Bandpass Filter



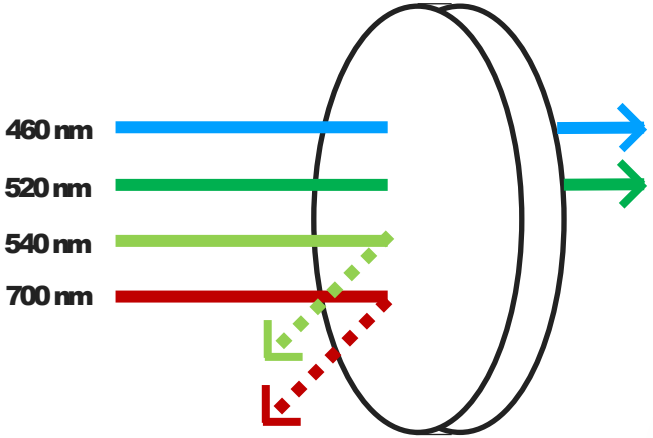
BP 530/30

Longpass Filter



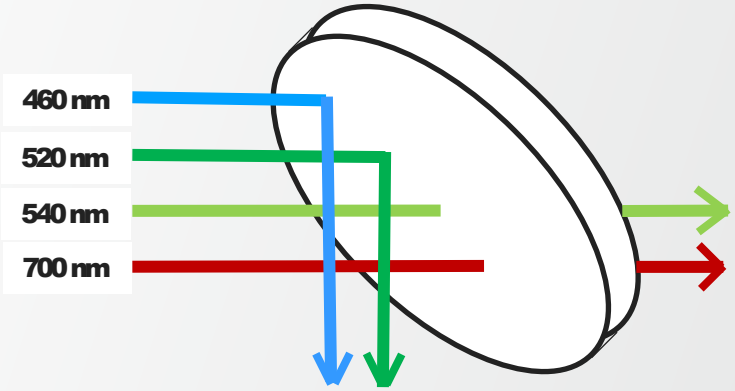
LP 530

Shortpass Filter



SP 530

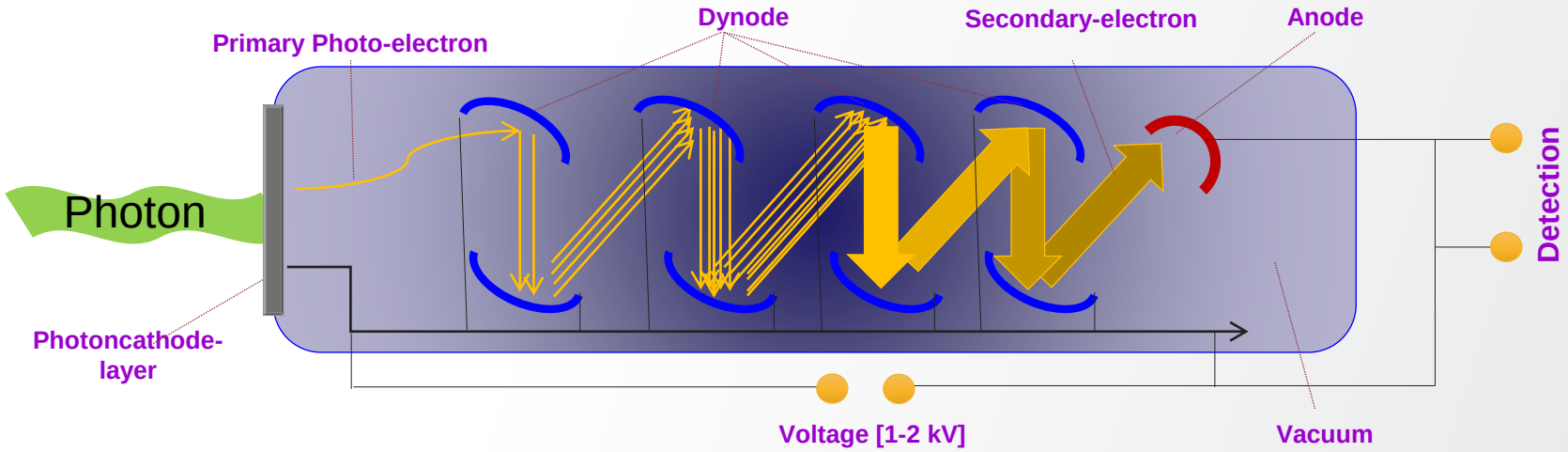
Dichroic Mirror



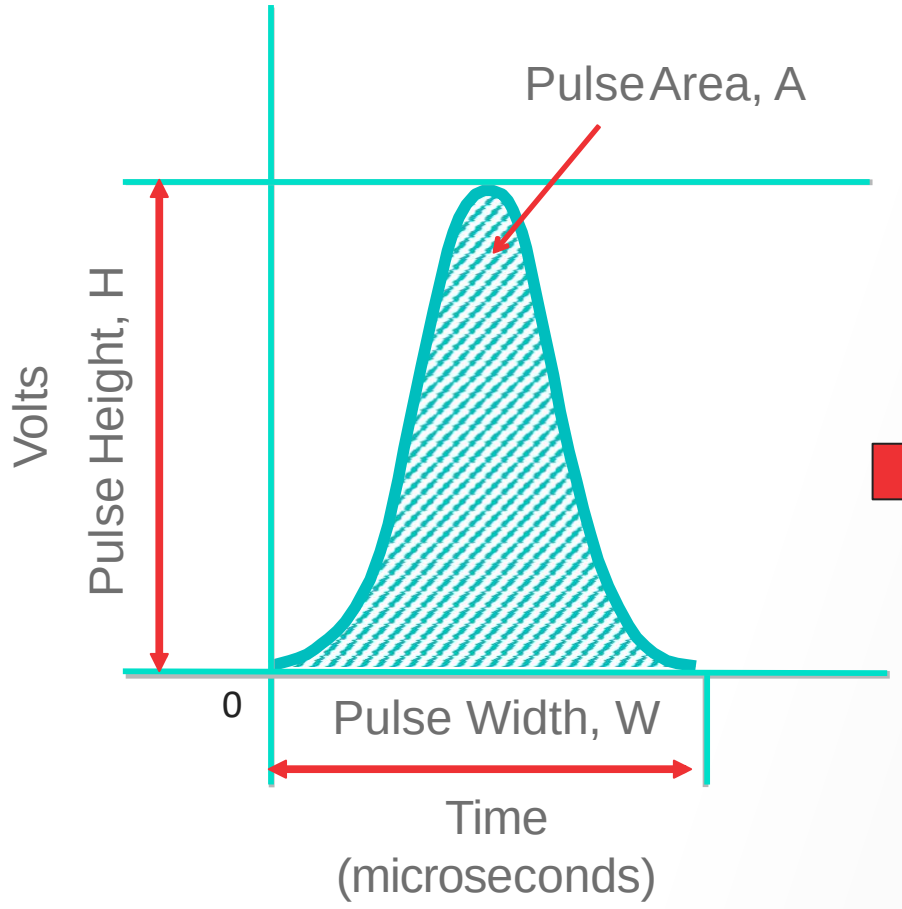
DLP 530

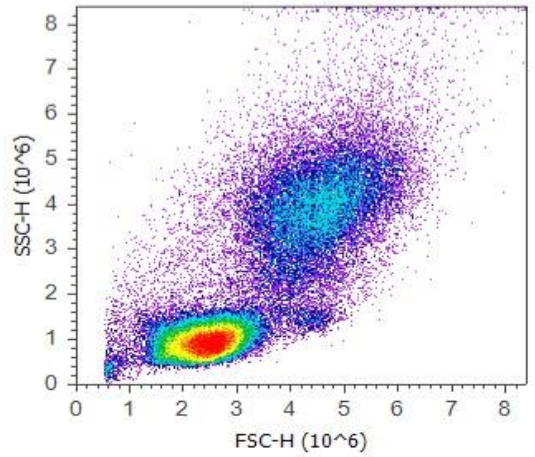
Detectors

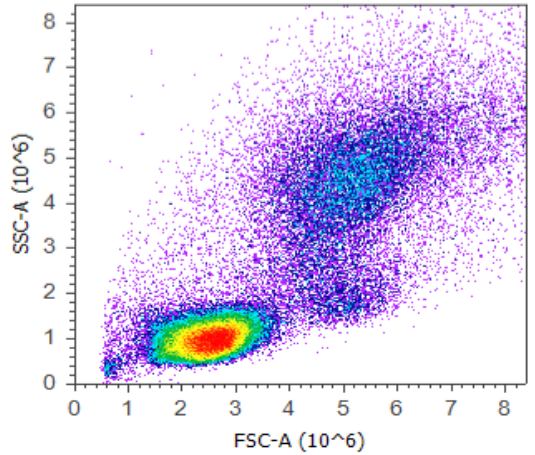
Photomultiplier tube (PMT):
PMT convert photons into electrons and amplify them to create a voltage pulse. Often referred as the “detector”



Sample Presentation: Voltage Pulse







Attune NxT Flow Cytometer

Flow Product line – Analyzer and Cell Sorter Highlight

Invitrogen™ Attune™ NxT Flow Cytometer

- **2014** Attune NxT Flow Cytometer introduced to the market
- **2016** Green laser introduced
- **2017** Invitrogen™ Attune™ violet 6-channel option released
- **2018** Integration of Thermo Scientific™ Orbitor™ RS2 Microplate Mover
- **2020** 21 CFR Part 11 compliance; Invitrogen™ CytKick™ Autosampler and CytKick™ Max Autosampler with Microsoft™ Windows™ 10 software compatibility released

Invitrogen™ Attune™ CytPix™ Flow Cytometer

- **2021** Introducing the Attune CytPix Flow Cytometer
- **2023 Q2** **Introducing Attune SW 6.0.1 enable AIA (Auto Image analysis)**

Invitrogen™ Bigfoot Spectral Cell Sorter

- **2022** **Introducing Bigfoot Spectral Cell Sorter into APJ**
- **2023** **New SKU 4L+ SPD activated**

The Attune NxT Flow Cytometer

Designed for efficiency, speed and accuracy

With acoustic-assisted hydrodynamic focusing, the Attune NxT Flow Cytometer avoids compromise between data quality and higher sample rates by uncoupling cell alignment from sheath flow. Acoustic-assisted hydrodynamic focusing precisely aligns cells using ultrasonic radiation pressure (>2 MHz) to transport particles into the center of the sample stream. This pre-focused stream is then injected into the sheath stream, resulting in a narrow particle stream and uniform laser illumination, regardless of the sample input rate

acquisition speed **35,000** events/sec	**1000 mL/min** max sample input flow rate
Max electronic speed **65,000** events/sec	**10x faster** than conventional cytometers

Benefits

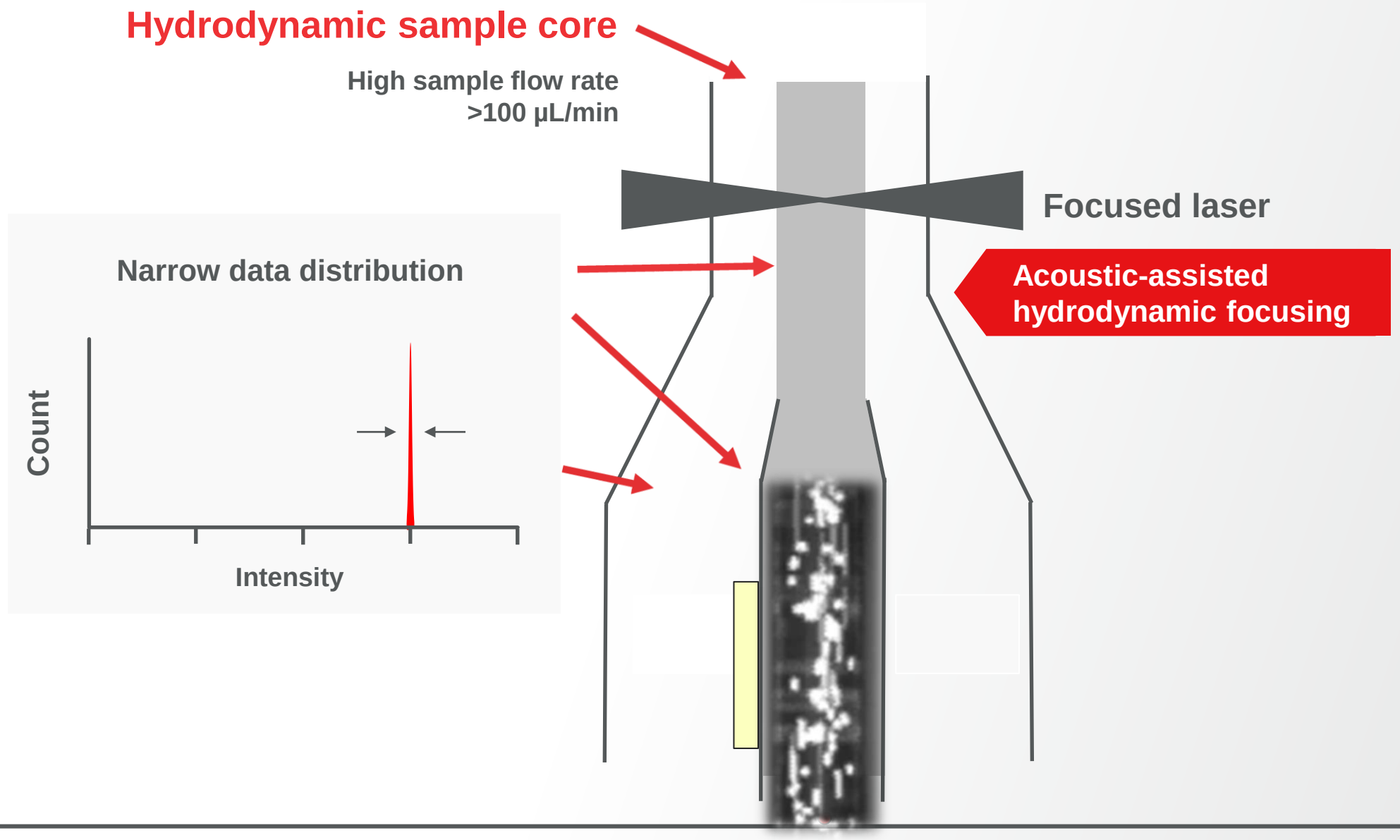
- Greater reproducibility and consistency in data
- Maintain consistent concentration results across all flow rates
- Process very dilute or concentrated samples while maintaining low coefficient of variations (CVs)

"The ability to run very dilute samples is quite amazing and might be a life saver on many occasions where you have little-to-no sample left."

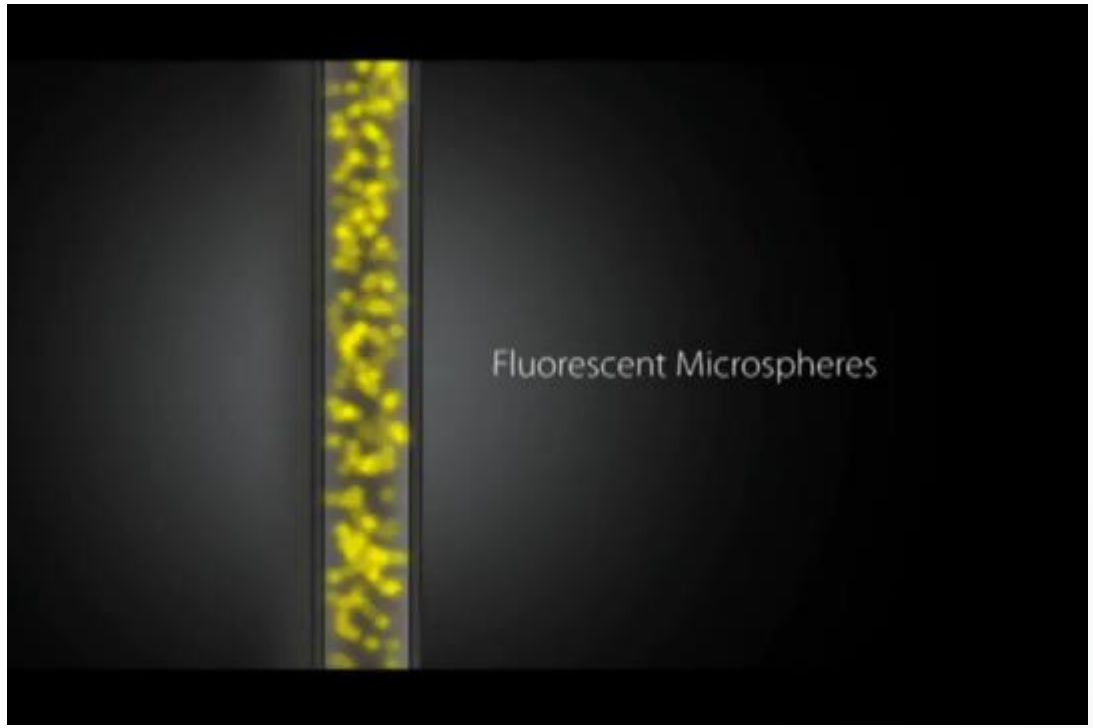
– J. P. Robinson, PhD
Purdue University

Speed and accuracy

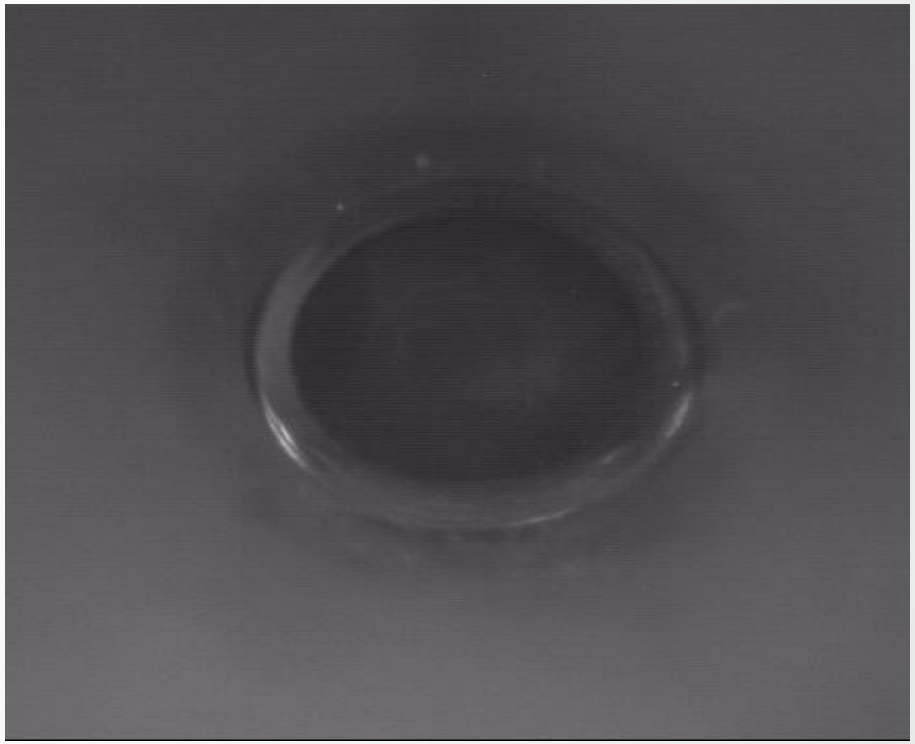
Acoustic Focusing and Hydrodynamic Focusing



Acoustic Focusing



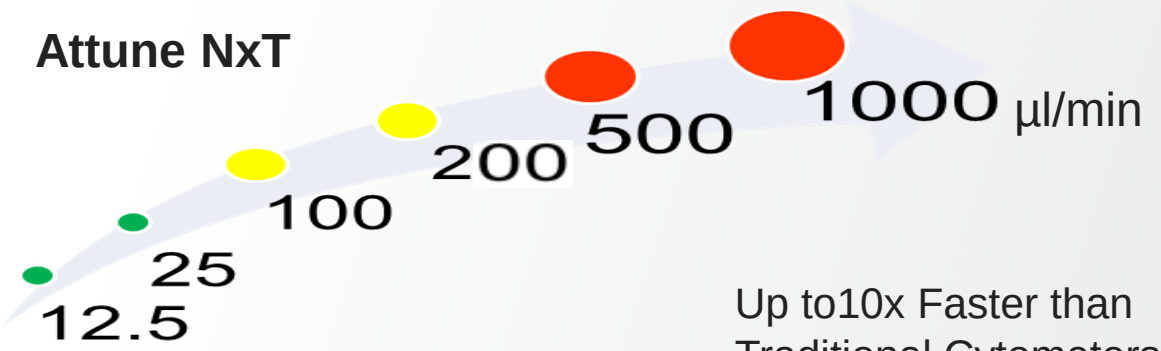
End-on view of capillary



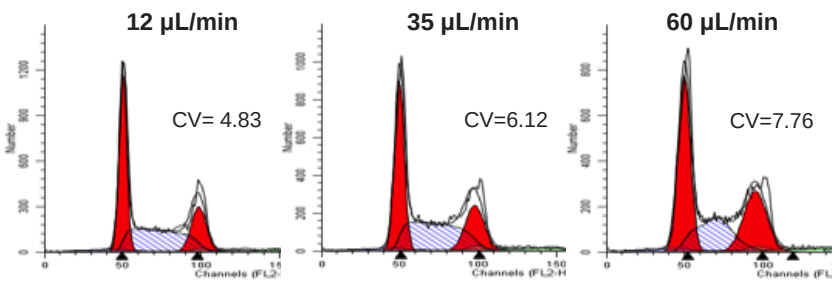
Comparable Results at All Flow Rates

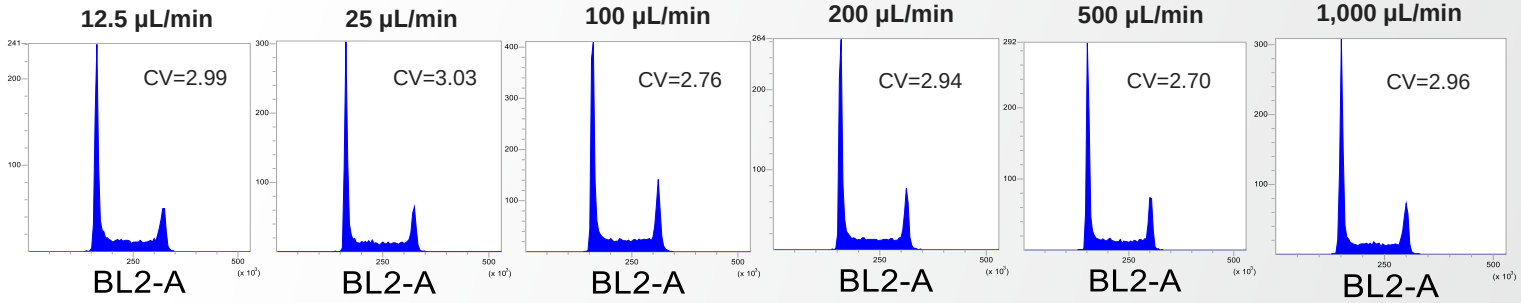
Traditional Cytometers

Attune NxT



Up to10x Faster than Traditional Cytometers





Hydrodynamic Focusing Only

Acoustically Enhanced Hydrodynamic Focusing

		Competitor A		Competitor B		Competitor C		Attune NxT	
	Cells	14 ul/min	66 ul/min	12 ul/min	120 ul/min	12 ul/min	60 ul/min	500 ul/min	1000 ul/min
Seconds	10,000	42.8	9	50	5	50	10	1.2	0.6
Minutes	100,000	7.1	1.5	8.3	0.8	8.3	1.7	0.2	0.1
Minutes	1,000,000	71.3	15.0	83.3	8.3	83.3	16.7	2.0	1.0
Hours	10,000,000	11.9	2.5	13.9	1.4	13.9	2.8	0.3	0.2

The Attune NxT Flow Cytometer

Transformative, clog-resistant system

Why Customer Care:

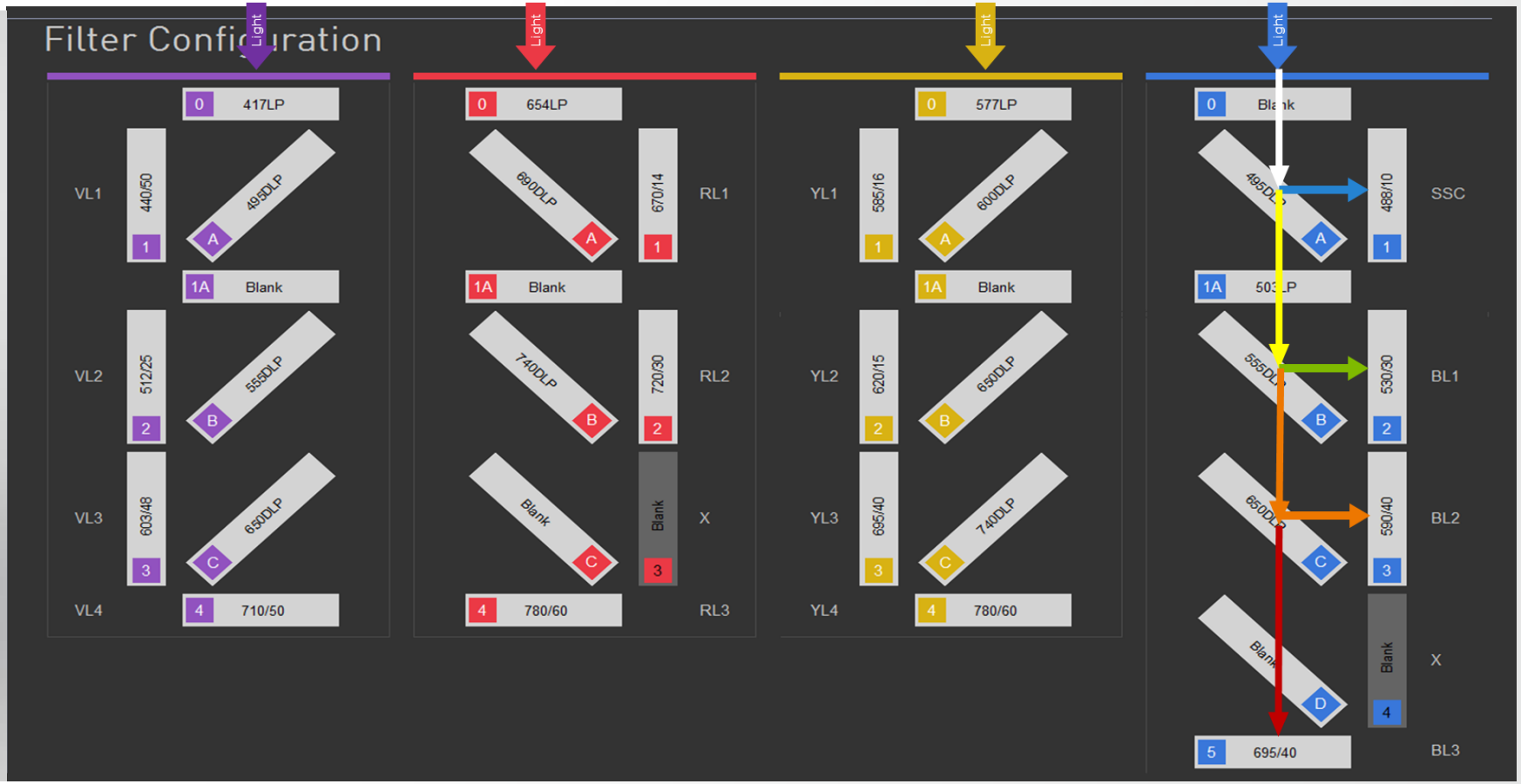
- Clogging number 1 reported issue
- Protect precious samples
- Difficult Sample Types
  - Processed tissue samples, solid tumor samples, adherent cells, HeLa cells, brain tissue, cardiomyocytes, milk, others
- Aggregation tends to occur as cell concentration increases
  - No need to concentrate cells with the Attune NxT, just dilute and run faster!

With the acoustic focusing and sample uptake technology, the Attune rarely clogs or encounters other common fluidic problems as seen with other cytometers that require sheath pressure. During its first year of operation with multiple users we only once required servicing for a problem that was readily solved."

Laurie Kennedy
Flow Core Manager, University of Calgary

Protect Precious Samples – Analyze Complex Cells

Optical Filters



Attune NxT accessory filter kits available

- Violet Side Scatter Kit- Small Particles
- Fluorescent Protein Filter Kit- Transfection
- No-wash, No-lyse Filter Kit- Blood

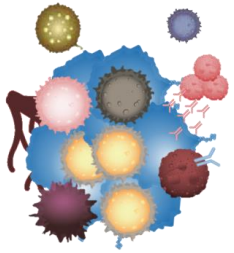
Choosing Which Channel to Use (BRVY)

Excitation Laser	Emission Filter (nm)	Channel	Common Fluorophores	Fluorescent Proteins
Violet-405 nm	440/50	VL1	SB436, BV421, Pacific Blue, eFluor 450, Alexa Fluor 405	Azurite, Cerulean, eBFP, eCFP, mTurquoise, Sirius
	512/25	VL2	Pacific Green, Qdot 525	T-Sapphire, CFP, vGFP
	603/48	VL3	SB600, BV605, Pacific Orange, Qdot 605	
	710/50	VL4	SB702, Qdot 705	
Blue-488 nm	530/30	BL1	Alexa Fluor 488, FITC, GFP, SYTOX Green	eGFP, Emerald, eYFP
	590/40	BL2	PE, PE-Alexa Fluor 610, PE-Texas Red, Qdot 585	
	695/40	BL3	PE-Alexa Fluor 700, PE-Cy5.5, PerCP, PerCP-Cy5.5, Qdot 705	
Yellow-561 nm	585/16	YL1	Alexa Fluor 568, PE, Qdot 565, PI, SYTOX Orange	mOrange, RFP, dTomato
	620/15	YL2	PE-Alexa Fluor 610, PE-Texas Red	mCherry, DsRed, mKate, mStrawberry
	695/40	YL3	PE-Alexa Fluor 700, PE-Cy5.5, Qdot 705	
	780/60	YL4	PE-Alexa Fluor 750, PE-Cy7, Qdot 800	
Red-637 nm	670/14	RL1	APC, Alexa Fluor 647, SYTOX Red, Qdot 655	
	720/30	RL2	Alexa 700, APC-Alexa Fluor 700, Qdot 705	
	780/60	RL3	APC-Cy7, APC-Alexa Fluor 750, Qdot 800	

Panel Builder & Experiment Considerations

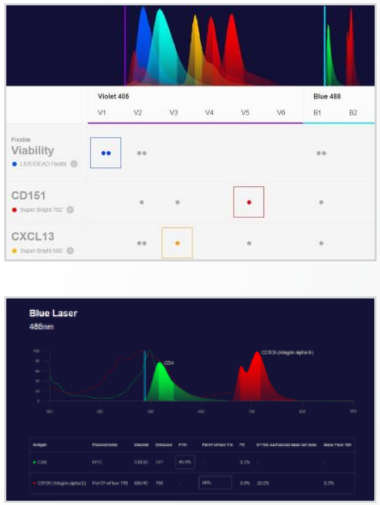
Multicolor Analysis Workflow

1 Target determination

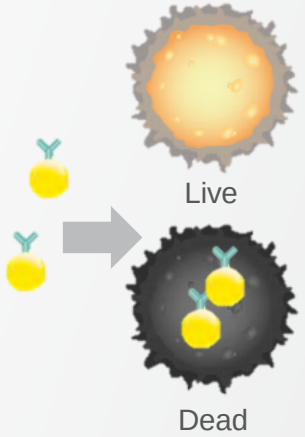


2 Buffer selection

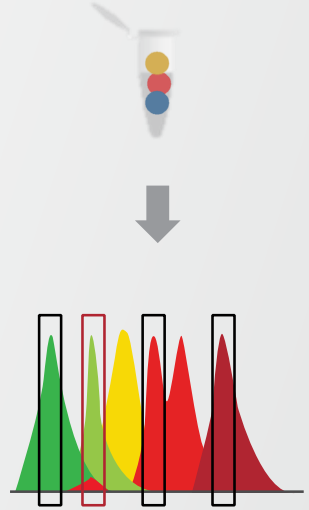
3 Panel design



4 Biological controls

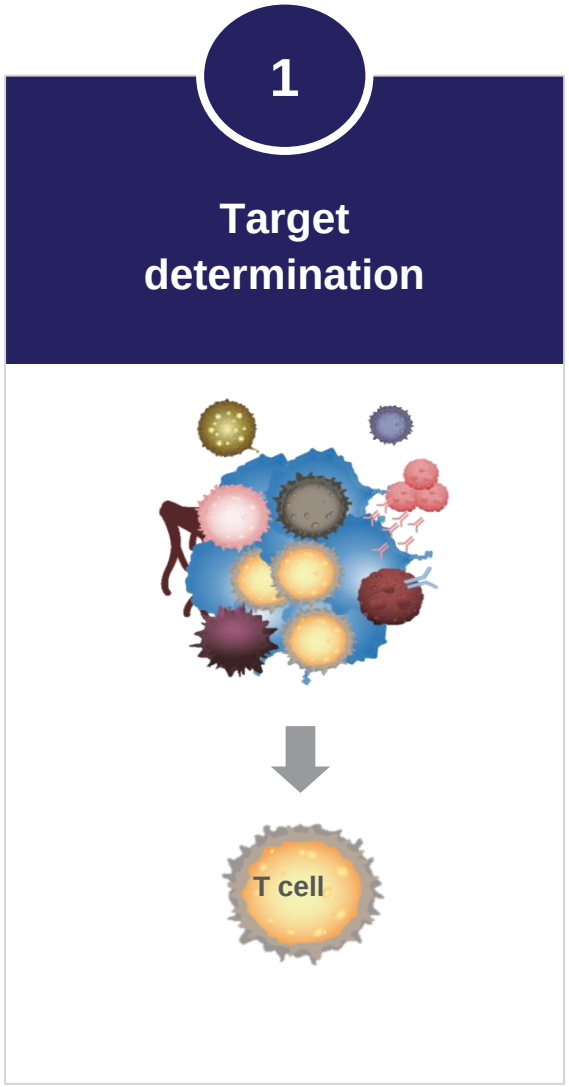


5 Experimental controls



thermofisher.com/flowpanel for Invitrogen™ Flow Cytometry Panel Builder
thermofisher.com/5stepsicfc for additional resources

Step 1: Target Determination

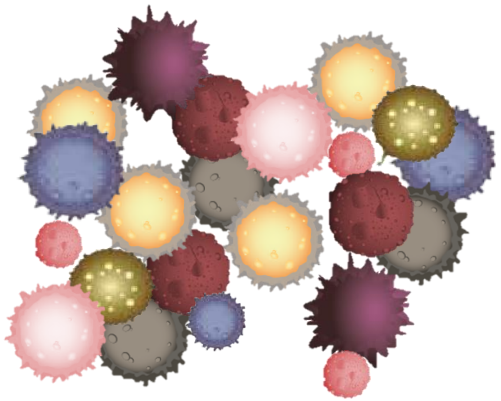


- Choose which markers will best answer your research questions
- Which antibodies are needed for markers of interest
- Example T cell panel that includes:
  - Phenotyping markers
  - Cytokines
  - Signaling markers

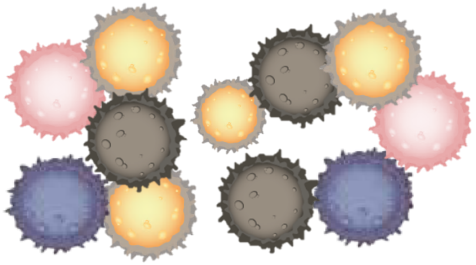
Step 1: Target Determination

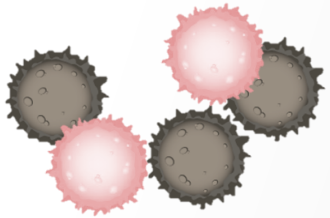
Phenotyping Markers

T cell identification	T cell subsets	Naive vs. memory T cells

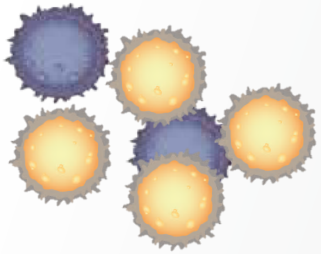


CD3

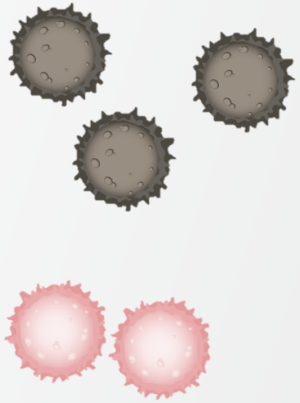




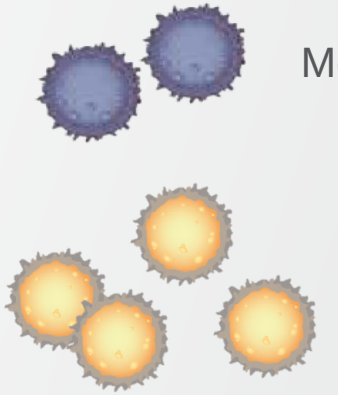
CD8
Cytotoxic T cells



CD4
T helper cells



CD45RA
Naive T cells

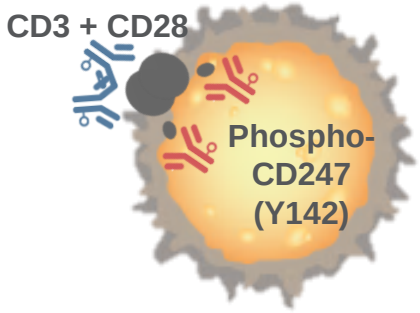


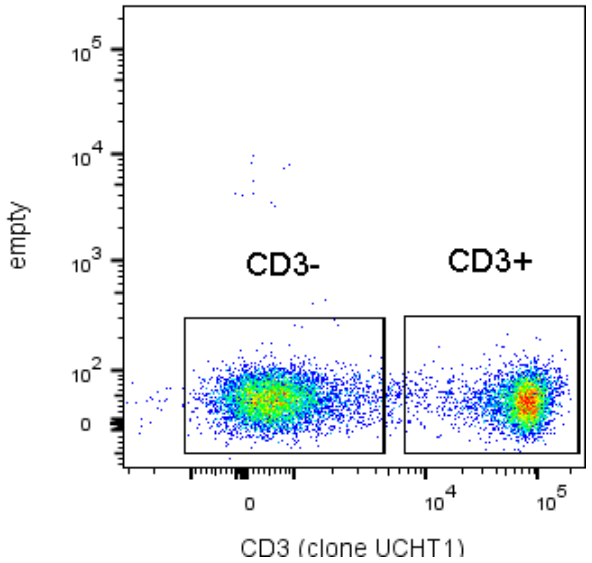
CCR7
Memory T cells

Step 1: Target Determination

Signaling Marker

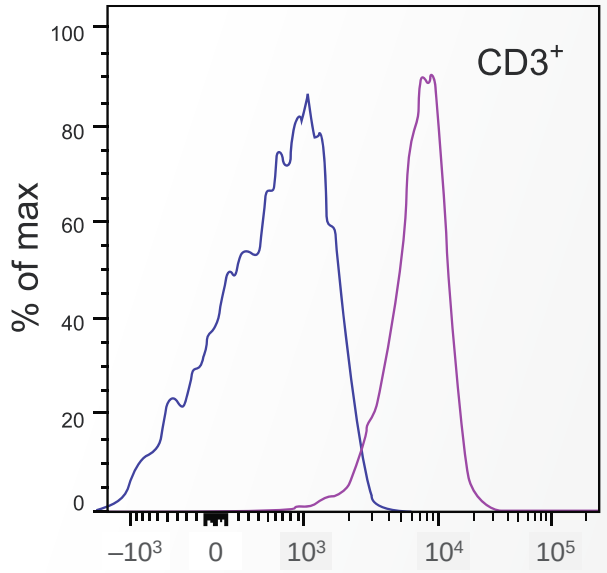
CD3 and CD28 Abs for TCR engagement

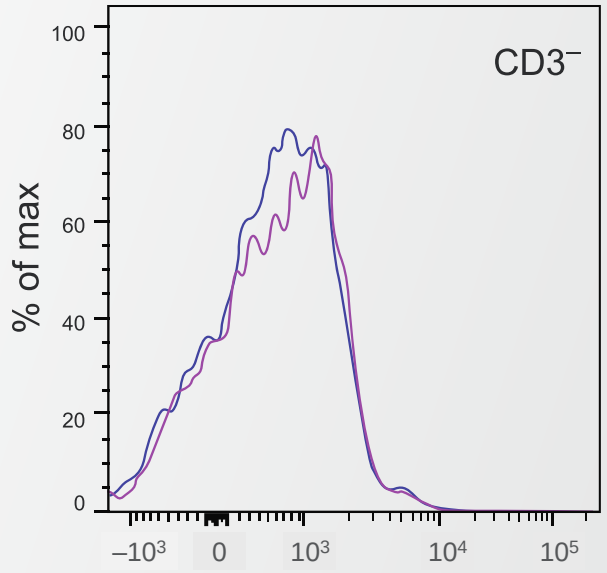




Staining of phospho-CD247 (Y142) following stimulation with functional anti-CD3 and anti-CD28 antibodies

No staining observed in unstimulated or CD3⁻ cells





Unstimulated

TCR stimulation

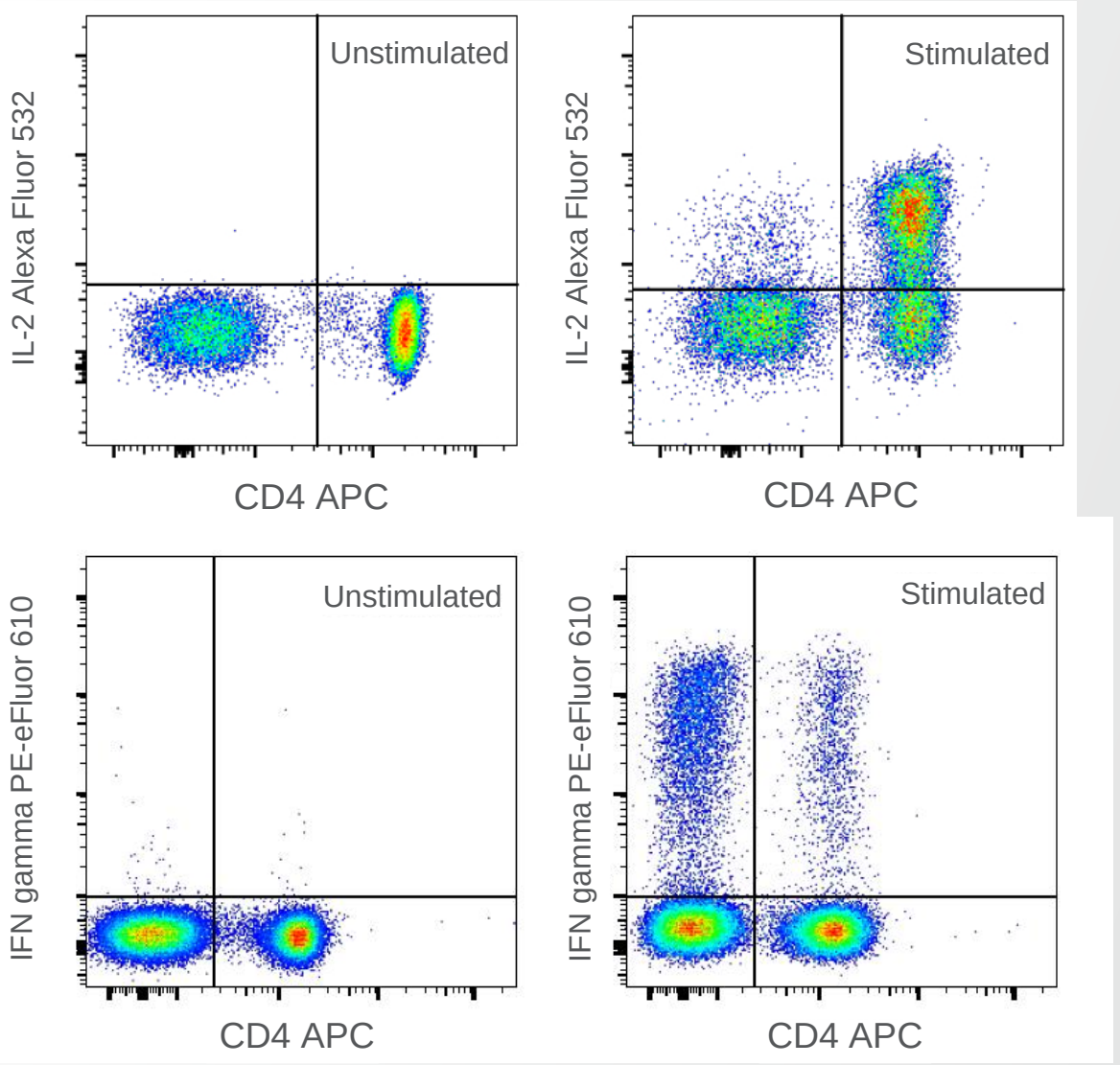
Step 1: Target Determination

Effector Function

Examples of cytokine staining on stimulated cells

IL-2: essential for proliferation and function of activated T cells

IFN gamma: anti-viral, anti-tumor cytokine with a wide variety of immune effects



Step 1: Target Determination

Optimized Multicolor Immunofluorescence Panels (OMIPs)

- Optimized Multicolor Immunophenotyping Panels (OMIPs) are an additional resource for target selection
- Validated panels for various cell types with gating strategies, clones, and fluorochromes

Home › Life Sciences › Cell Analysis › Flow Cytometry › Flow Cytometry Learning Center › Flow Cytometry Resource Library › Optimized Flow Cytometry Multiplex Panels

Optimized Flow Cytometry Multiplex Panels

‹ Flow Cytometry Resource Library

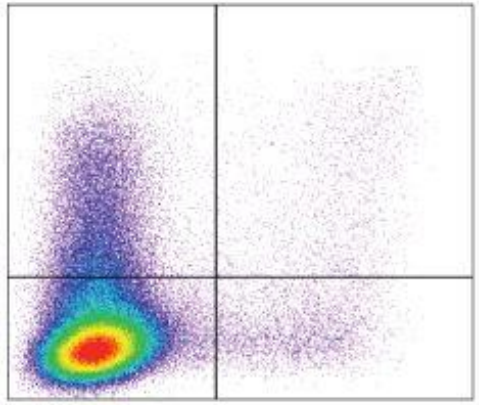
Flow Cytometry Application Notes

Flow Cytometry Research Tools

Flow Cytometry Educational Videos & Webinars

Optimized Flow Cytometry Multiplex Panels

Flow Cytometry Guided Learning



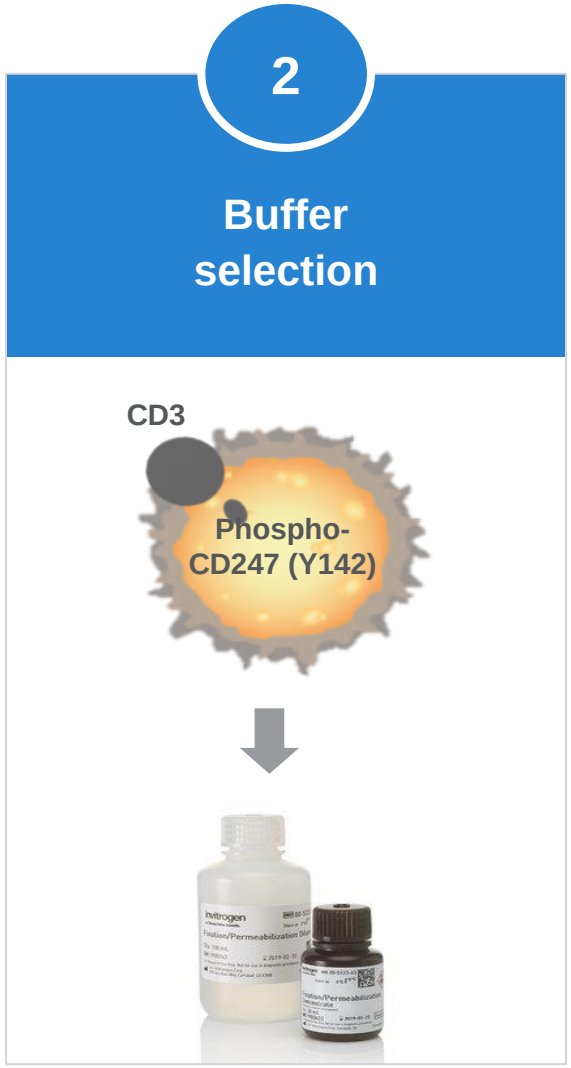
Validated antibody panels for flow cytometry

Coined in 2010, the term "optimized multicolor immunofluorescence panel", or OMIP, refers to a thoroughly tested and validated set of antibodies and reagents that can be used together for the multicolor characterization of a specific cell state or response. Published in the journal *Cytometry Part A* (Wiley Online Library), these OMIPs are designed for flow cytometry, but OMIPs may potentially be defined for image cytometry, fluorescence microscopy, and other polychromatic fluorescence–based methods.

Learn more about Optimized Multicolor Immunofluorescence Panels (OMIPS) in BioProbes 74.

More targets are not necessarily better!

Step 2: Buffer Selection



Choose the appropriate buffers for sample prep and staining

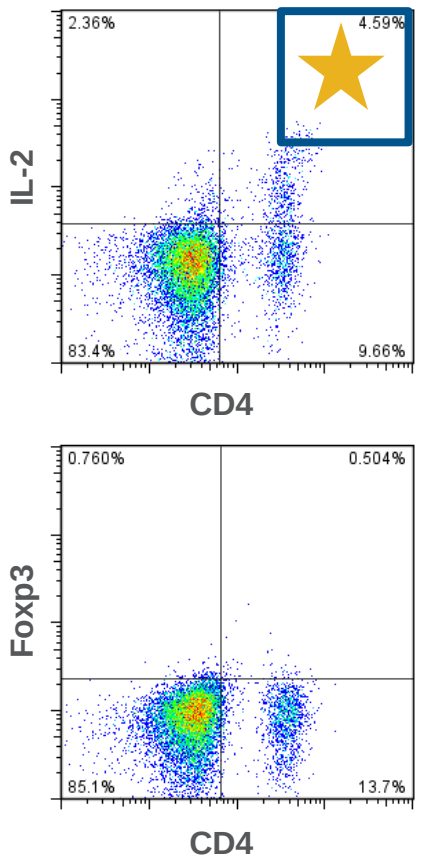
Intracellular staining always requires fixation and permeabilization of cells

Various options are available, so selecting appropriate option is critical for optimal staining

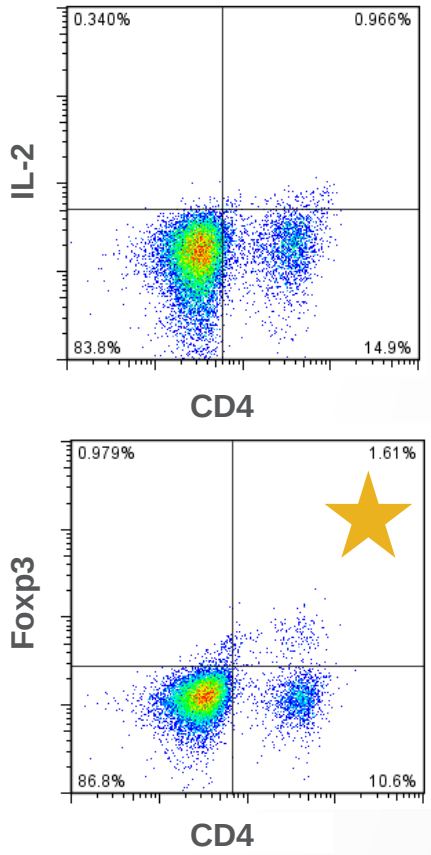
Step 2: Buffer Selection

Optimal Protein Detection

Intracellular Fixation & Permeabilization Set



Transcription Factor Staining Buffer Set



IL-2 staining (cytoplasmic target)

- Optimal with Invitrogen™ eBioscience™ Intracellular (IC) Fixation and Permeabilization Set

Foxp3 staining (nuclear target)

- Optimal with Invitrogen™ eBioscience™ Foxp3 Transcription Factor Staining Buffer Set

If targeting cytoplasmic IC proteins, first option is most appropriate

Some targets are compatible in both buffers

Step 2: Buffer Selection

Why Is Buffer Important?

- The fixation / permeabilization kit allows for access to intracellular targets without damaging epitopes of interest

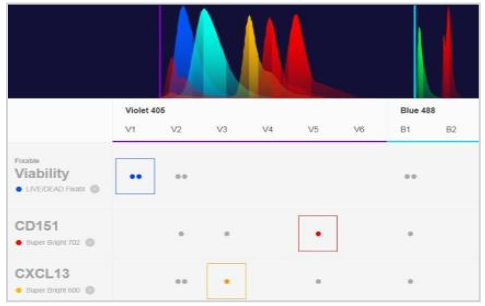
- General guidelines:
  - Invitrogen™ eBioscience™ Intracellular (IC) Fixation and Permeabilization Set will not allow access to nuclear targets
  - Invitrogen™ eBioscience™ Foxp3 Transcription Factor Staining Buffer Set may not work with some cytoplasmic targets
  - Some phospho-flow targets will require methanol permeabilization (eg. pSTATs)
  - Check product data pages or other online resources

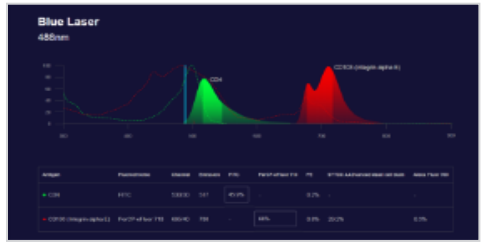
- What if you have targets of interest with different buffer requirements?
  - **Divide experiment into two panels**

Step 3: Panel Design

3

Panel design





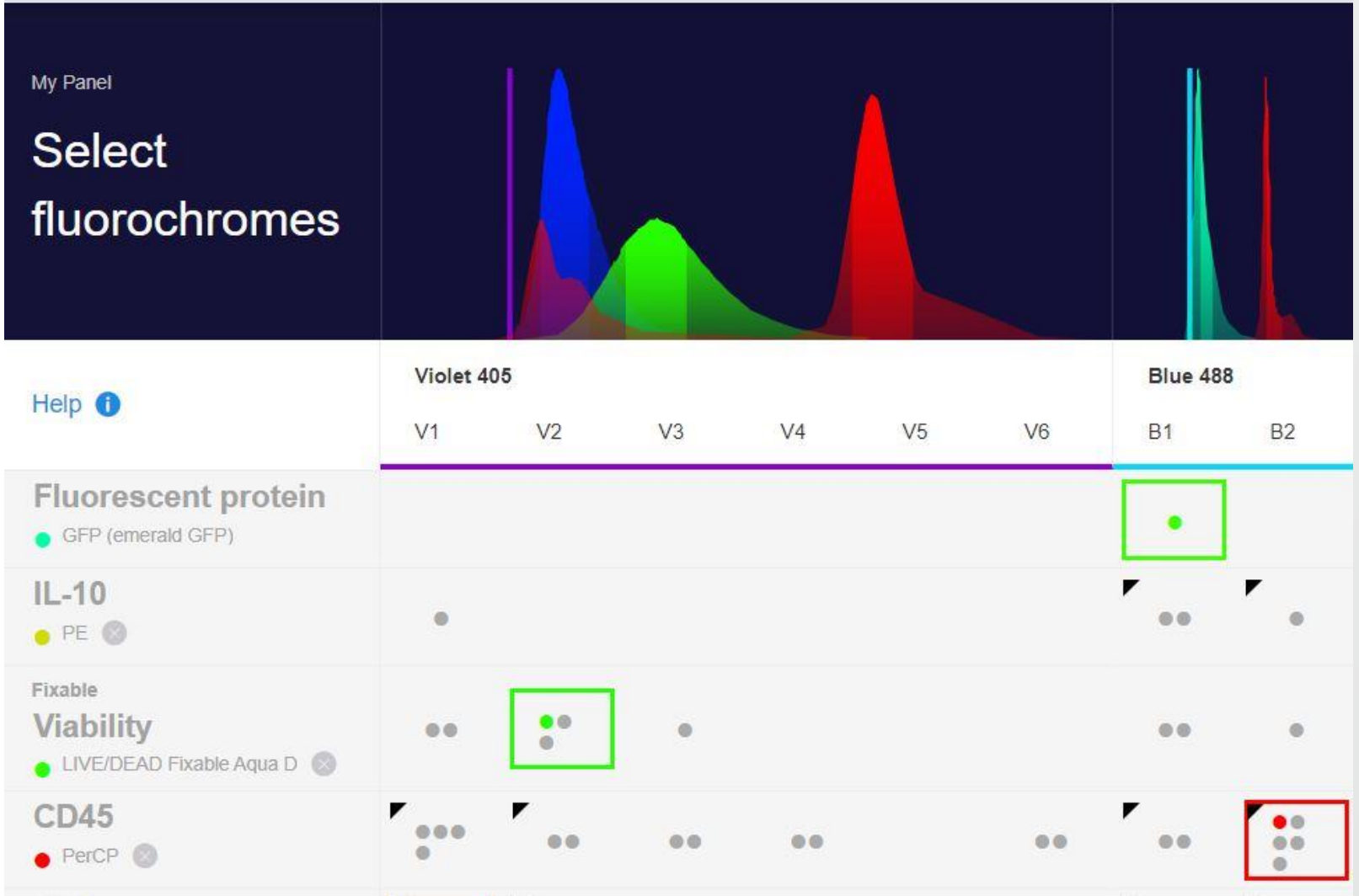
Understand differences in fluorochrome performance

Understand the biology of your targets of interest, such as expression levels, co-expression, and gating strategy

Use online resources to help build your panel

Building Your Panel—Using the Panel Builder

- A quick and easy-to-use web tool
- Allows incorporation of antibodies using your flow cytometer's configuration
- Fluorochrome selection built on spectral visualization of all fluorochromes per laser

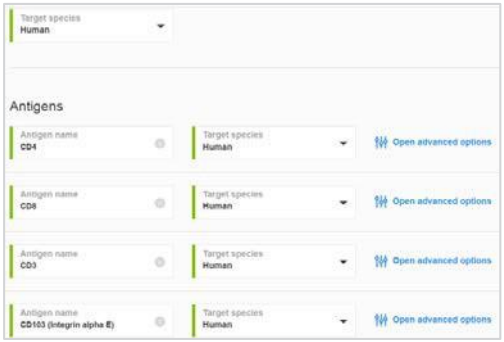


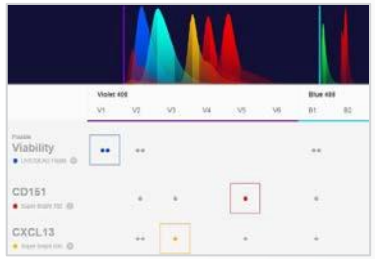
thermofisher.com/flowpanel

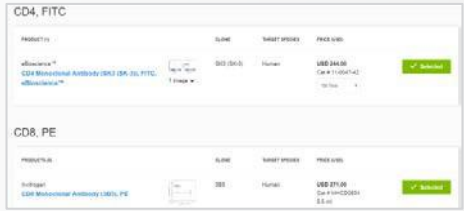
Flow Cytometry Panel Builder

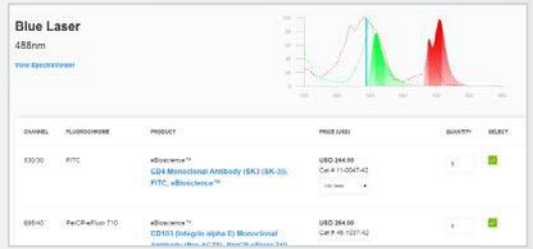
Simplified panel design with a 5-step panel design strategy

Step 1: Cytometer | **Step 2:** Antigens | **Step 3:** Fluorophores | **Step 4:** Products | **Step 5:** Summary









Find out more at: **thermofisher.com/flowpanel**

For Research Use Only. Not for use in diagnostic procedures. Not for resale.

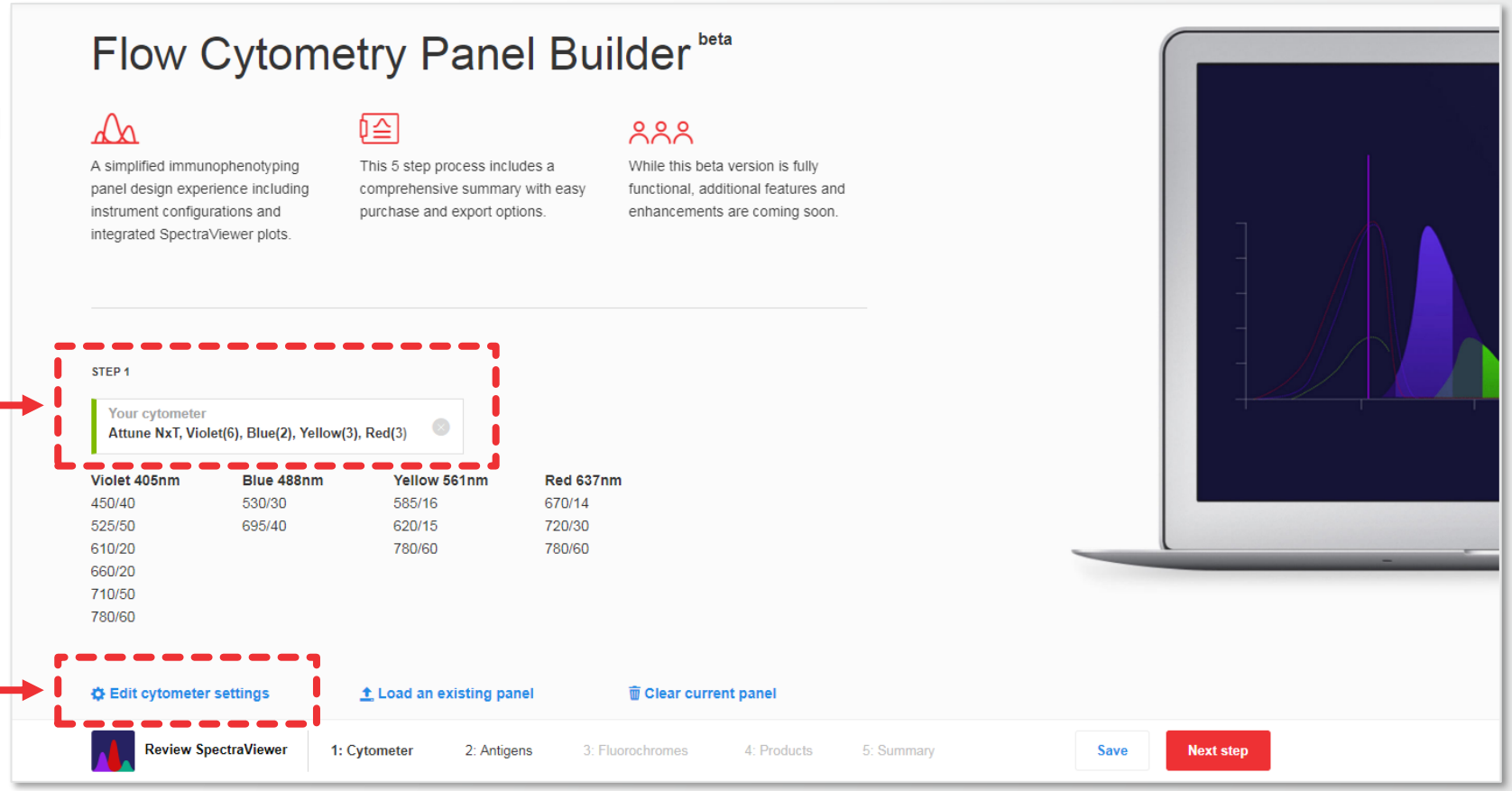
Building Your Panel

Selecting Your Cytometer

- Extensive list of instruments
- Edit instrument settings or build from scratch
- Remembers settings

Select your cytometer from the drop-down list

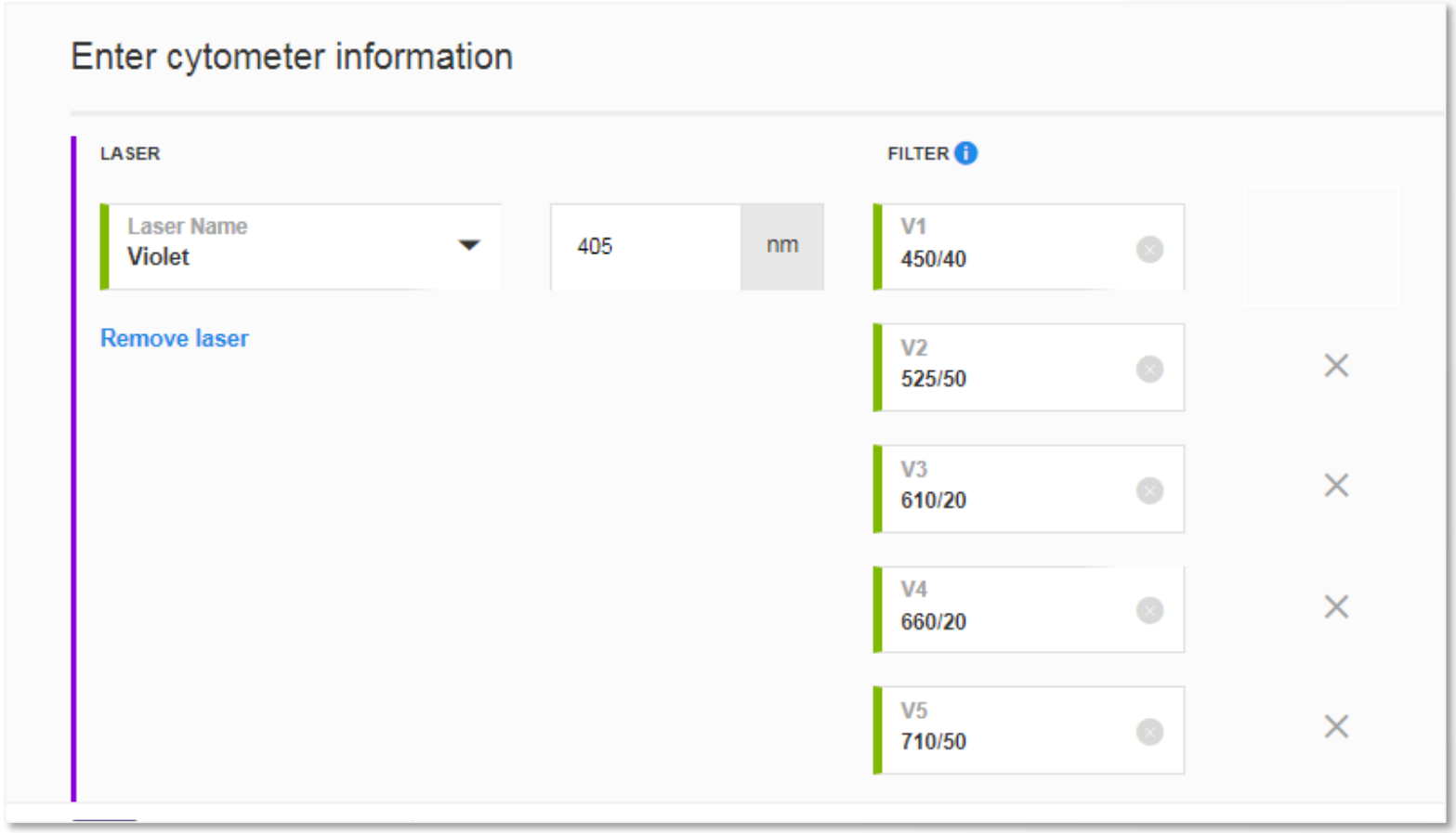
Cytometer settings can be customized after selection



This first step lets the panel builder know what fluorochromes your flow cytometer can detect.

Building Your Panel

Modify Instrument Settings



Manually specify:

- Lasers
- Fluorochrome/channel names
- Bandpass filters

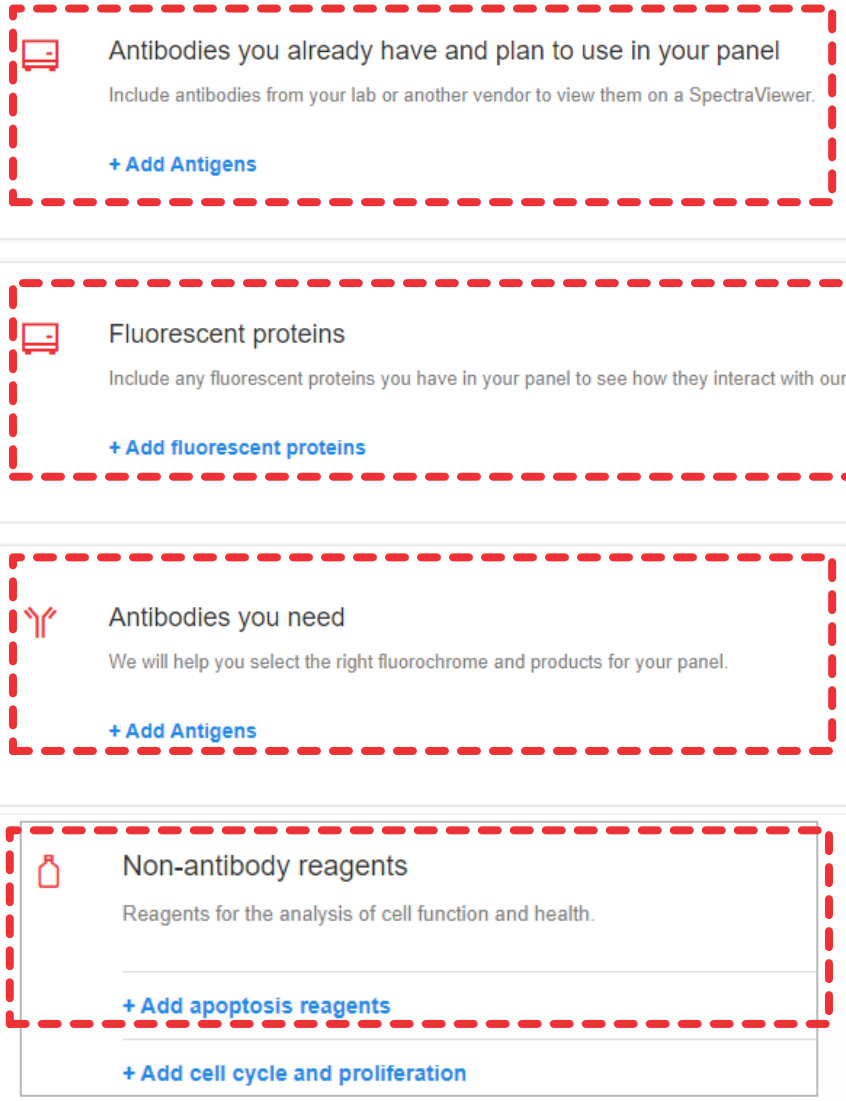
Editing cytometer from the list and manual editing follow similar designs

Configuration parameters on the cytometers can be saved

Ensure that your filter settings match those of your cytometer so that you can detect your chosen fluorophores

Building Your Panel

Selecting Your Antibodies and Reagents



Placeholder for antibodies already in the fridge

Fluorescent proteins

- GFP and other FPs occupy channels and spectrums

New antibody for panel

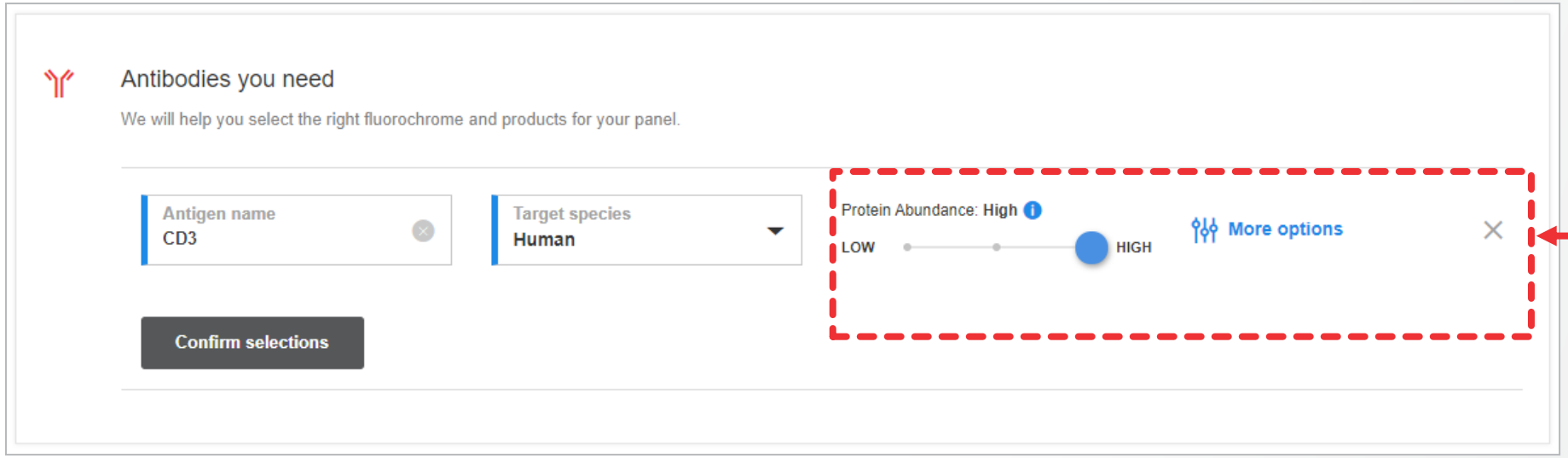
- Clone
- Species
- Dump channel

Non-antibody reagents

- Viability
- Proteins
- Cell cycle
- Others

Building Your Panel

Slider for Protein Abundance



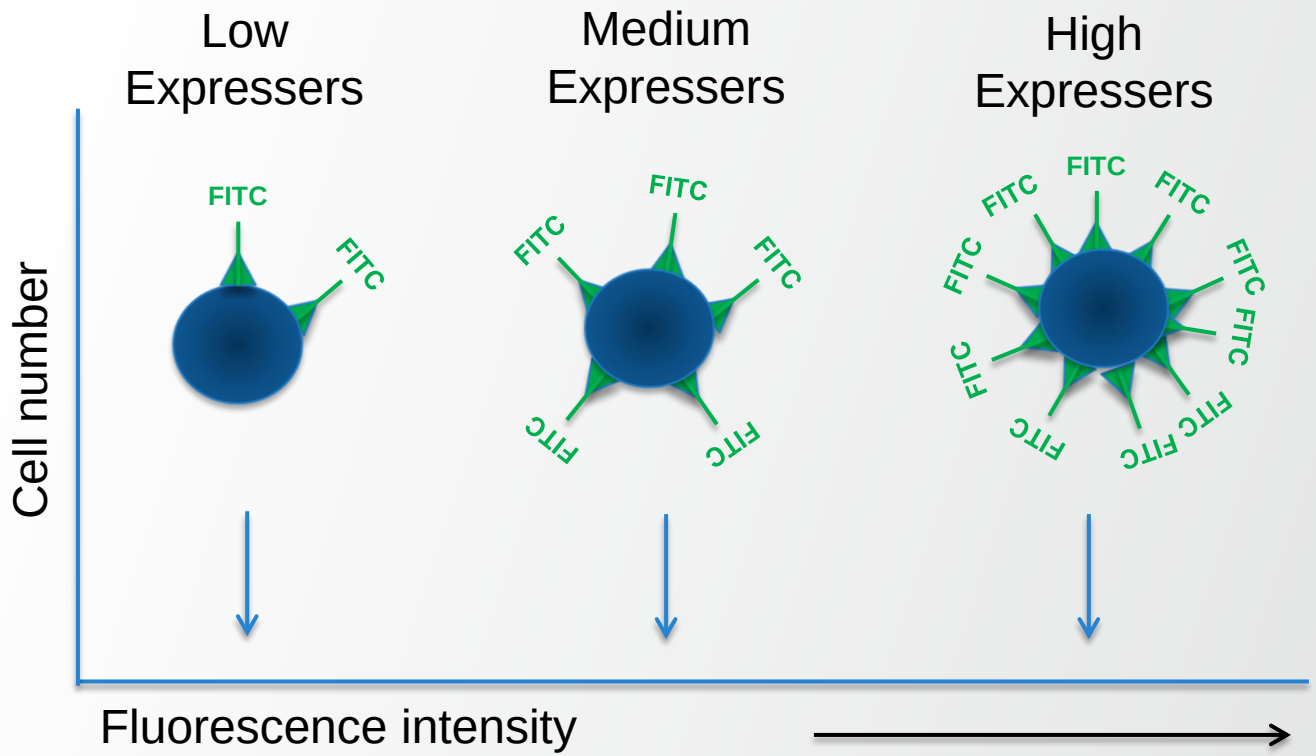
Keep on medium if you do not know the density

Antigen density along with fluorochrome brightness are used to recommend fluorochromes

Building Your Panel

Antigen Expression Levels: High

- Markers with **High** expression
  - Well-characterized markers
  - Identify broad subsets of cells
  - Expression is usually positive or negative
  - Often used as parent gate
- Assign these to the “dimmer” fluorochromes, or those most affected by spillover

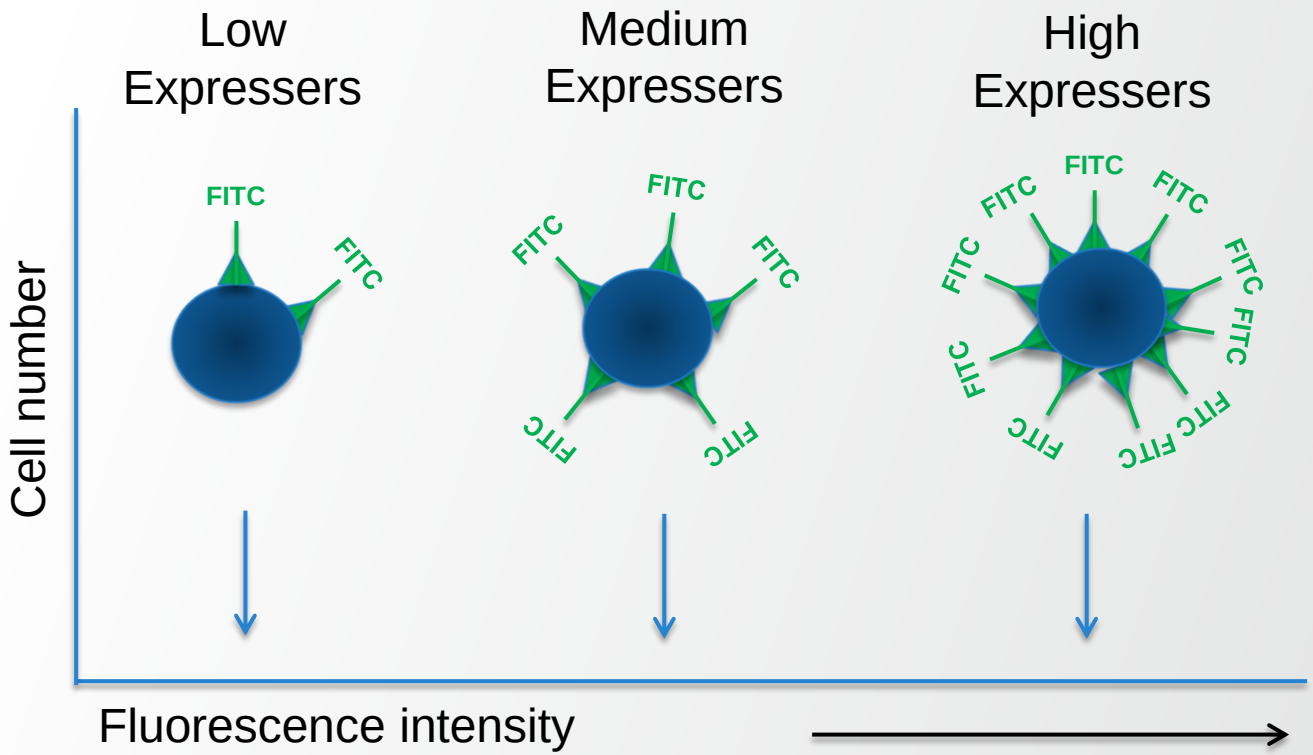


Building Your Panel

Antigen Expression Levels: Medium

- Markers with **Medium** expression
  - Well-characterized markers
  - Defined expression patterns
  - Expression may be on a continuum

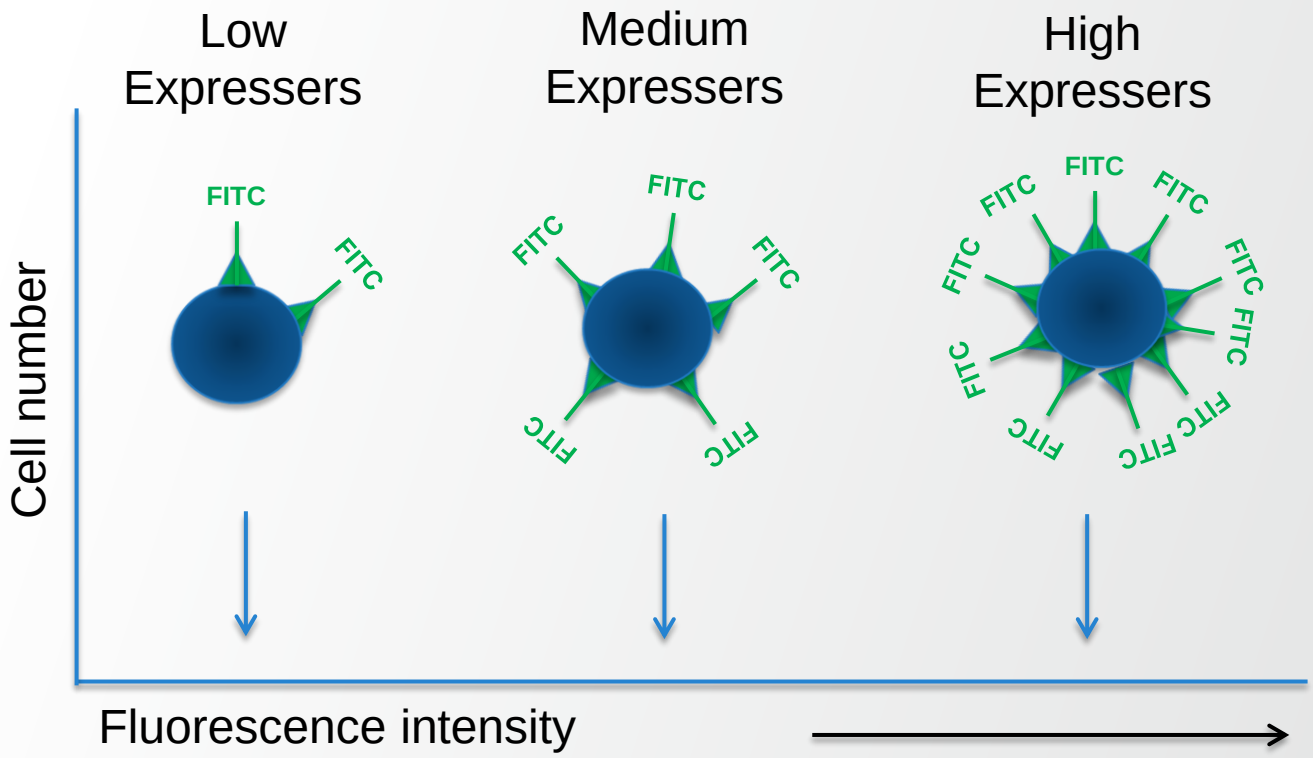
- Assign these to the next tier of fluorochromes
  - Intermediate brightness
  - Few spillover problems



Building Your Panel

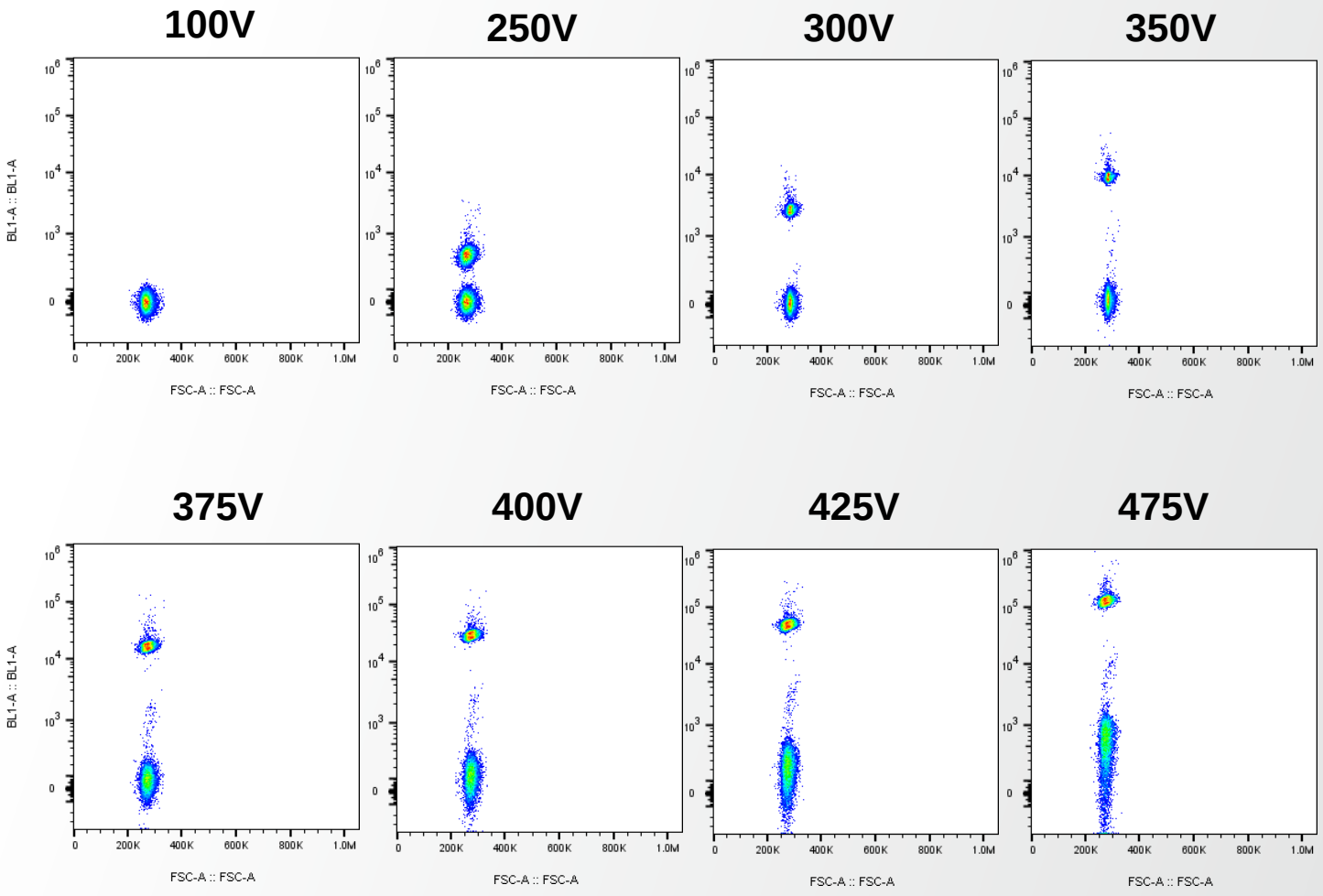
Antigen Expression Levels: Low or Undefined

- Markers with **Low or Undefined** expression
  - There are often fewer color choices for these
  - Often the “critical target” in the panel
- Save your brightest fluorochromes for these!
  - Minimize spillover from other channels
- **Intracellular antigen** expression
  - Staining tends to be dimmer
  - Populations may be less discrete
- Expression levels may vary by donor or activation



Voltage Optimization

- Also known as voltage walk or voltration
- Why do it?
  - Assures the best sensitivity, resolution, and dynamic range of each PMT detector (and fluorophore)
  - Assures linearity
  - If less than optimal, the resolution of dim populations can be lost
  - More than the optimal voltage gives no advantage to resolution
- When to do it:
  - New machine
  - New filter
  - New alignment
  - New PMT or a Laser
  - New dye (possibly)



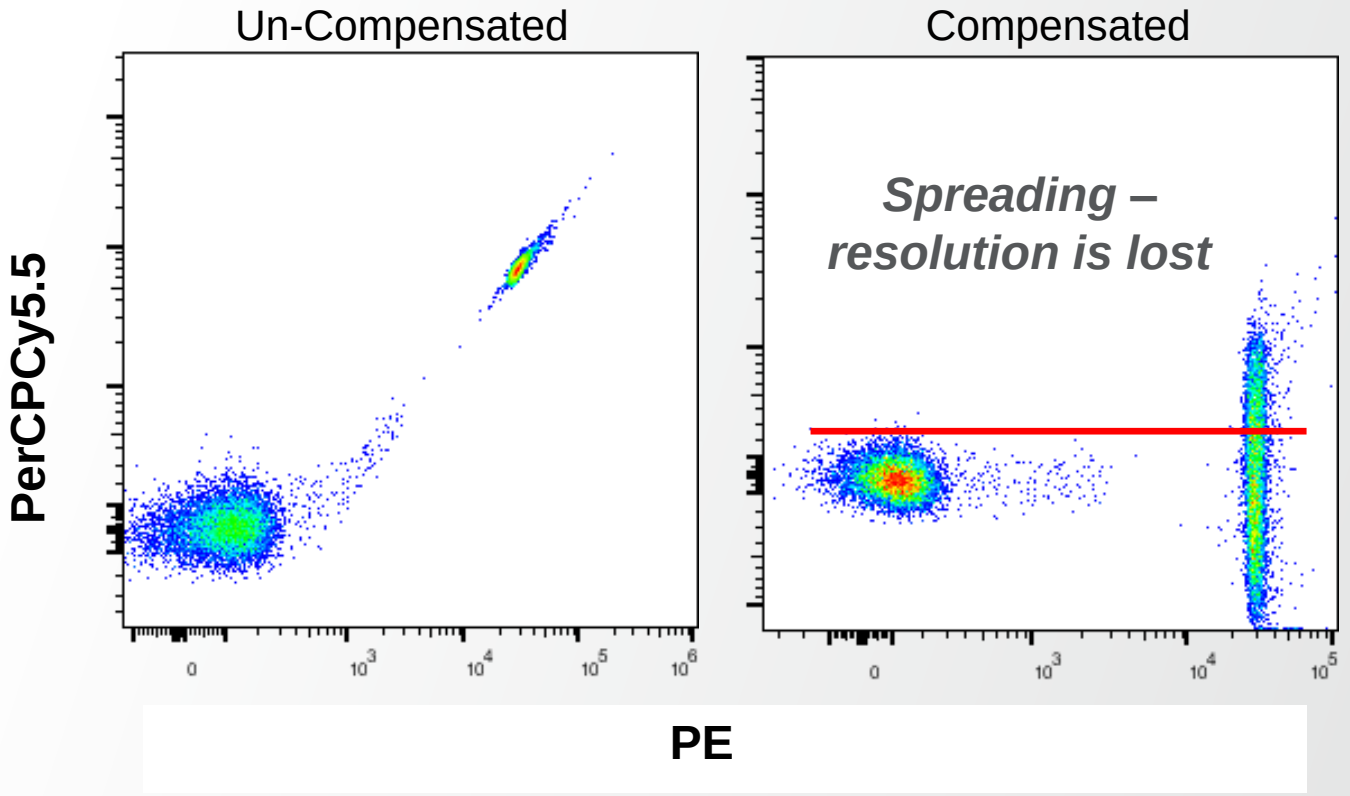
Increasing voltages until there is no increase in resolution

Spreading Error

Brighter is Not Always Better

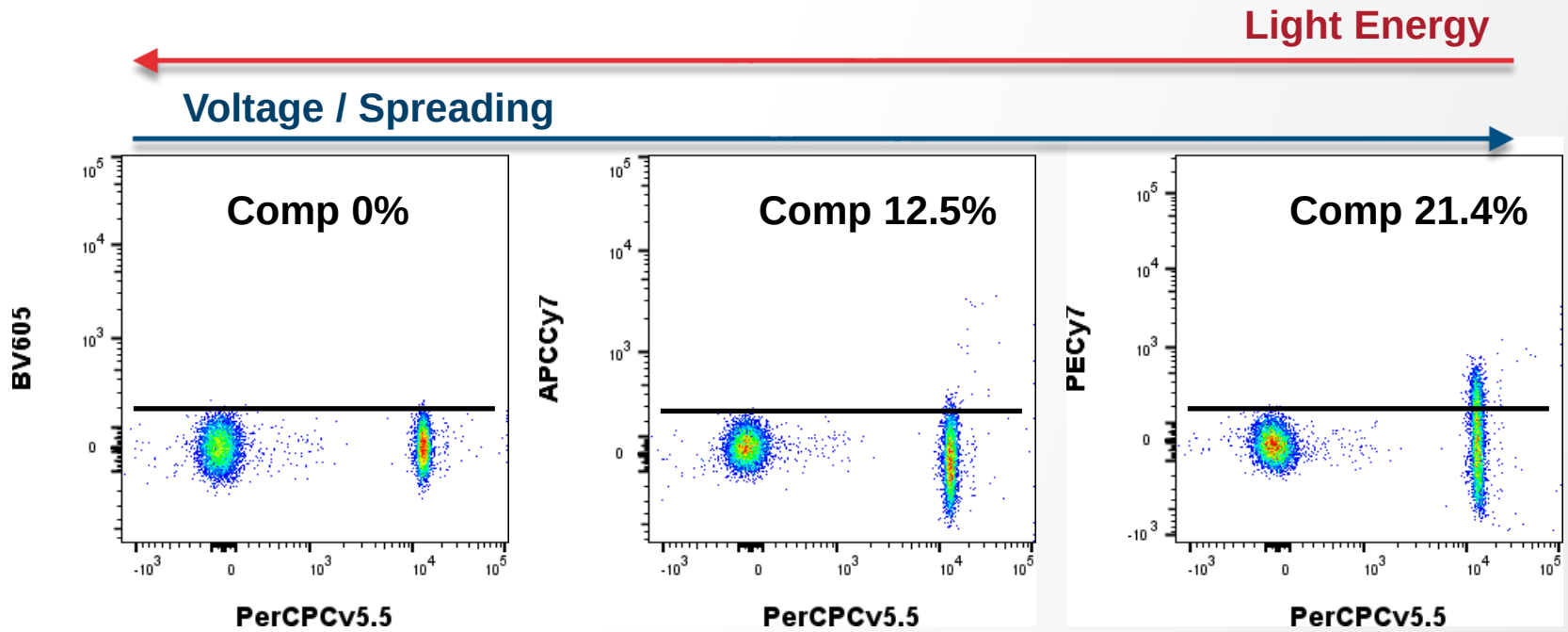
- Spreading Error is spreading of cell populations into other channels
- Spreading Effect is a result of the quantity and the energy of the spillover photons
- Factors that cause Spreading Error
  - Spillover of a fluorophore into adjacent detector
    (Broad fluorophore emission = Higher Spillover)
  - Balance of Antigen density and Dye Brightness
    (Bright dye on High expressor = High Spillover)
  - Amplification of photons in adjacent detector
    (Increasing voltage = Higher Spreading)
  - Energy of Light
    (Low energy of light = High amplification = High Spreading)

PE into PerCP-Cyanine5.5



Compensation is not responsible for spread effect, compensation only makes spreading visible

Spreading Error Depends on Several Factors



- Higher compensation values tend to correlate with higher spreading error
- High compensation values are linked to a loss of resolution
- Detector efficiency/amplification of photons = Higher spreading
- Low voltages = Can help with dim population resolution
- Avoid using dim or low stain in channels with high spreading error (Avoid Red and Far Red)

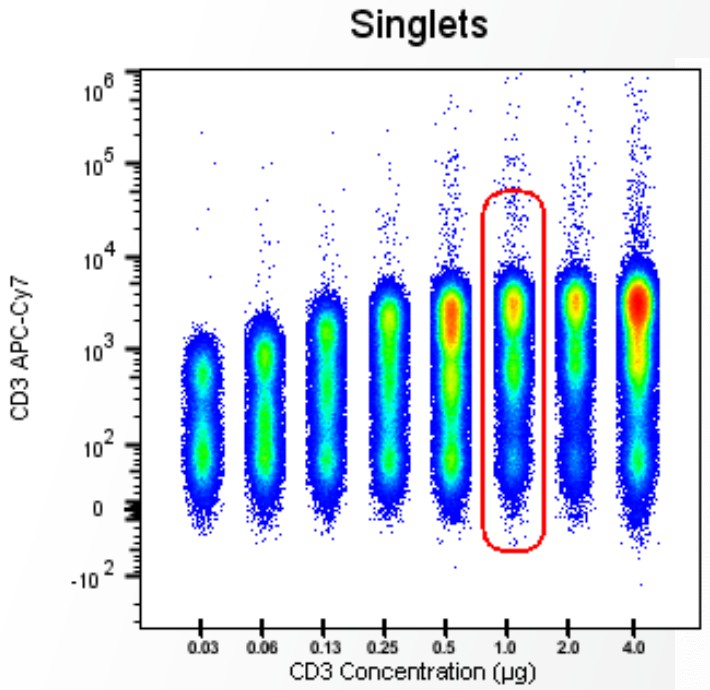
Antibody Titration – What and Why?

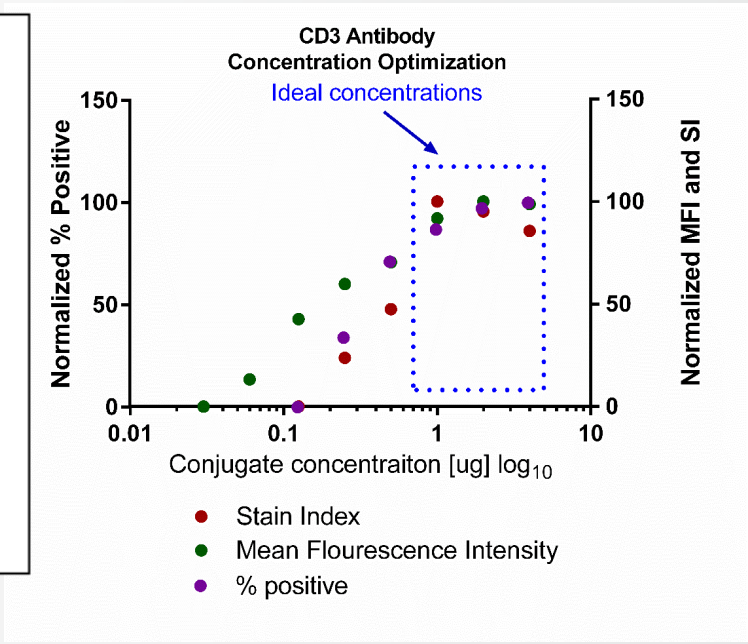
➢ What is it?

- Serial dilution of the antibody to determine the concentration for optimal performance
- Can save on reagent usage – compare cost per test
- Recommended working concentrations are just a starting point

➢ Why do it?

- Determine target saturation
- Improve resolution
- Eliminate or reduce false positives



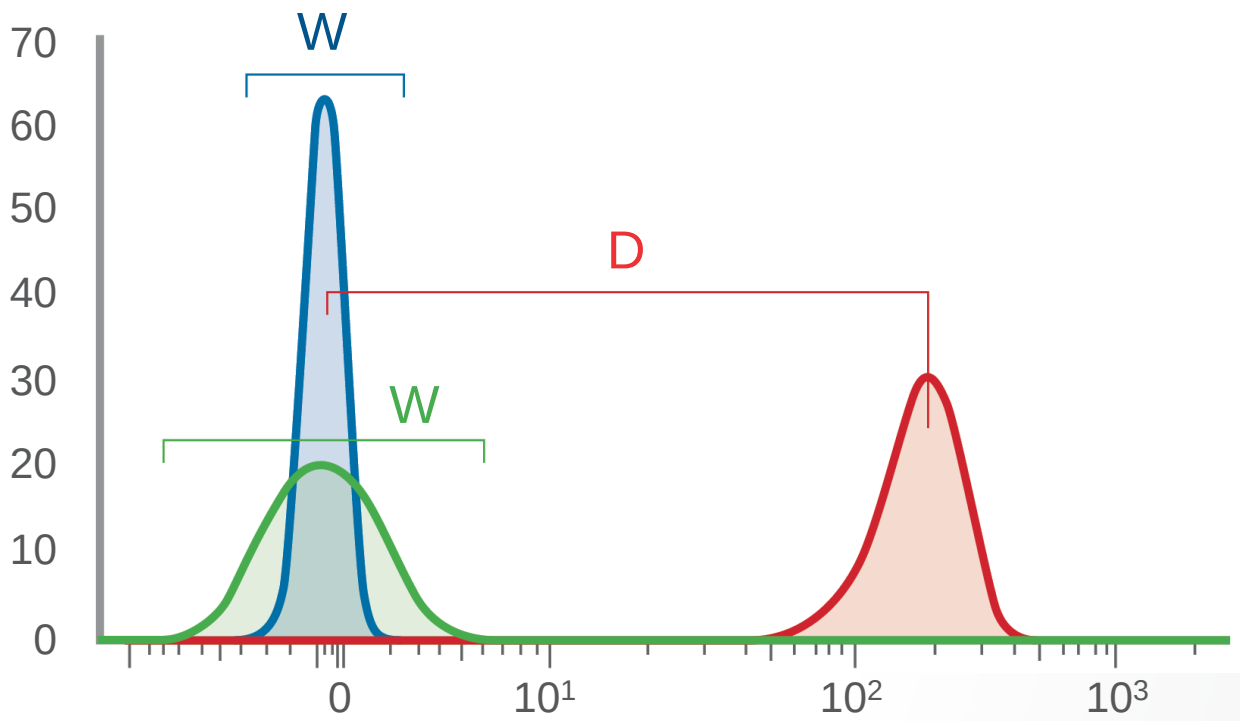


Stain Index (SI): $\frac{Median\ (pos) - Median\ (neg)}{2\ x\ SD\ (neg)}$

- Calculate staining index to determine best resolution
- Complete for each channel being used

Stain Index

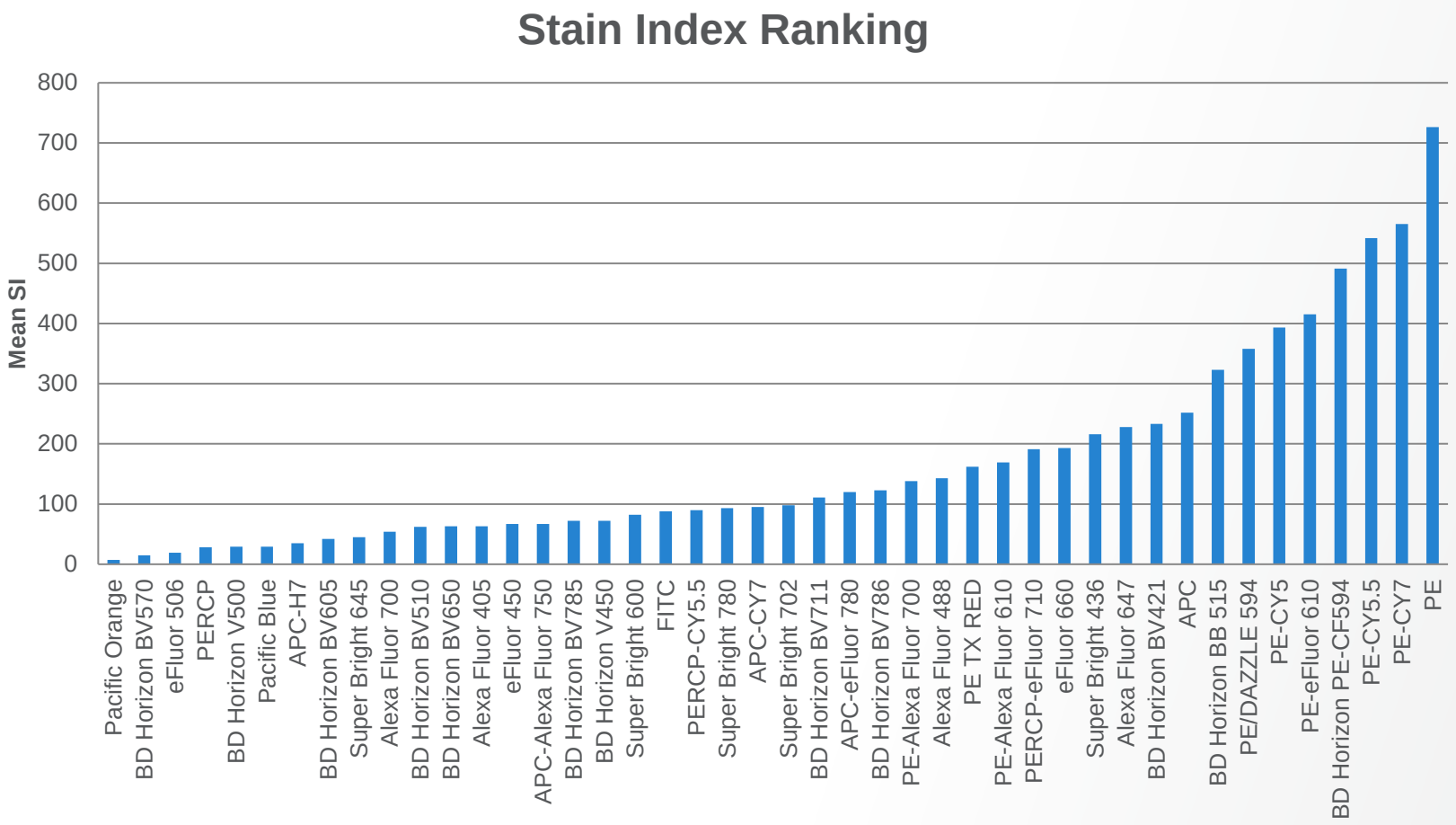
- Understand fluorochrome performance in order to optimize core markers in your panel
- Save the brightest fluorochromes for antigens with low, variable, or unknown expression
- Stain index (SI) defines fluorochrome brightness



Stain index = D/W

- D = Difference between positive and negative peak medians
- W = Spread of negative peak, 2 x rSD (robust standard deviation)
- SI is more useful for accessing fluorochrome brightness than the signal-to-background ratio, as it takes W into account

Brightness of Fluorochromes



	Fluorochrome	Laser	Filter	Stain Index
Brightest	PE	561	585/16	726
	PE-CY7	561	780/60	565
	PE-CY5.5	488	695/40	542
	BD Horizon PE-CF594	561	620/15	491
	PE-eFluor 610	561	620/15	415
	PE-CY5	488	695/40	393
	PE/DAZZLE 594	561	620/15	358
	BD Horizon BB 515	488	530/30	323
	APC	637	670/14	252
	BD Horizon BV421	405	450/40	233
	Alexa Fluor 647	637	670/14	228
	Super Bright 436	405	440/40	216
Bright	eFluor 660	637	670/14	193
	PERCP-eFluor 710	488	695/40	191
	PE-Alexa Fluor 610	561	620/15	169
	PE TX RED	561	620/15	162
	Alexa Fluor 488	488	530/30	143
	PE-Alexa Fluor 700	488	695/40	138
	BD Horizon BV786	405	780/60	123
	APC-eFluor 780	637	780/60	120
	BD Horizon BV711	405	710/50	111
Moderate	Super Bright 702	405	710/50	98
	APC-CY7	637	780/60	95
	Super Bright 780	405	780/60	93
	PERCP-CY5.5	488	695/40	90
	FITC	488	530/30	88
	Super Bright 600	405	610/20	82
	BD Horizon V450	405	440/40	72
	BD Horizon BV785	405	780/60	72
	APC-Alexa Fluor 750	637	780/60	67
	eFluor 450	405	440/40	67
	Alexa Fluor 405	405	440/40	63
	BD Horizon BV650	405	660/20	63
	BD Horizon BV510	405	512/50	62
	Alexa Fluor 700	637	670/14	54
Dim	Super Bright 645	405	660/20	45
	BD Horizon BV605	405	610/20	42
	APC-H7	637	780/60	35
	Pacific Blue	405	450/40	29
	BD Horizon V500	405	525/50	29
	PERCP	488	695/40	28
	eFluor 506	405	525/50	19
	BD Horizon BV570	405	610/20	15
	Pacific Orange	405	610/20	7

Collected using the Invitrogen™ Attune™ NxT Flow Cytometer, with four laser configuration

PBMCs were labeled with anti-human CD4 antibody conjugated to various fluorochromes, and SI was calculated.

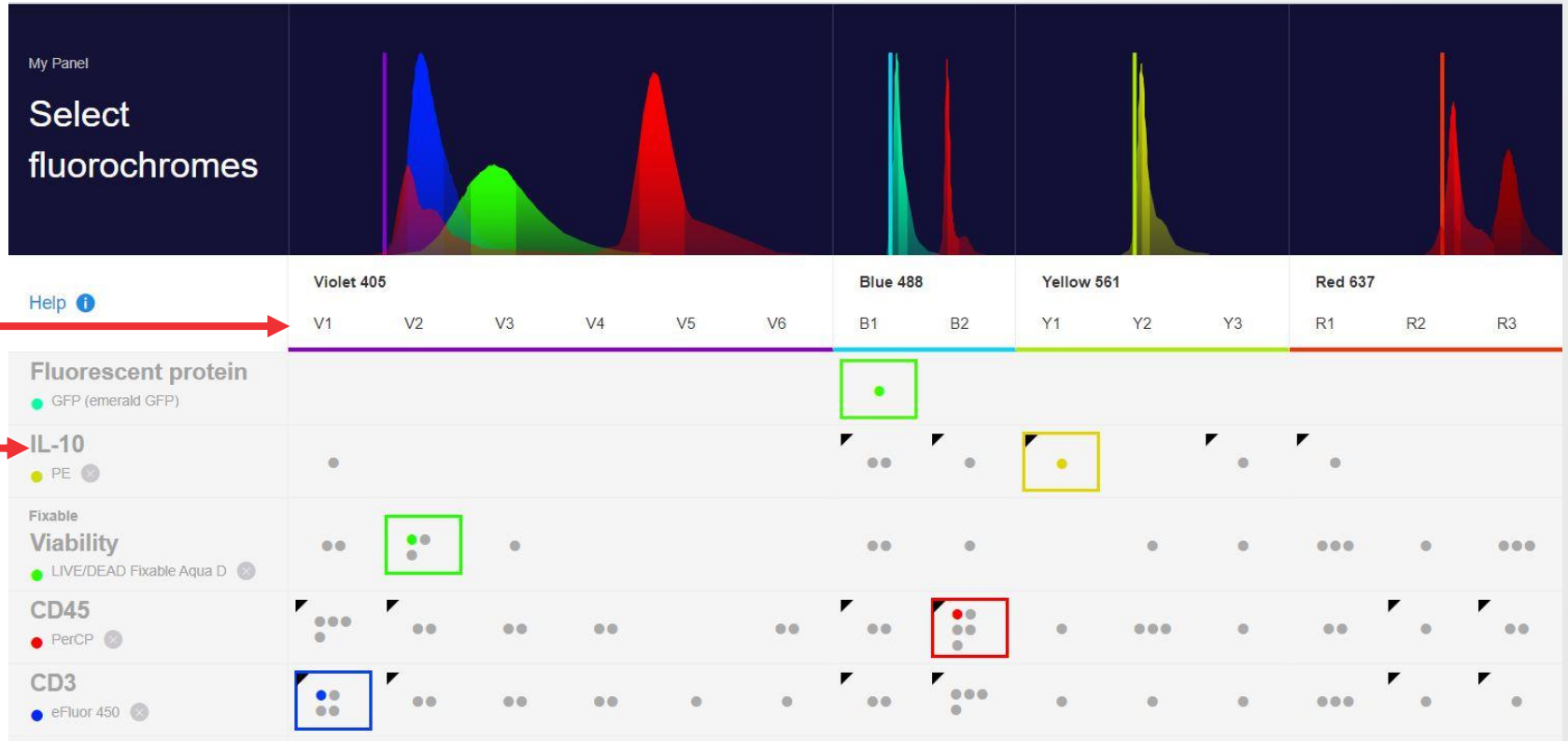
Building Your Panel

Selecting Your Fluorochromes

Matrix view of options

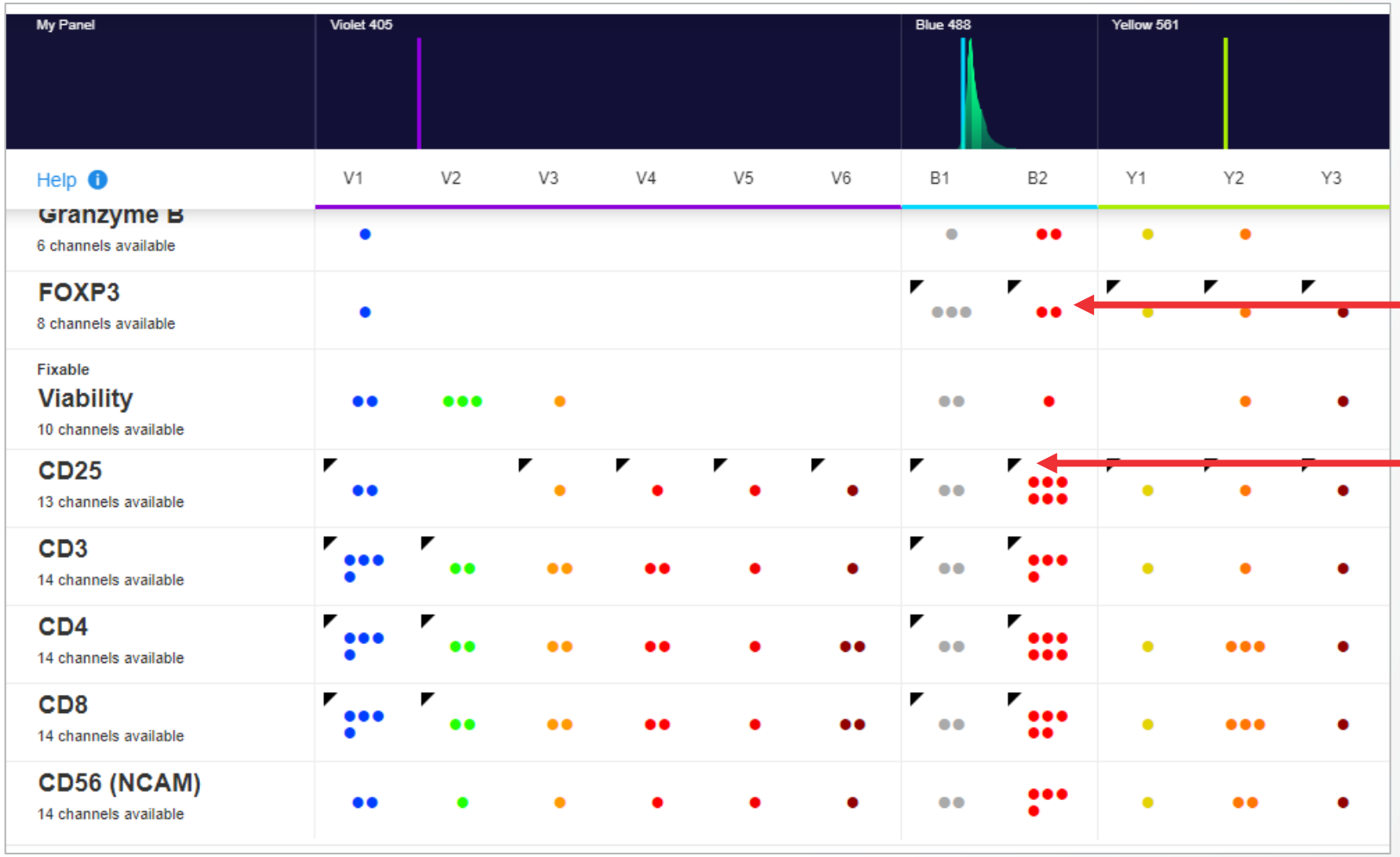
Channels: columns

Antigens: rows



Building Your Panel

Selecting Your Fluorochromes



Many channels will have more than one fluorochrome option (multiple dots)

Colored dots

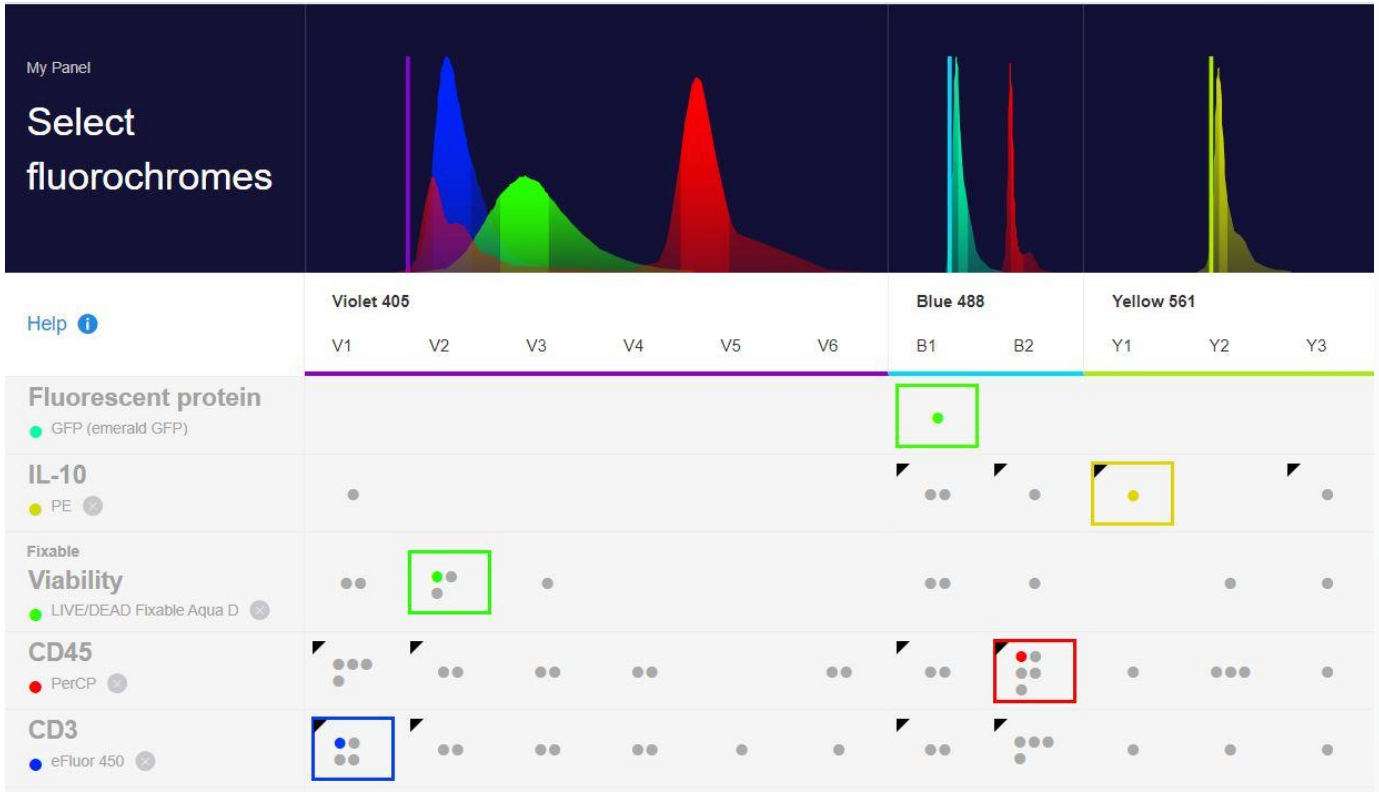
- Indicate available fluorochromes in each channel
- Black flag: recommended channel

Recommendations based on:

- Protein abundance from antigen slider
- Fluorochrome brightness

Building Your Panel

Filling in Your Panel



As panel is filled in:

- Colored box indicates chosen channel for the target
- Grayed out column indicates channel is used
- Grayed out row indicates target selection
- Emission curves are filled into SpectraViewer tool as fluorochromes are selected (top)
- Use spillover information from spectraviewer with recommendations based on protein abundance to make the best choices

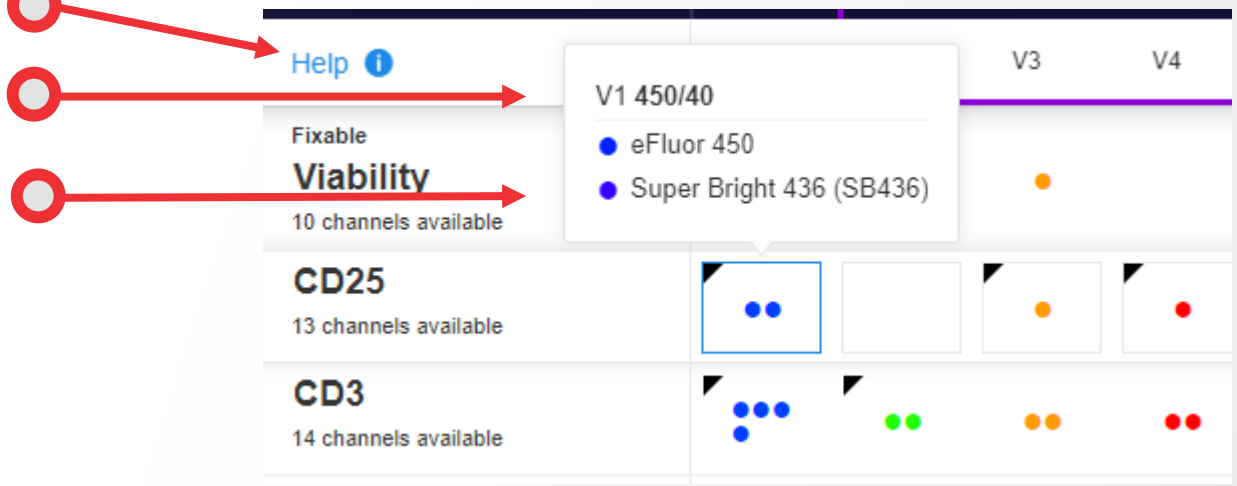
Building Your Panel

Selecting Your Fluorochromes

Tutorial to explain features

Bandpass wavelength

Fluorophore choices



Black Flag:

Chose “low” protein abundance → bright and medium-bright fluors are flagged
Chose “high” protein abundance → dim and medium-dim fluors are flagged
Chose “medium” or makes no choice → no fluors are flagged

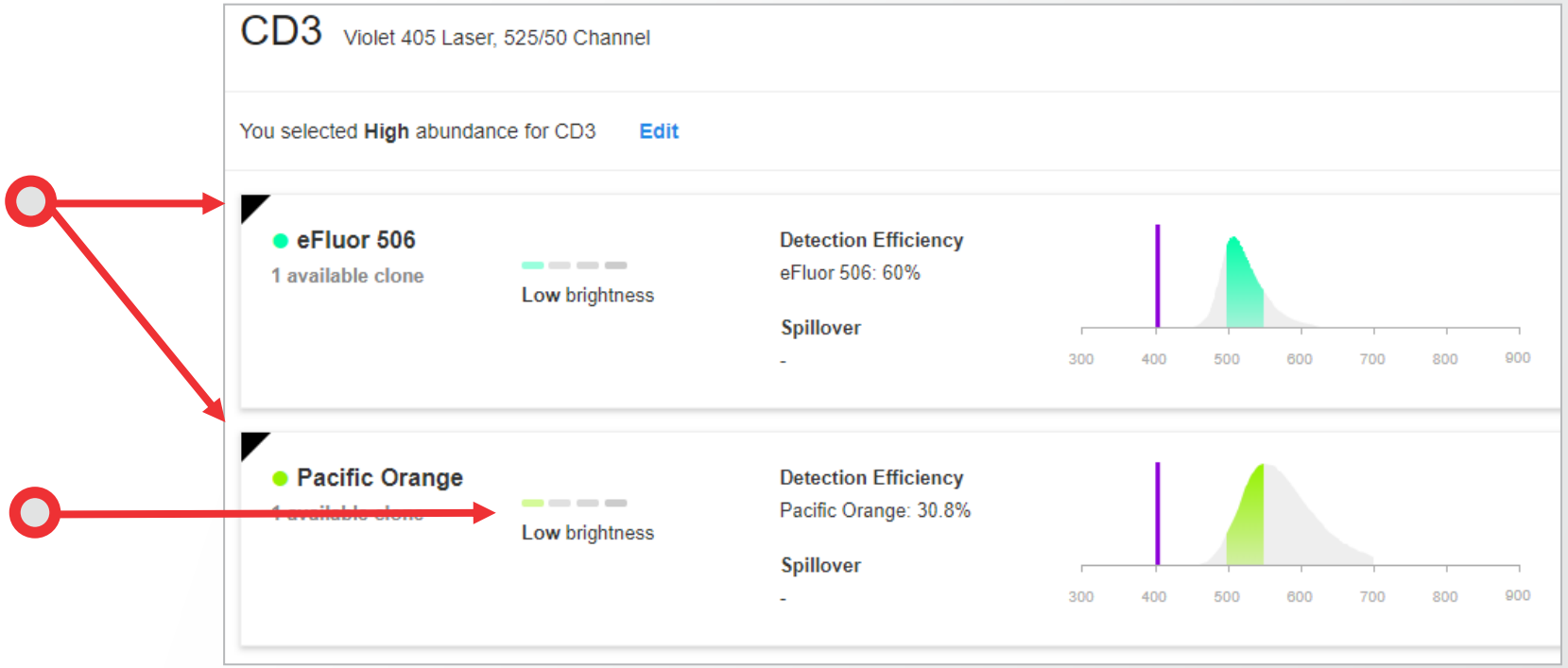
Building Your Panel

Selecting Your Fluorochromes

Black Flags

Flags indicate preferred fluorophores

Brightness indicator for fluorophore



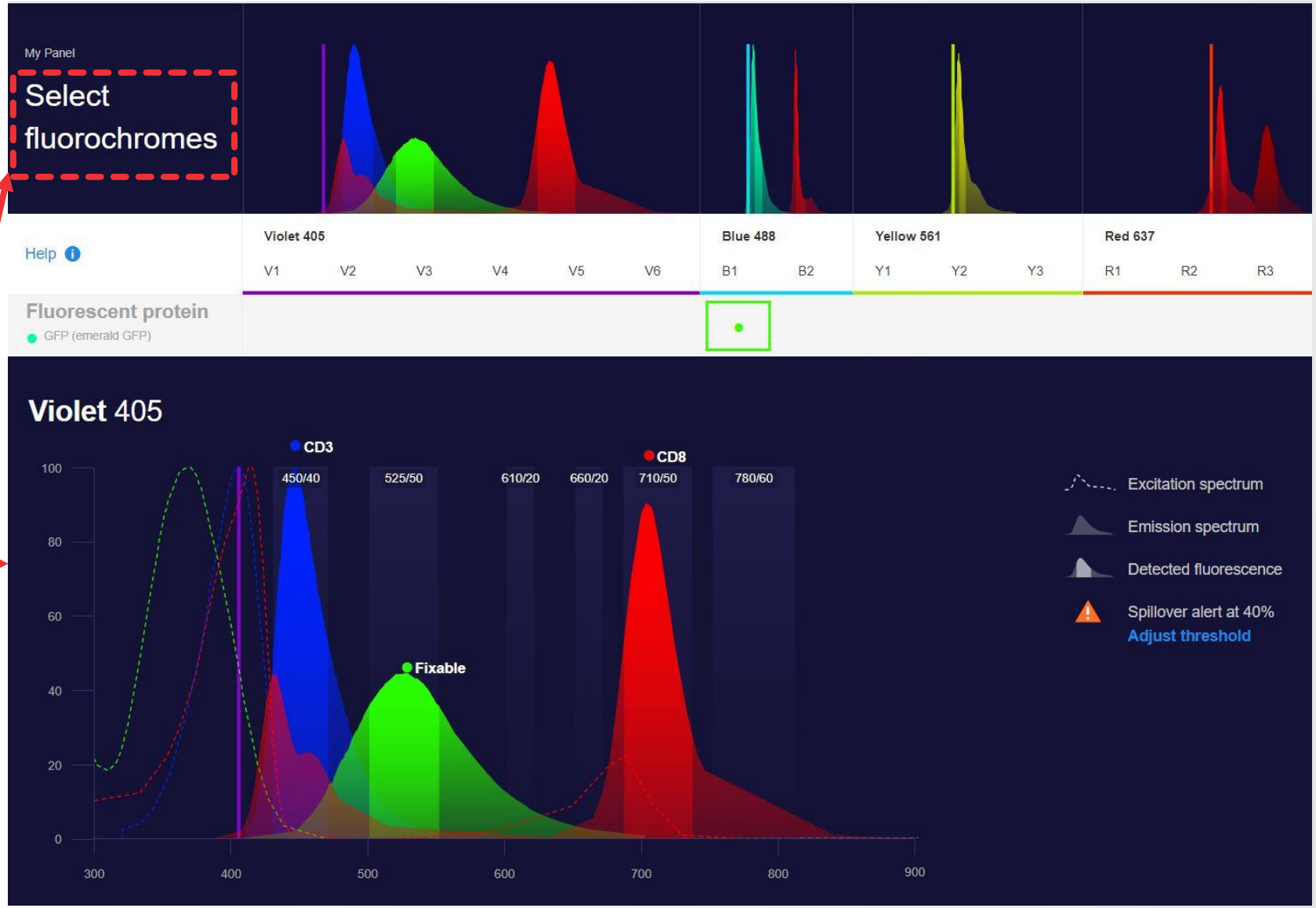
Building Your Panel

Visualize Your Fluorochrome Spectrums

Full-page SpectraViewer on top of page

- Separated by laser
- Spillover values in table under each emission for a given laser line
- Populates as selections are made

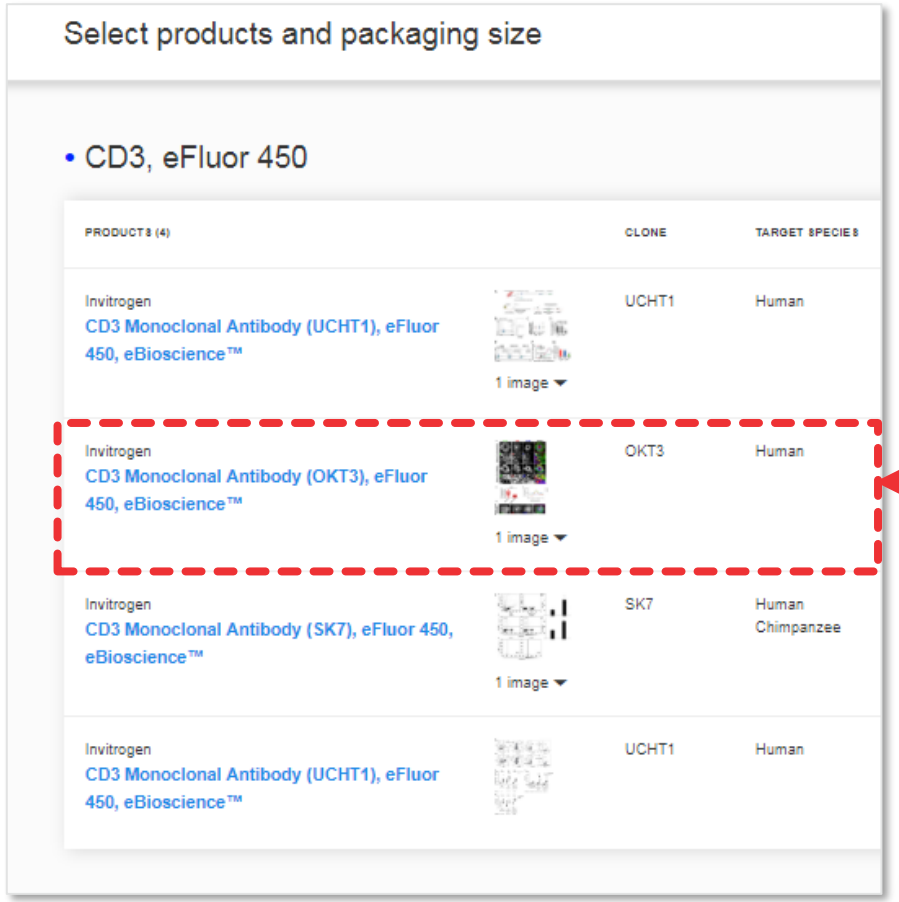
Expands to full-screen



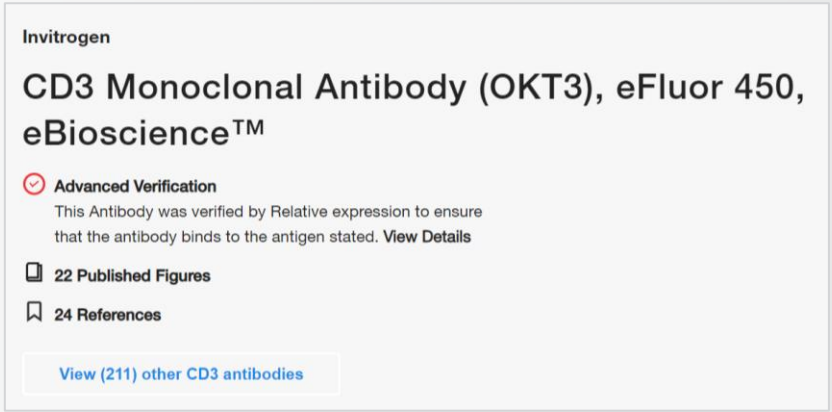
Building Your Panel

Examine Your Options

Choose the products that work best for you



1. Antigen/fluorochrome combinations may have multiple product options
2. Different clones
3. Different sizes
4. Clicking on product name takes you to the product data page that includes:
   - Data
   - References
   - Detailed product information



Building Your Panel

Select Your Clone

Invitrogen
CD3 Monoclonal Antibody (OKT3), Super Bright 600, eBioscience™

16 References

28 figures

OKT3

Human

USD 335.00
Cat # 63-0037-42

100 Tests

Select

Antibody Testing Data (1) | Advanced Verification (1) | Published Figures (26)

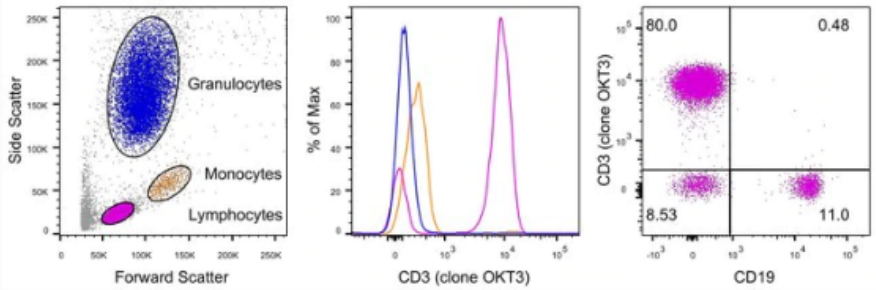


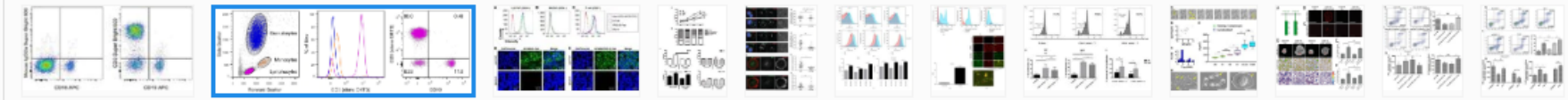
FIGURE 2/28

Relative expression

This Antibody was verified by Relative expression to ensure that the antibody binds to the antigen stated.

CD3 Antibody (63-0037-42)

Staining of human peripheral blood cells. As expected based on known relative expression patterns, CD3 clone OKT3 stains a subset of lymphocytes (T cells) and does not stain monocytes and granulocytes (middle plot). Additional analysis of lymphocytes shows that CD3 clone OKT3 does not stain any CD19+ B cells (right plot). Details: Normal human whole blood was surface stained with CD3 (clone OKT3) and CD19 (... View more



Internal testing data

Published figures

Building Your Panel

Export Your List of Antibodies

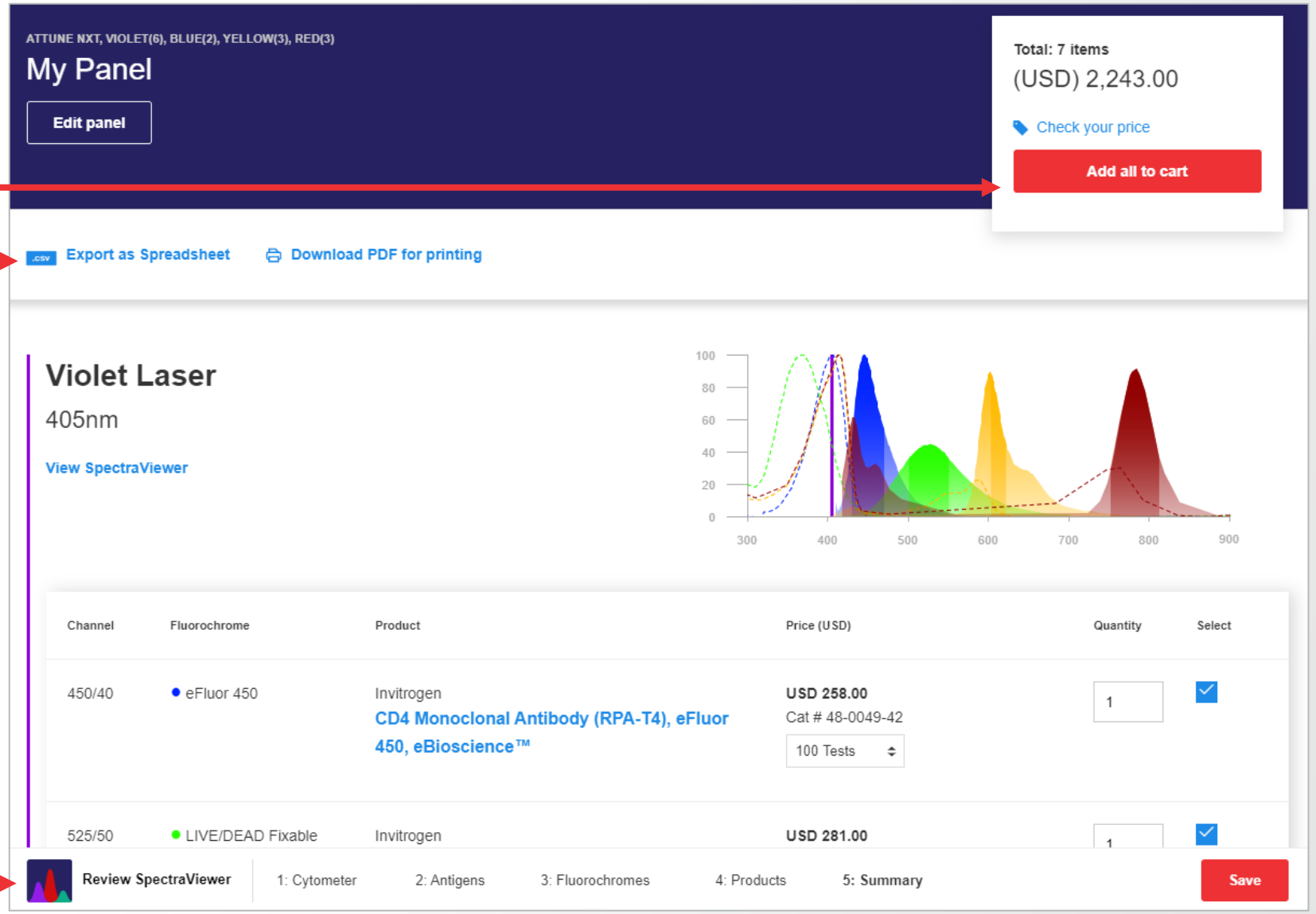
Order

Export as spreadsheet or PDF

Final panel includes:

- Selected products
- Placeholder reagents
- Other reagents (beads, buffers, and isotype controls)
- SpectraViewer

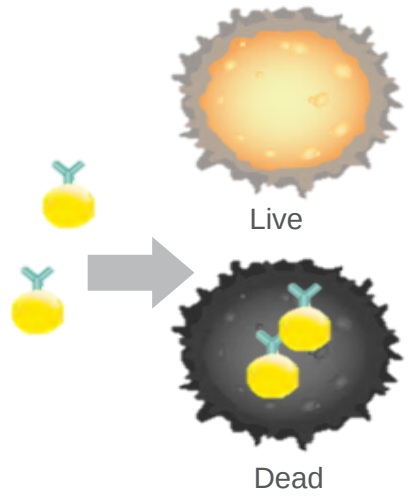
Can go back to other steps



Step 4: Biological Controls

4

Biological controls



Biological controls assist with experimental analysis

Help ensure that gates are drawn appropriately

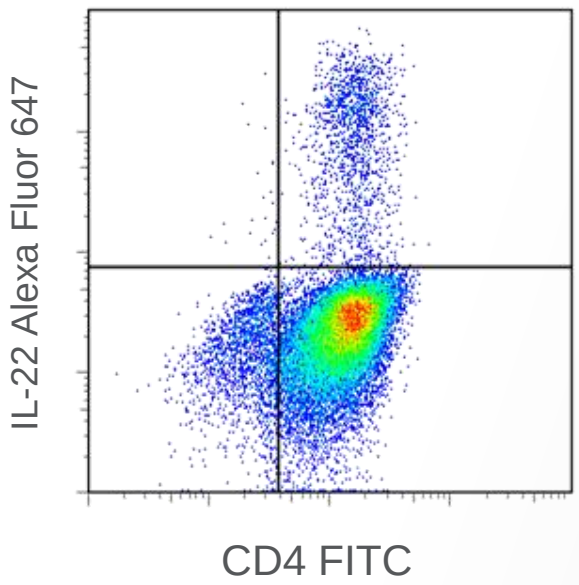
Eliminate false positives and ensure antigen specificity

Step 4: Biological Controls

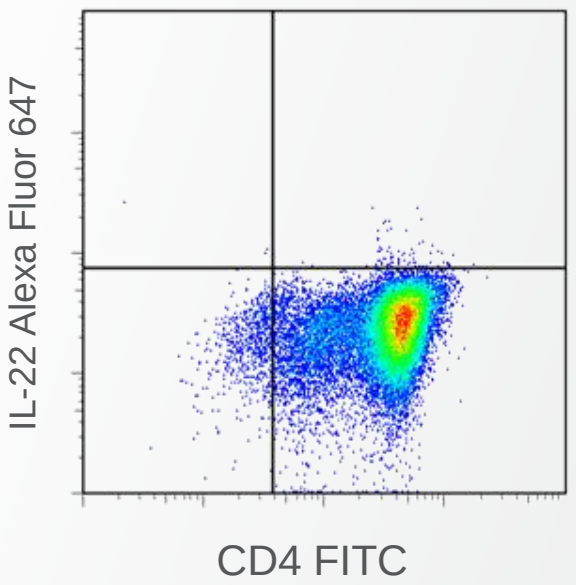
Stimulation Controls

Use unstimulated cells as the control for any cell treatment

Stimulated



Unstimulated

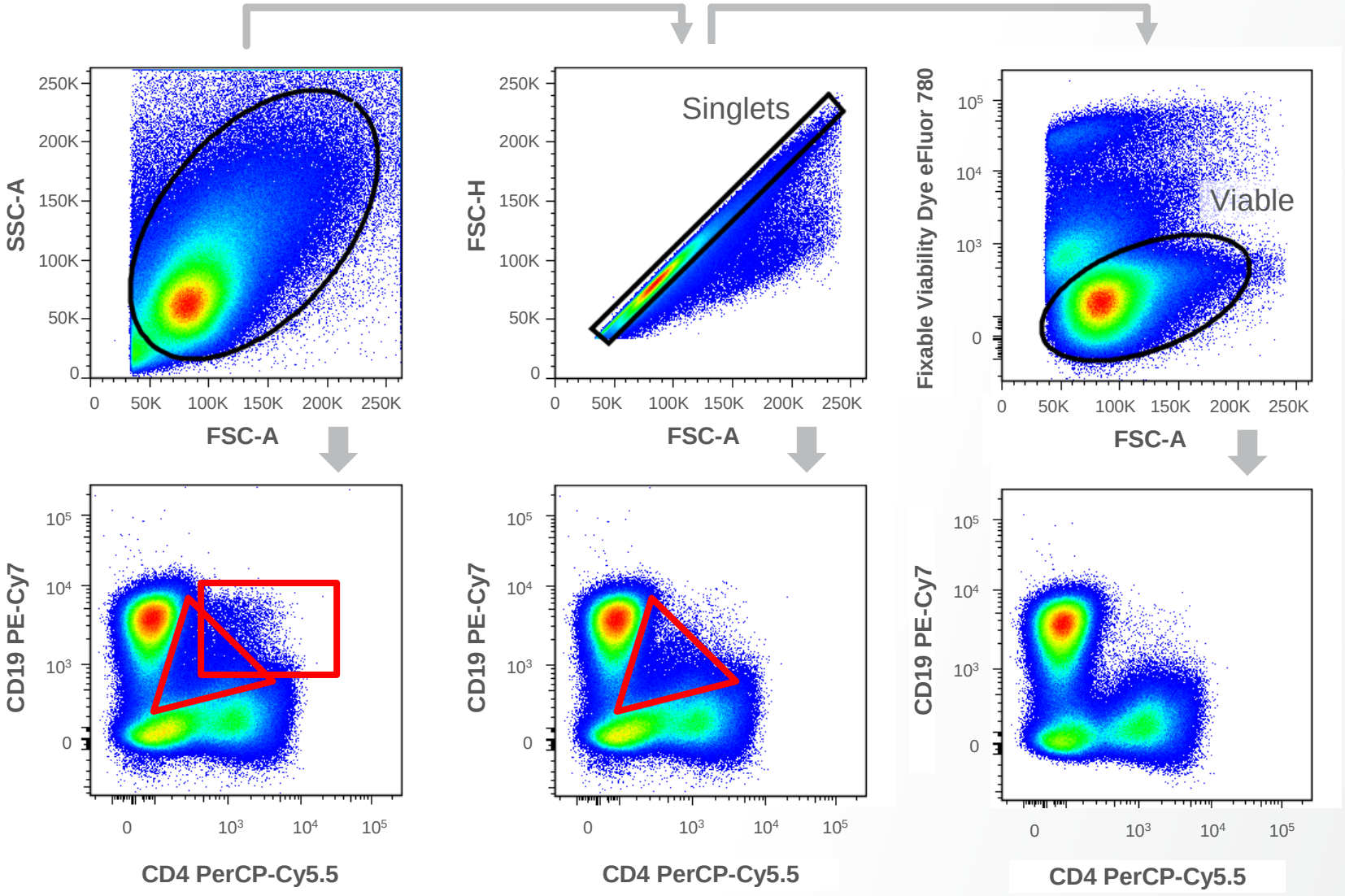


An internal negative control may be more appropriate in some experiments.

The more controls, the better!

Step 4: Biological Controls

Viability and Singlet Gating

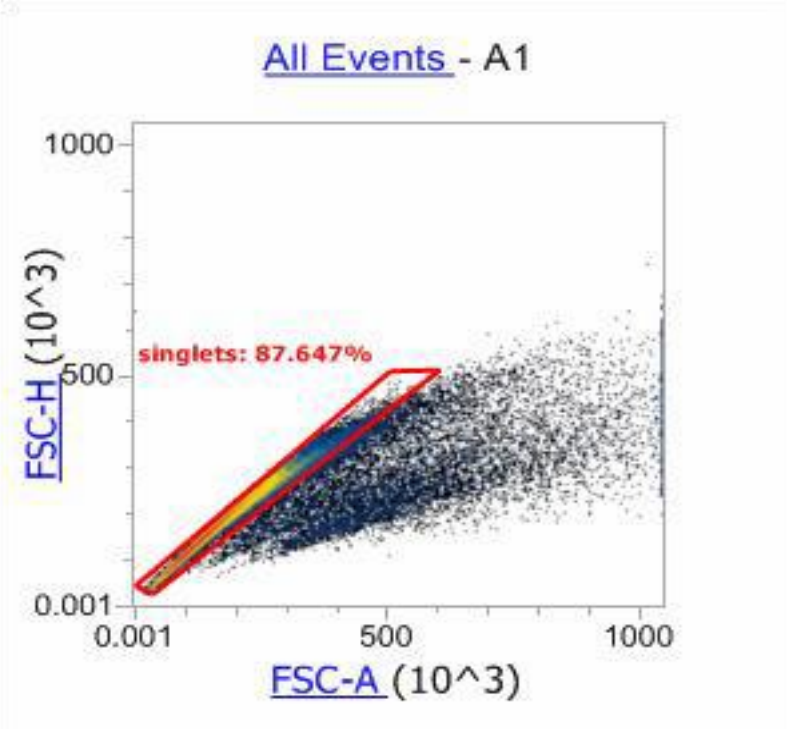


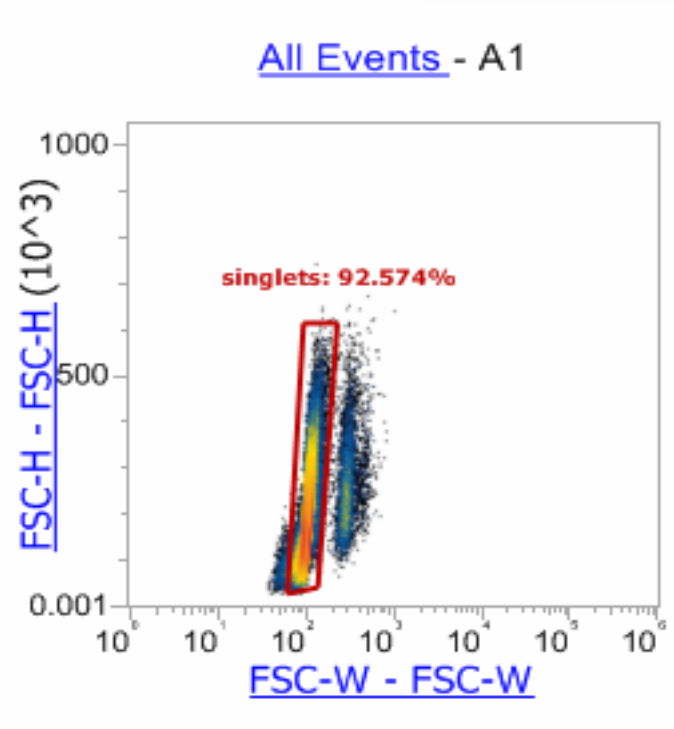
- Dead cells, debris, and coincident events can result in false positives
- Applying a singlet gate removes false positives caused by coincident events
- Using a viability dye excludes dead cells and debris

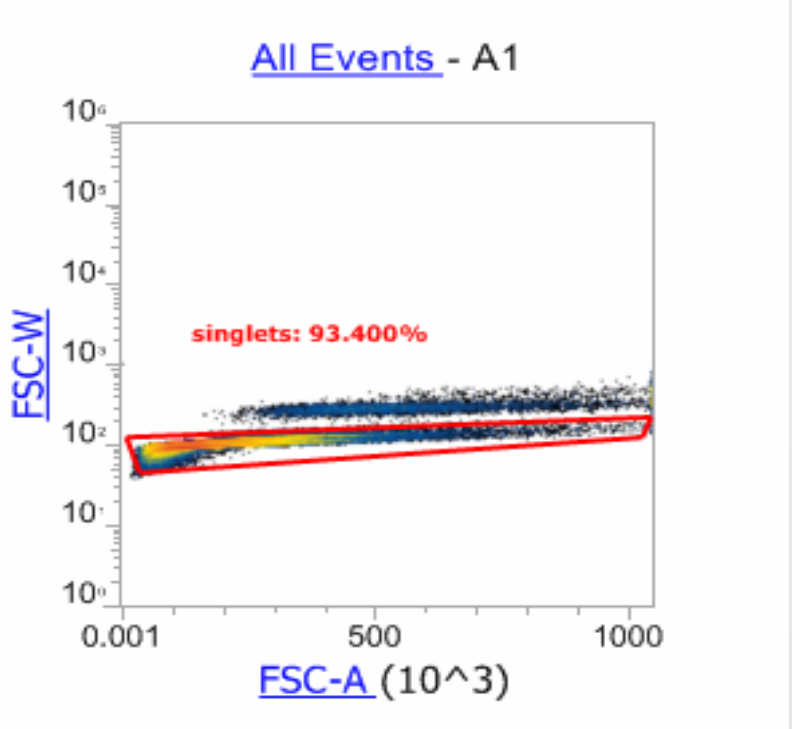
Doublet Discrimination

Examples of Determination

In most cases, data analysis should include gating on single cells.



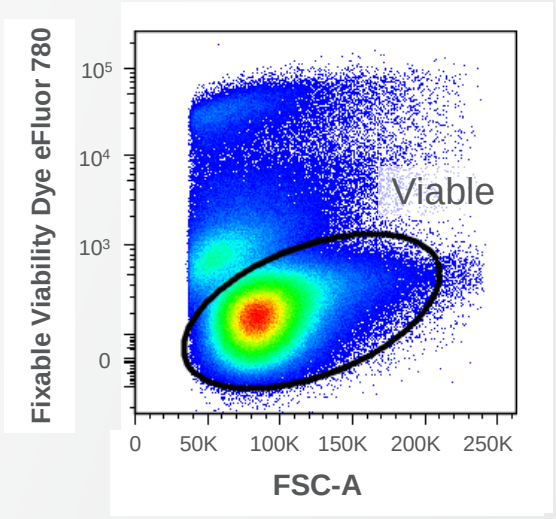




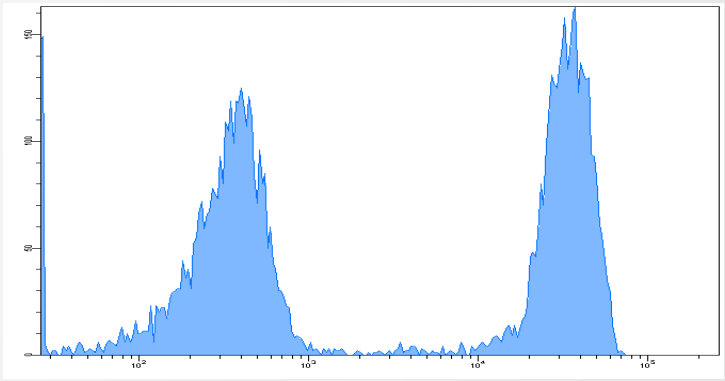
Step 4: Biological Controls

Viability Dye Options

- Exclusion stain, so negative (or dim) population has cells of interest
- Viability dyes detect integrity of the plasma membrane
- Nucleic acid dyes:
  - Can cross compromised membrane to bind nucleic acid
  - Not compatible with cell fixation
- Amine-reactive dyes:
  - Bind surface amines in viable cells (dim staining)
  - Bind IC amines when membrane is compromised (bright staining)
  - Staining is preserved after fixation
- Many options across multiple laser lines
- Can also be part of a dump channel



Live Cells **Dead Cells**

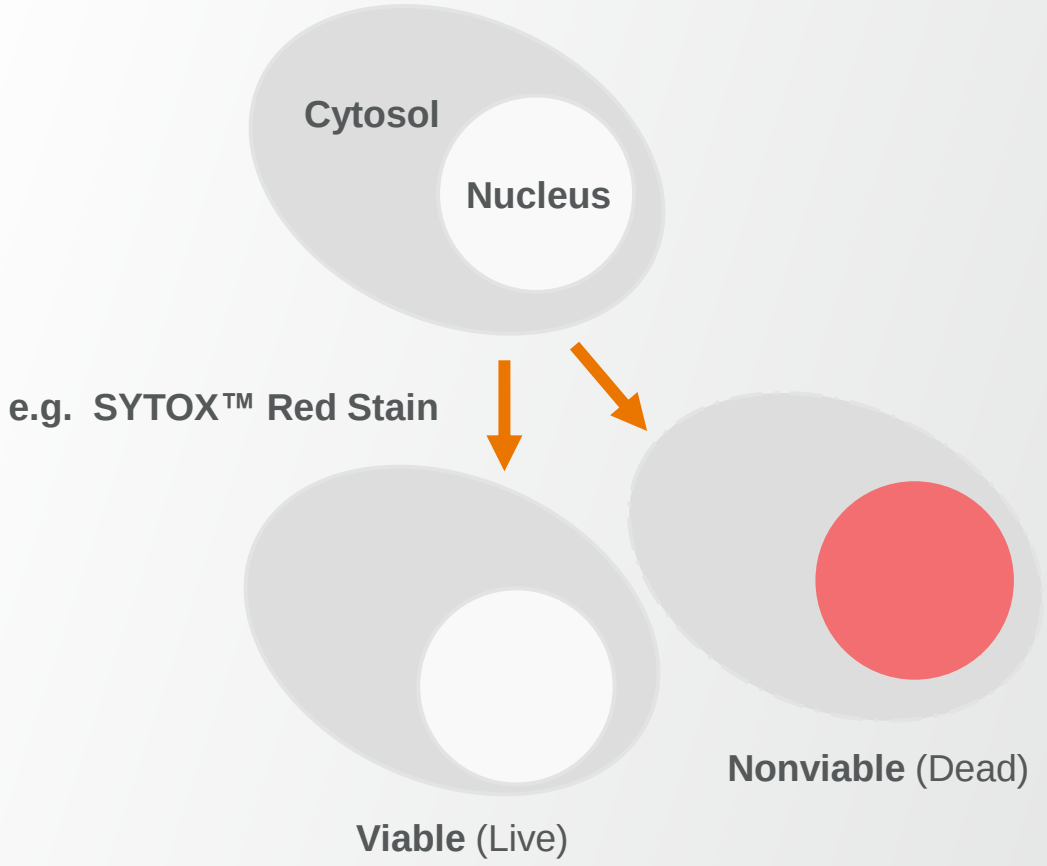


LIVE/DEAD™ Fixable Aqua

Impermeant Nucleic Acid Dyes, Flow Cytometry

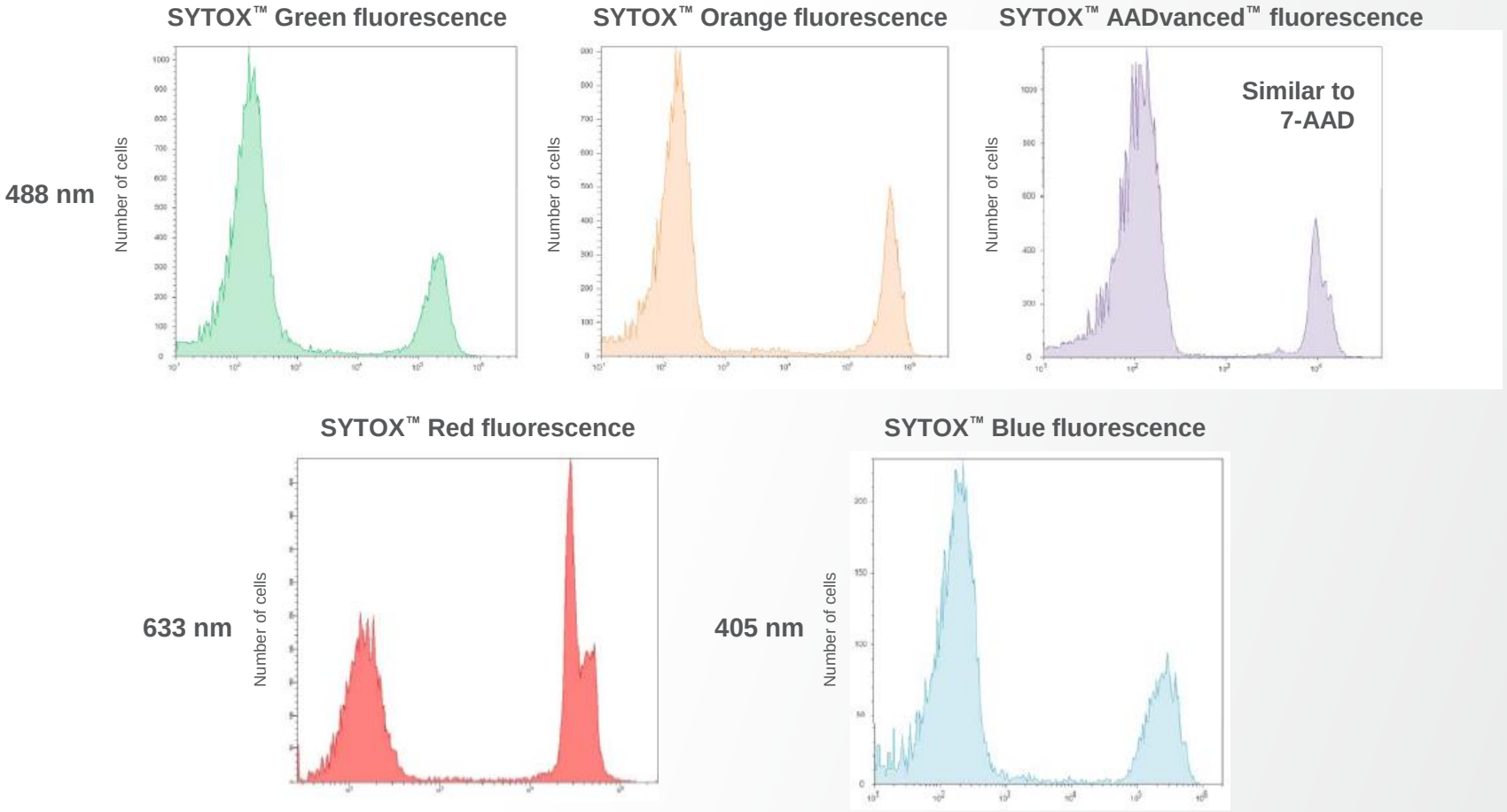
Dyes which penetrate cells with a compromised cell membrane to stain nucleic acids, but do not cross the membranes of live cells

- Can be used to **identify dead cells** in a population
- Can be used to **quantitate DNA content** in fixed cells
  - Propidium Iodide (488 nm ex)
  - 7-AAD (488 nm ex)
  - DAPI
  - SYTOX™ AADvanced™ dead cell stain (488 nm ex)
  - SYTOX™ Green dead cell stain (488 nm ex)
  - SYTOX™ Orange dead cell stain (488/532/561 ex)
  - SYTOX™ Blue dead cell stain (405 nm ex)
  - SYTOX™ Red dead cell stain (633 nm ex)



SYTOX™ Dead Cell Stains

Five different colors for flexibility in multicolor panels

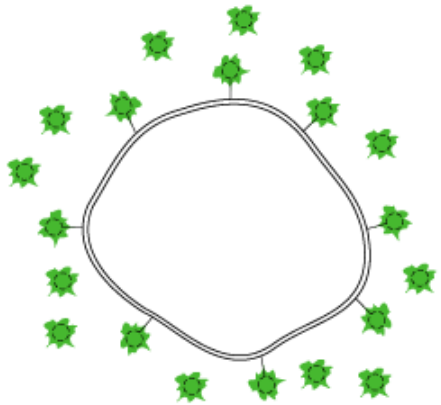


Propidium Iodide ReadyProbes™ Reagent

Power and Simplicity

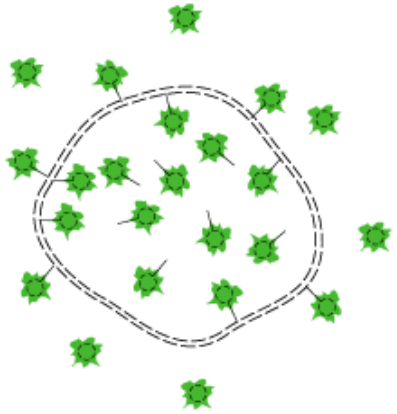
- Ready-to-use liquid propidium iodide formulation
- Rapid staining of dead cells without wash steps
- Ready-to-use liquid formulation in convenient dropper bottle—no need to dilute, weigh, or pipette
- **Stable at room temperature—**keep handy at your work station or cell culture area

LIVE/DEAD™ Fixable Dead Cell Stains



Live cells:

React with the fluorescent reactive dye only on their surface to yield weakly fluorescent cells.

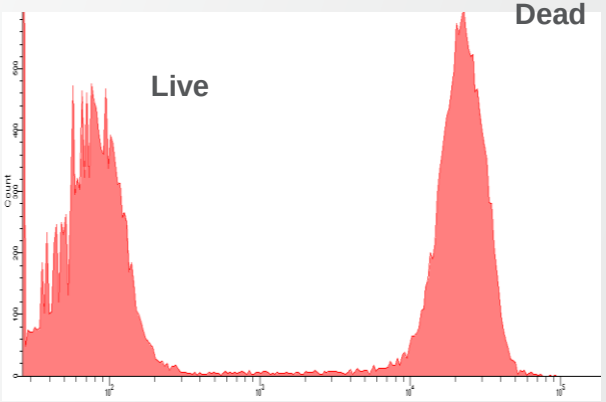


Cells with compromised membranes:

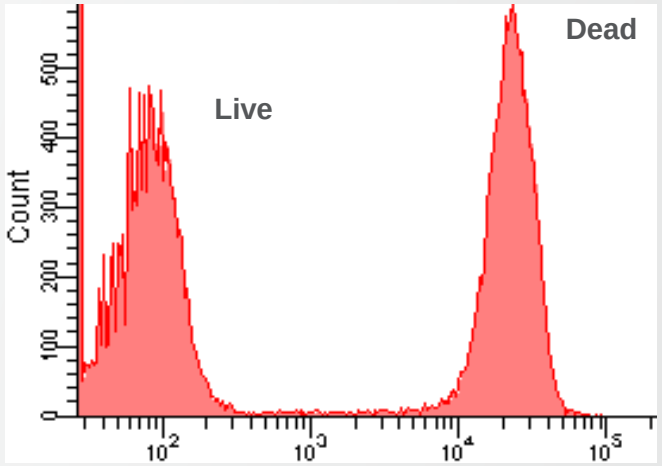
The dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining.

Viability = membrane integrity

Before fixation



After fixation



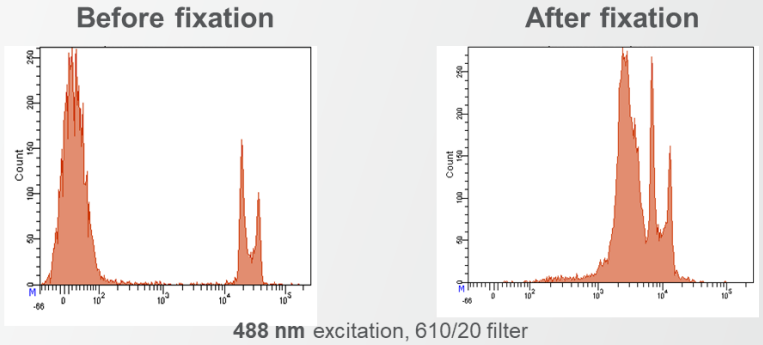
Fixable violet dead cell stain

405 nm Violet Excitation (440/40 BP)

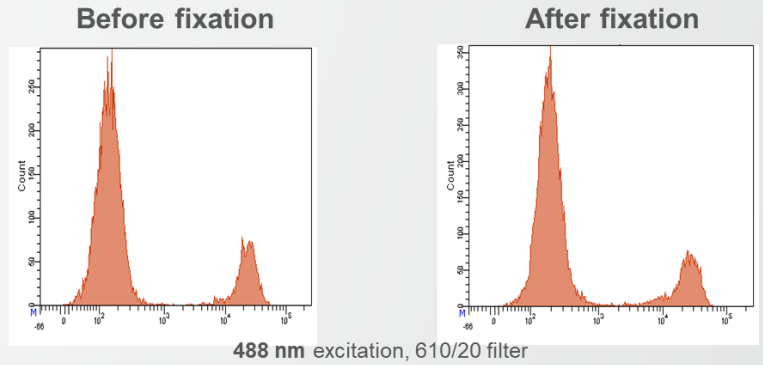
LIVE/DEAD™ Fixable Dead Cell Stains

- **Compatible with Fix and Perm Procedures**
  Useful for dead cell identification with intracellular targets
- **Specificity and reliability**
  Staining pattern is similar before and after fixation
- **Reduced handling risks**
  Formaldehydes are known to reduce risks of viruses
- **Convenience**
  Cells can be stained and fixed at various times during the experiment, and the results can be analyzed several hours later, without loss of the discrimination pattern

Propidium Iodide

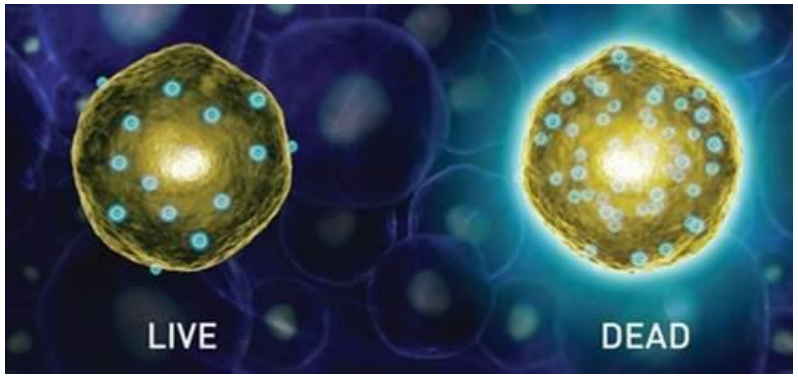


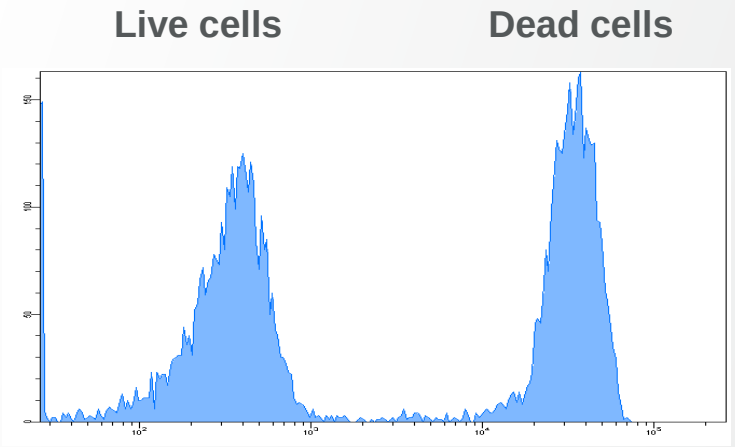
LIVE/DEAD™ Fixable Red Stain



Amine Reactive Dyes for Dead Cell Identification

8 Color Options, Packaged for Stability





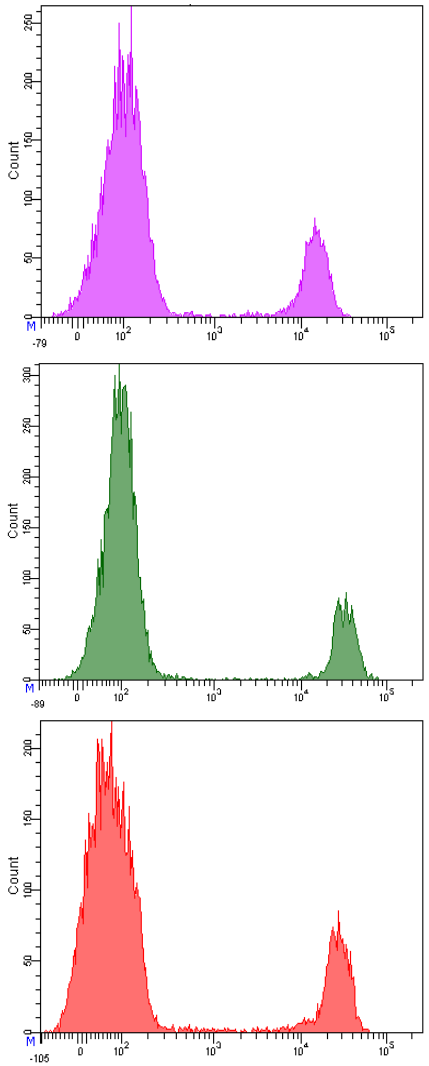
Aqua-fluorescent reactive dye

LIVE/DEAD™ Fixable Dead Cell Stain Kits

Reactive dye	Excitation source	Ex*	Em*
Blue fluorescent reactive dye (L23105)	UV	350	450
Violet fluorescent reactive dye (L34955)	405 nm	416	451
Aqua fluorescent reactive dye (L34957)	405 nm	367	526
Yellow fluorescent reactive dye (L34959)	405 nm	400	575
Green fluorescent reactive dye (L23101)	488 nm	495	520
Red fluorescent reactive dye (L23102)	488 nm	595	615
Far red fluorescent reactive dye (L10120)	633/635 nm	650	665
Near-IR fluorescent reactive dye (L10119)	633/635 nm	750	775

* Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.

Tips and Tricks



Impermeant DNA dyes

- Add at final step, do not wash out
- Emission is broad, consider for multicolor applications
- Dead/Fixed cells can be used for compensation control

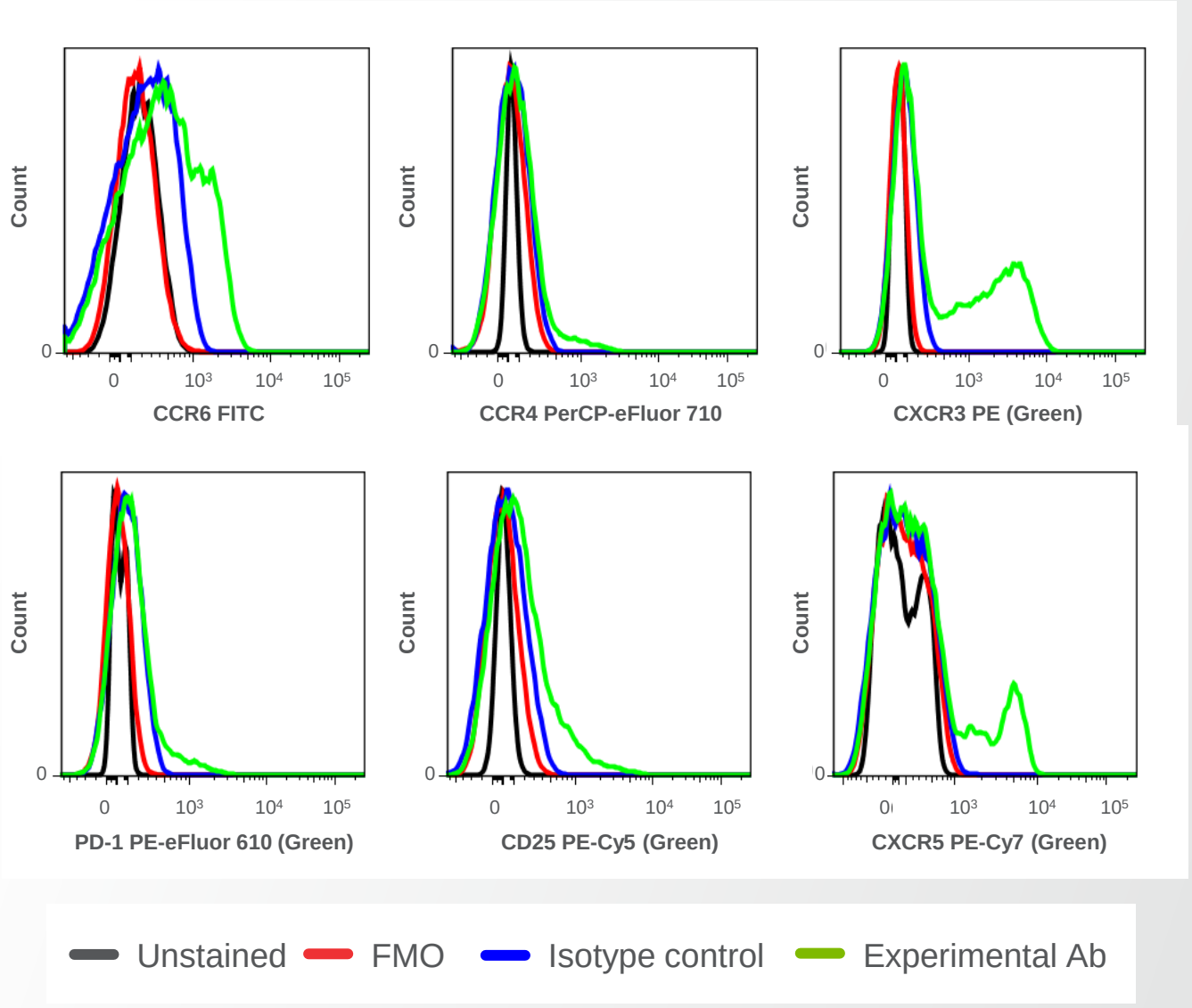
Amine-reactive dyes

- Do not use protein in buffers
- Live cells have dim fluorescence
- Use with -aldehyde fixatives
- Can be used without fixing cells too
- ArC™ compensation beads useful

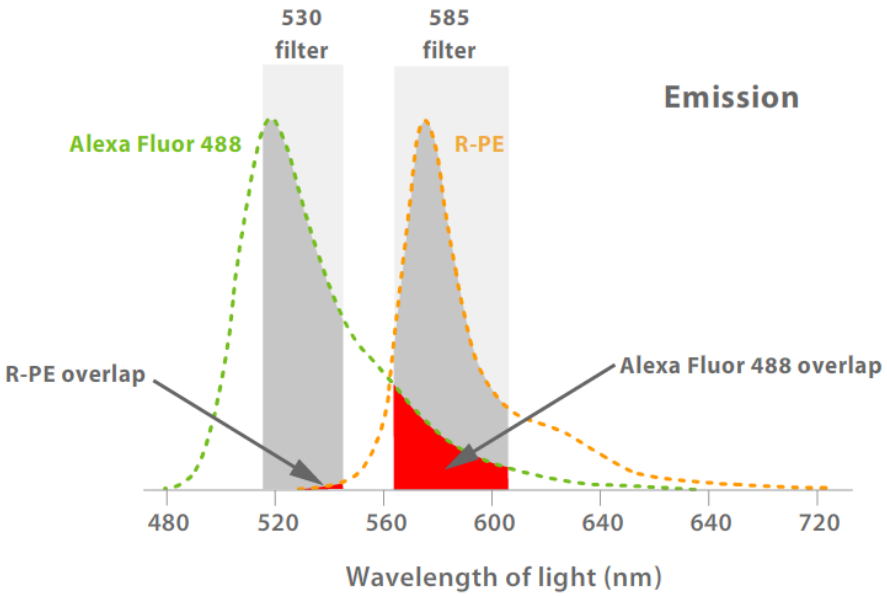
Step 5: Experimental Controls

- **Unstained cells:** to assess AF of cells
- **Single-color controls:** to set compensation and optimize voltages
- **FMO controls:** to assess the effect of other fluorochromes and compensation on background
- **Isotype controls:** to assess the stickiness of antibodies
- **Biological controls:** viability for removing dead cells and debris; unstimulated for correct gating

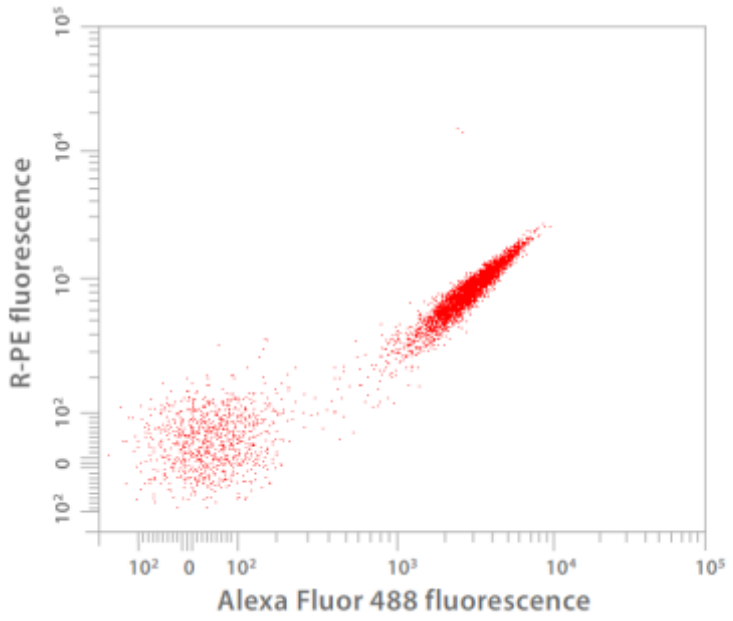
The more controls, the better!



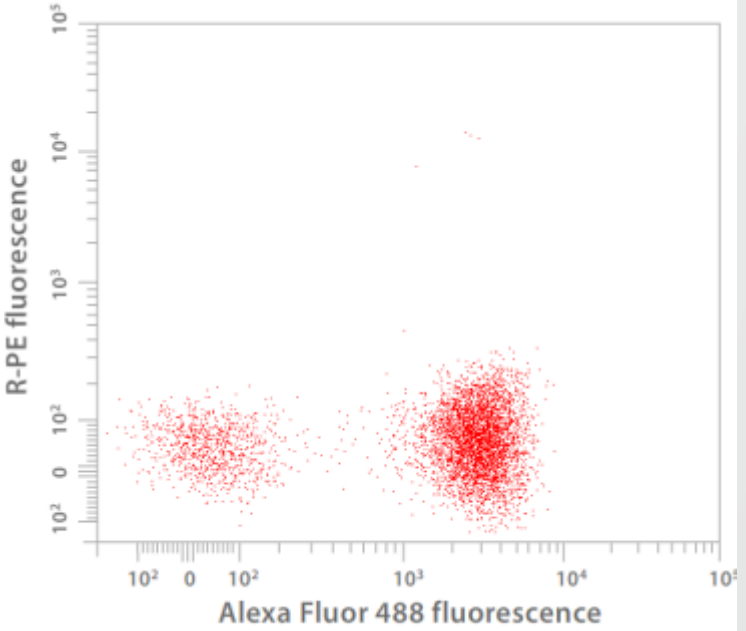
Compensation: Eliminates Spectral Overlap



Un-Compensated
Single Color Control



Compensated
Single Color Control



R-PE channel requires more compensation than Alexa Fluor 488 channel

Basic Rules of Compensation

- Unstained cells
- Single color controls are required
- Controls need to be at least as bright as the brightest positive sample
- Background fluorescence should be the same for the positive and negative control populations
- Compensation color must be matched to your experimental color (FITC cannot substitute for GFP)
- The actual tandem dye being used in the sample staining must be used in the single-color control
- Collect enough events to be statistically relevant

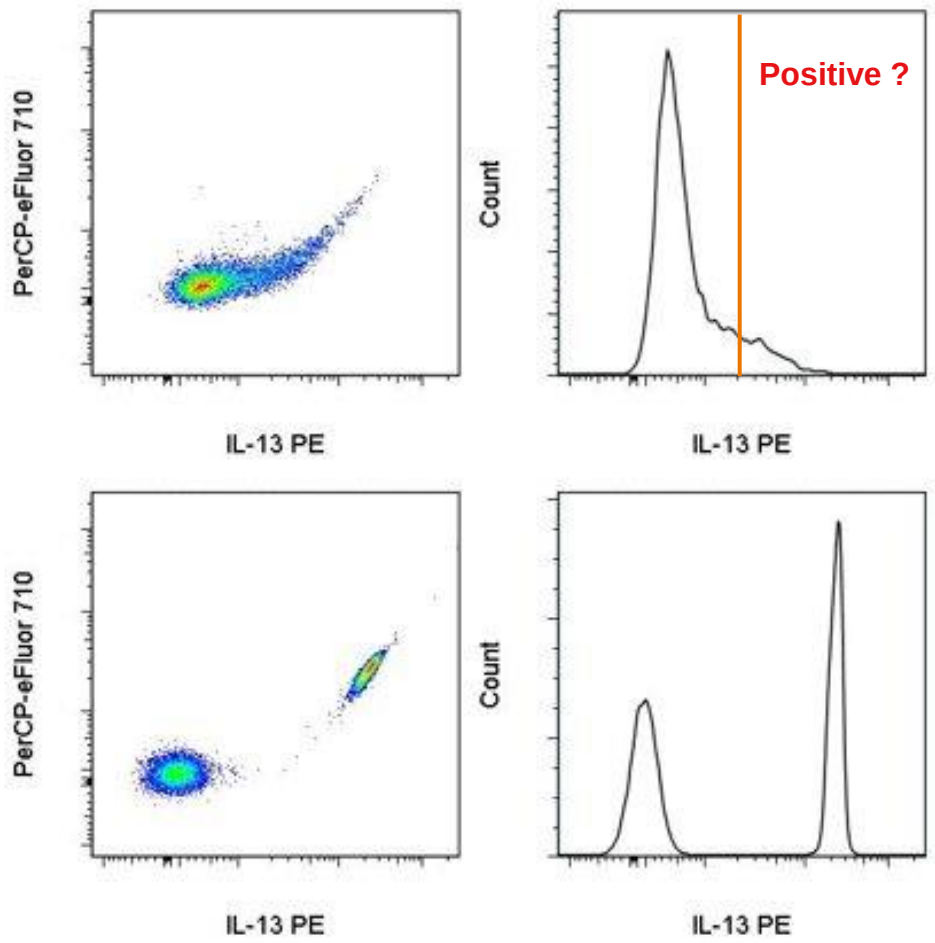
Compensation Beads

Do I need compensation beads?

- Cells may be in limited supply – reserve for experimental use
- Antigen may be dimly expressed by the cells
- Compensate with the antibody used in experiment
- Few cells in sample express the antigen of interest
- Correct compensation guaranteed with positive & negative populations available

Are You Simply Guessing?

Good compensation controls contain distinct positive and negative populations



IL-13 staining on cells

- Broad distribution
- Few positive events
- Difficult to identify positive population

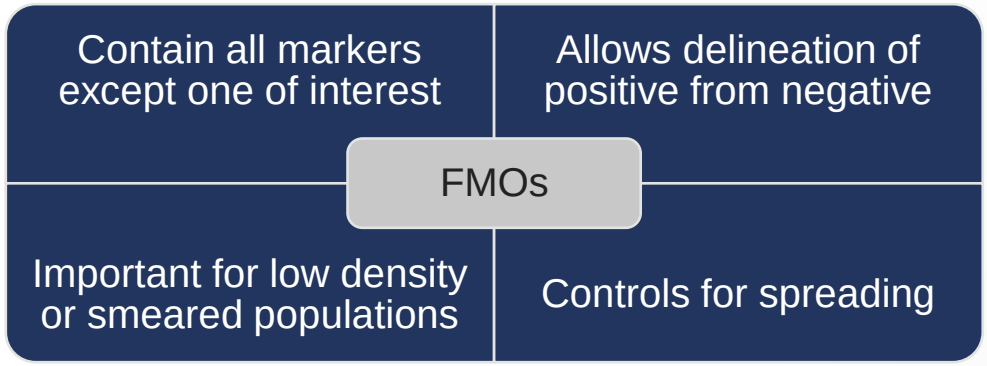
IL-13 staining on OneComp eBeads

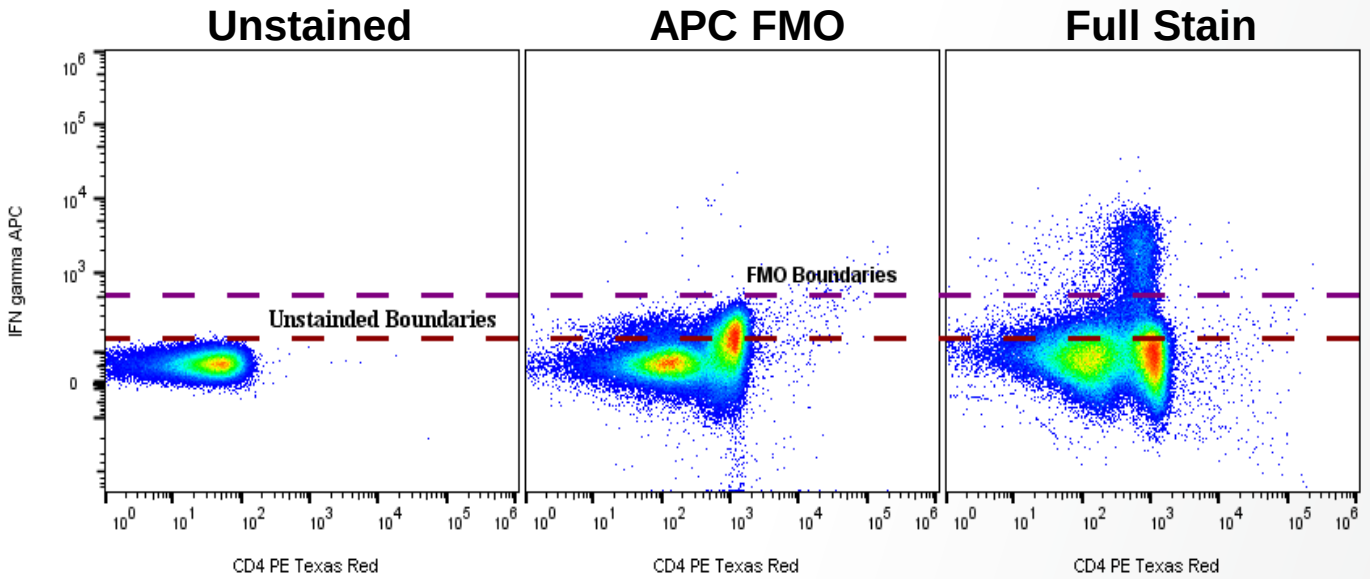
- Discrete distribution
- 50% positive
- Easy to identify positive population

Compensation Beads

	UltraComp eBeads Plus compensation beads	UltraComp eBeads compensation beads	OneComp eBeads compensation beads	AbC™ Total Antibody Compensation Bead Kit
Reactivity	Human, rabbit, hamster, mouse, and rat antibodies with recognition of the kappa and lambda chains.	Hamster, mouse, and rat antibodies with recognition of the kappa and lambda chains.	Hamster, mouse, and rat antibodies with recognition of the kappa and lambda chains.	Mouse, rat, hamster, and rabbit antibodies with recognition of the kappa and lambda chains.
Laser compatibility	UV to 633 nm Improved for polymer dye use from violet laser	UV to 633 nm	Not with UV or violet lasers.	UV to 633 nm

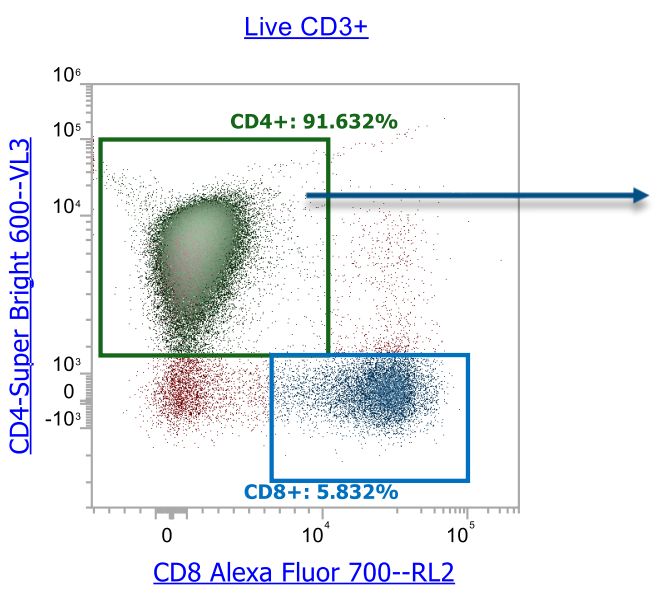
Fluorescence Minus One (FMO)

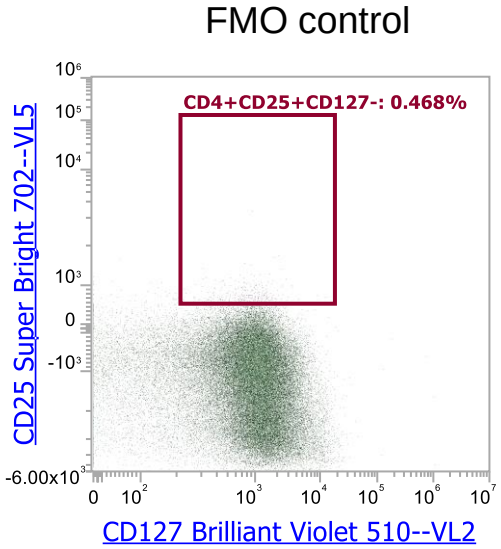


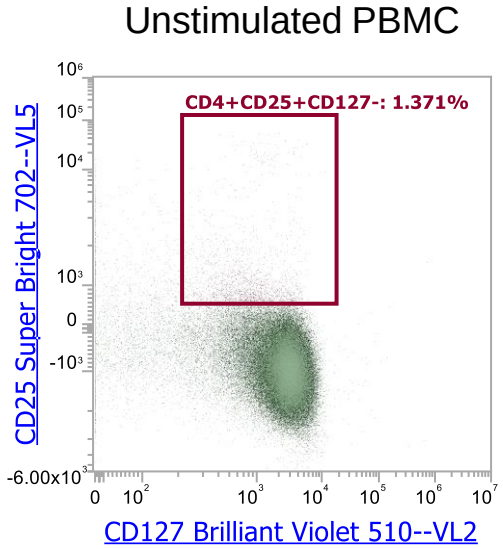


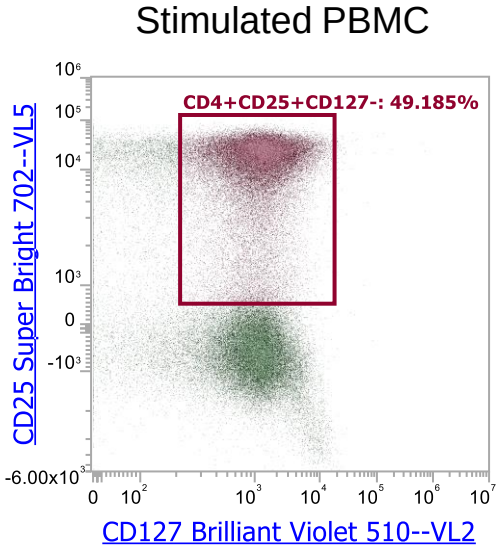
- Setting gates is not arbitrary!
- Don’t rely only on unstained
- Use FMO to take into account spreading from compensation
- Set up FMO for every color for pilot study
- Especially important when -/+ populations are not easily resolved

Un-Stimulated Controls Inform on Gating Strategy









Unstimulated cells is an important biological control for gating strategy

Summary

Steps:

1 Target determination

Choose the phenotyping and functional markers to analyze your cells of interest

2 Buffer selection

Determine which buffers will provide optimal staining

3 Panel design

Select fluorochromes to optimize core markers and minimize spillover

4 Biological controls

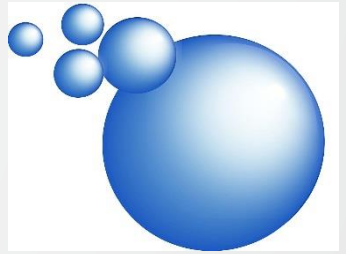
Include viability and stimulation controls to remove dead cells and analyze accurately

5 Experimental controls

Set up experimental controls to compensate for spillover

Analyze Extracellular Vesicles by Flow Cytometer

Flow cytometry: what does it mean?



CYTOMETRY

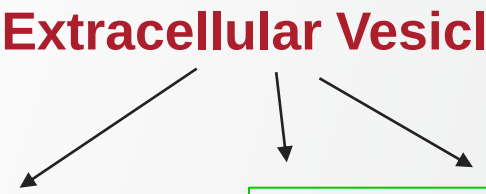
Cell Measurement

measurement of a ~~cell~~ particle

EV formation and release

Extracellular Vesicles

- Family of small membrane vesicles
- Found in the extracellular environment
- Carry information about their cell of origin
- Phospholipid bilayer
- Found in all body fluids
- Found in cell cultures



Exosomes

- Multi-Vesicular Body (MVB) formation
- MVB outer membrane fuse with the cell membrane
- Vesicles inside the MVB exit the cell
- Outside of the cell membrane is on the outside of the exosome

Microvesicles

- Outward budding of the cell membrane
- Outside of the cell membrane is on the outside of the microvesicle

Apoptotic Bodies

- Cell fragmentation, often called blebbing

Terminology

Journal of Extracellular Vesicles
Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the *International Society of Extracellular Vesicles*
https://doi.org/10.1080/20013078.2018.1535750 Open access

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

- ISEV, ISAC and ISTH joint working group EV guidelines

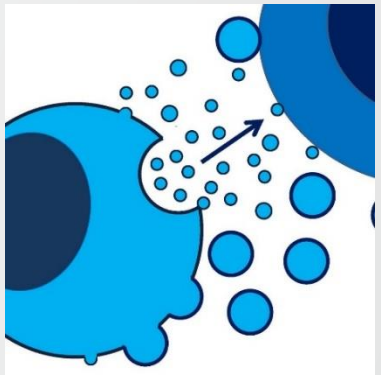
https://www.tandfonline.com/doi/full/10.1080/20013078.2020.1713526 Open access

"**ISEV endorses "extracellular vesicle" (EV) as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate.**

Since consensus has not yet emerged on specific markers of EV subtypes, such as endosome-origin "exosomes" and plasma membrane-derived "ectosomes" (microparticles/microvesicles) assigning an EV to a particular biogenesis pathway remains extraordinarily difficult unless, e.g. the EV is caught in the act of release by live imaging techniques."

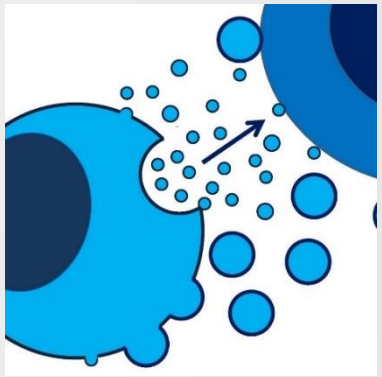
Extracellular Vesicles increasing interest

- Interest in studying EVs has grown exponentially in the last decade
- EVs come from many different cell types and fluid sources
  - Cultured Cells, Tumor and Cancer Cells, Normal Cells
  - BioFluids (plasma, urine, CSF)
- Evidence that EVs play an important role in health and disease
  - Involved in intercellular communication
  - May transfer cargo
- Measurement of EVs and their cargo help in understanding their origins, potential targets, and possible functions

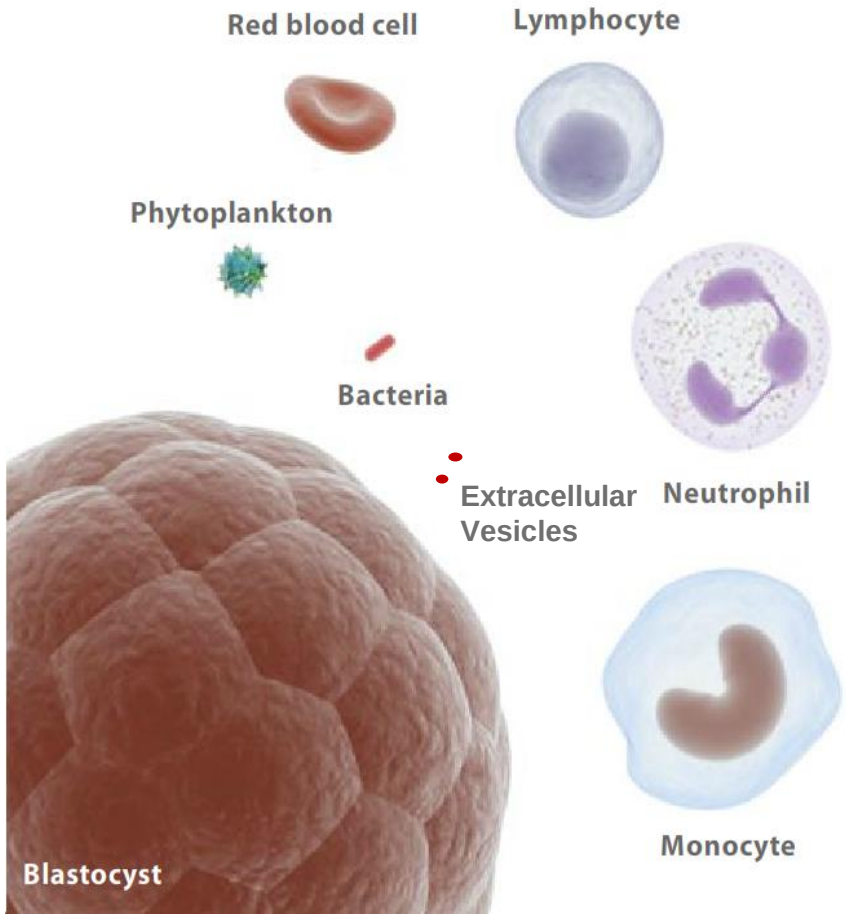


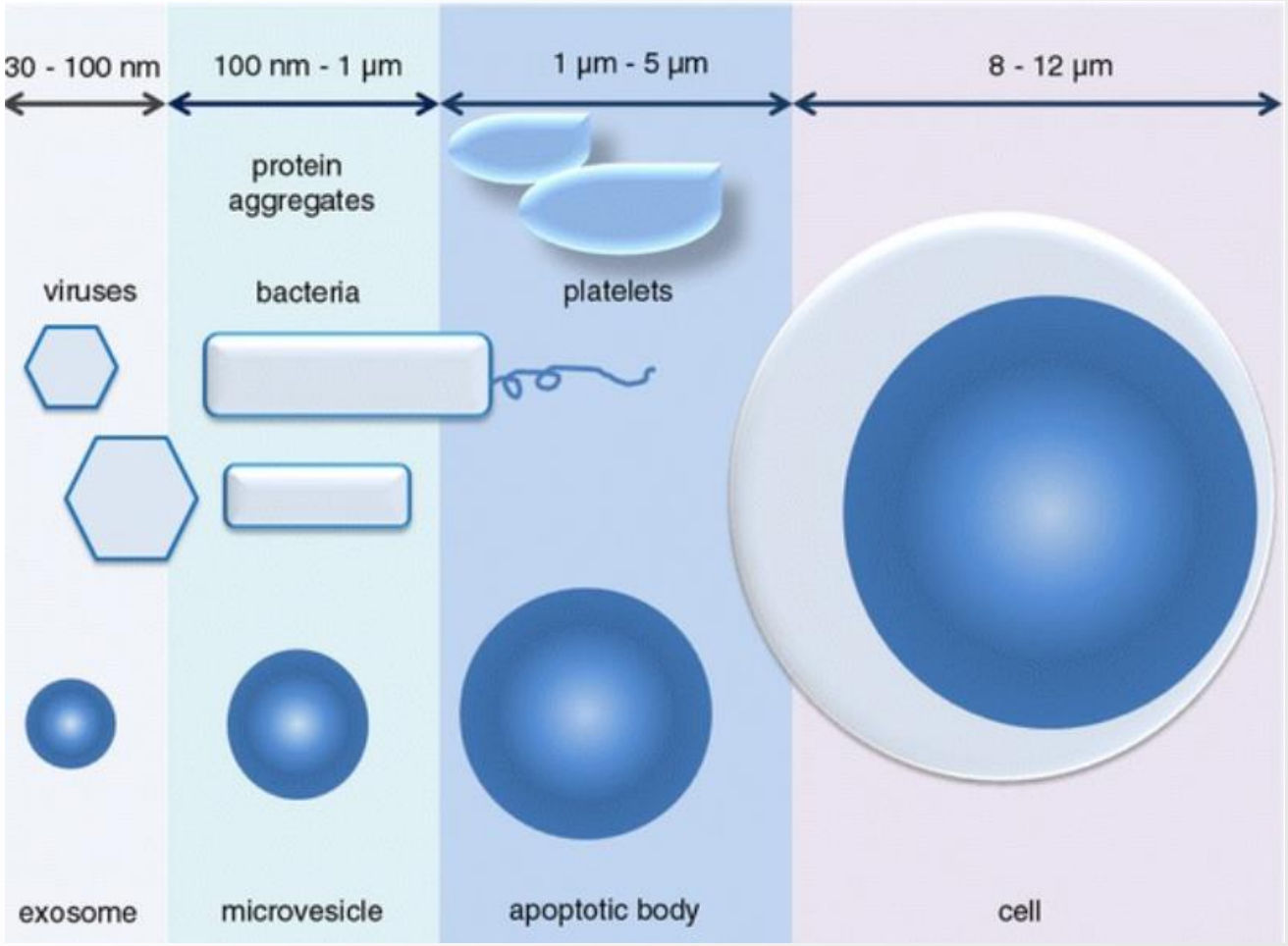
Extracellular Vesicles increasing interest

- EVs can be loaded with multiple proteins, nucleic acids, DNA, RNAs (mRNA, miRNA, ncRNA), lipids, and other molecules (i.e., biomolecules of viruses)
- Represent potential diagnostic and prognostic targets
  - Biomarker for cancer and immunosuppression
  - Cardiovascular disease
  - Infectious diseases
  - Inflammation



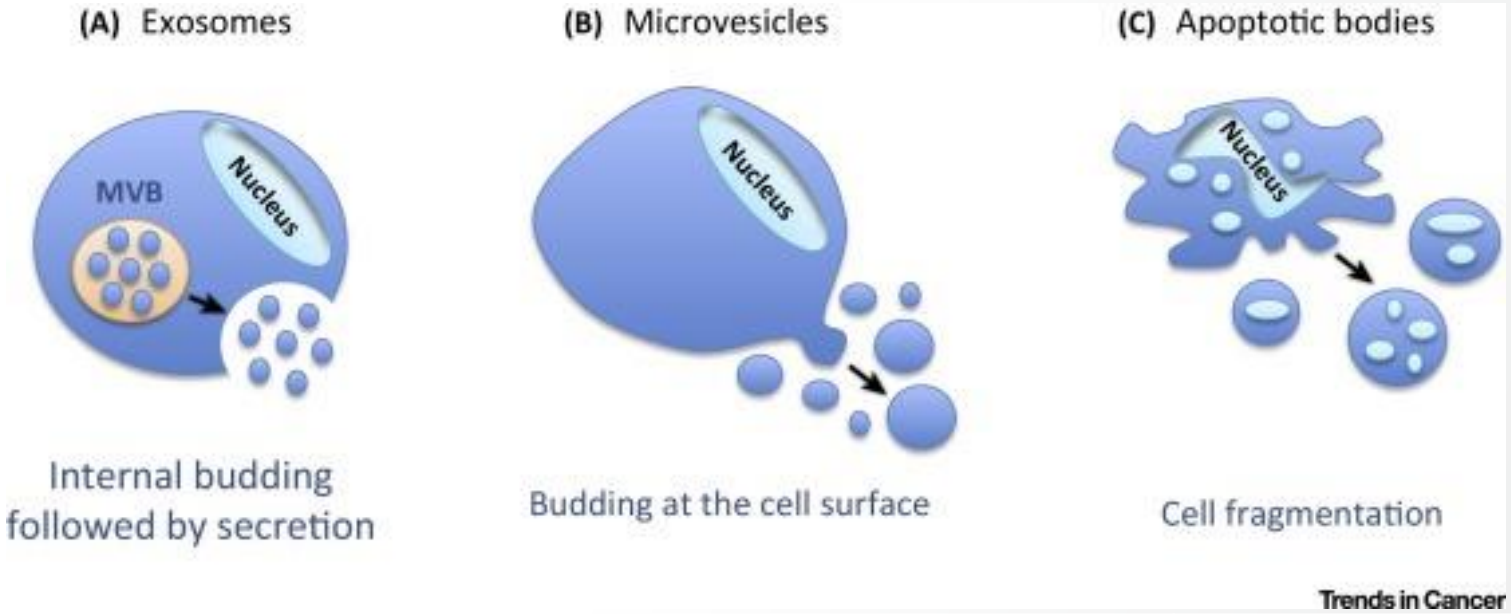
Relative sizes





György, B., Szabó, T.G., Pásztói, M. et al. Cell. Mol. Life Sci. (2011) 68: 2667. https://doi.org/10.1007/s00018-011-0689-3

Extracellular vesicles

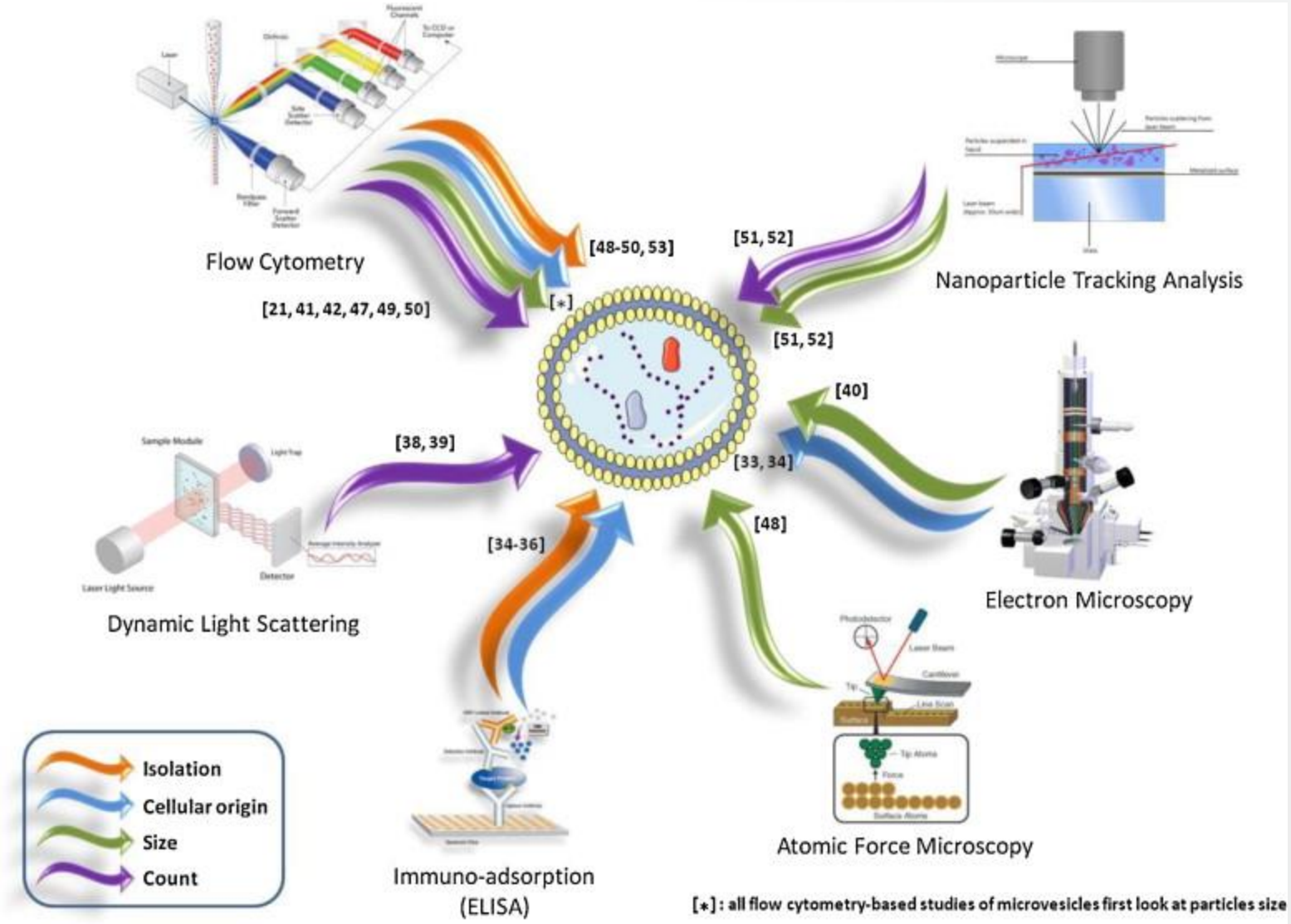


	Exosomes	Microvesicles	Apoptotic Bodies
Size	30-100 nm	100-1000 nm	1000-5000 nm
Shape	regular	irregular	irregular
Origin	Endosomal compartments	Cell Surface Plasma Membrane	Cells undergoing Apoptosis

Adapted from: Oncology Reports 35(35):1237-1224 Dec 2015

Benefits and Challenges of Small Particle Analysis

Extracellular Vesicle characterization methods



Jean-Daniel Tisson, et al, Translational Proteomics 2013, https://doi.org/10.1016/j.trprot.2013.04.004

Extracellular Vesicle characterization methods

Two Categories:

1. Total or aggregate EV measurements
2. Individual EV measurements: important in understanding heterogeneity

Characterization Method	Assay Type	Measurements
Flow Cytometry	Individual and Total Vesicles	Concentration, Cargo, Size*
Nanoparticle Tracking Analysis (NTA)	Individual Vesicles	Concentration**, Size
Resistive Pulse Sensing (RPS)	Individual Vesicles	Concentration**, Size
Dynamic Light Scattering (DLS)	Individual Vesicles	Size
Actomic Force Microscopy	Individual Vesicles	Visual Confirmation, Size
Electron Microscopy (EM)	Individual Vesicles	Visual Confirmation, Size
Western Blot	Total Vesicles	Cargo
ELISA	Total Vesicles	Cargo
* with appropriate size standards		
**Particles, not vesicle specific		

Benefits of flow cytometry

- Records data from single particles
- Makes measurements on large numbers of particles
- Thousands of particles can be analyzed rapidly
- Rich statistical analysis on populations
- Because single particles are measured, heterogeneity can be revealed
- Ability to archive standardized files
- Flexibility of data analysis with the ability to re-analyze data
- Ability for automated high throughput acquisition
- Ability to multiplex
- Counting ability

Benefits of flow cytometry in small particle testing

Single particle analysis

- Ability to analyze small particle rapidly and individually
  - Traditional methods of EV analysis are limited in the capacity to analyze individual particles
  - Time-consuming and expensive options limit EV studies
- Capable of analyzing thousands of particles per second, improving statistics, and allowing the quantification of unique and rare particle types.

Particle counting and concentration

- A significant advantage in both speed and accuracy for counting methods
- Obtain counts by direct volumetric counting with the Invitrogen™ Attune™ NxT Flow Cytometer

Ability to Multiplex

- More information can be obtained by simultaneous analysis with fluorescent labels such as surface proteins, membrane lipids, cellular esterase

Challenges with EV detection using flow cytometry

Identity

Small size of EVs, their heterogeneity, and low refractive index make them difficult to identify[1,3]

High Coincidence

Multiple small particles can be erroneously detected as single microvesicles[1,2]

Detection

Fluorescent labeling of EVs required for optimal detection[1]

1. Nolan, J.P. 2015. Flow cytometry of extracellular vesicles: potential, pitfalls, and prospects. *Curr. Protoc. Cytom.* 73:13.14.1-13.14.16. doi: 10.1002/0471142956.cy1314s73
2. Rousseau M, Belleannee C, Duchez A-C, et al. Detection and Quantification of Microparticles from Different Cellular Lineages Using Flow Cytometry. Evaluation of the Impact of Secreted Phospholipase A_2 on Microparticle Assessment. Combes V, ed. *PLoS ONE*. 2015;10(1):e0116812.
3. Pospichalova V, Svoboda J, Dave Z, et al. Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. *Journal of Extracellular Vesicles*. 2015;4:10.3402/jev.v4.25530.

Tips for Success

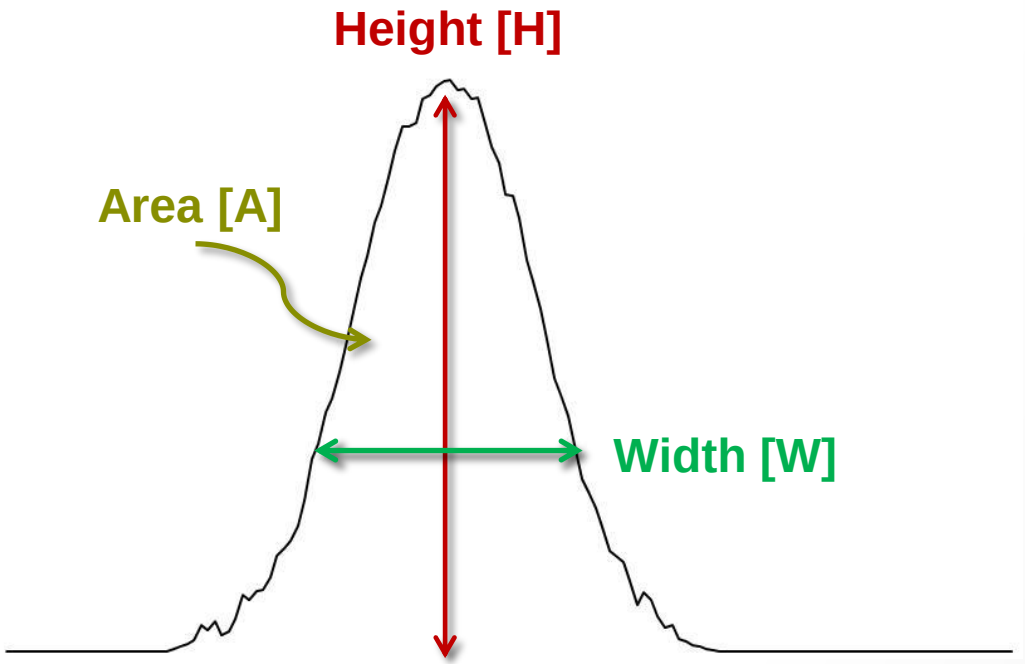
Anatomy of a light pulse

Particles flow through a focused spot of light

This generates a pulse of light

Anatomy of a light pulse

The Gaussian-shaped light pulses are measured



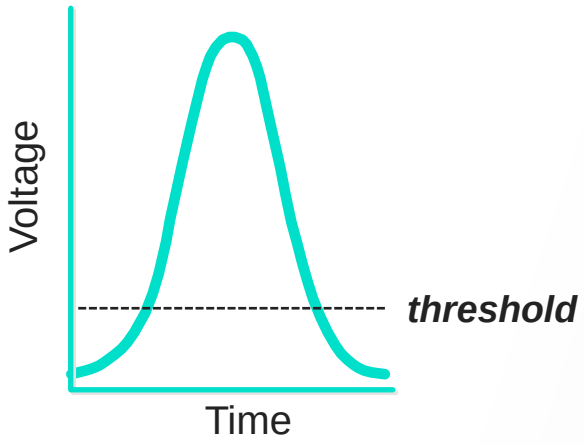
Data is derived from these light pulses

The Area, Height, and Width refer to the optical pulses

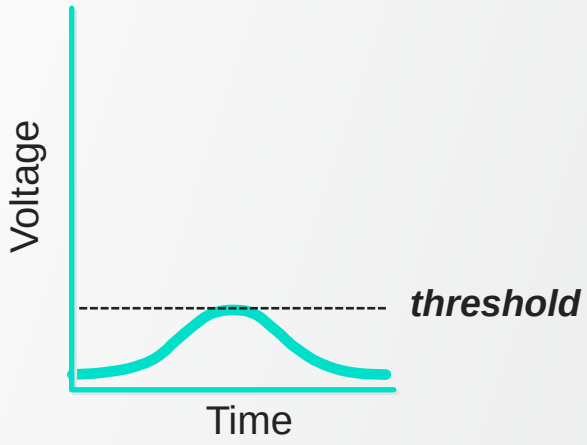
Threshold in flow cytometry

Threshold is an electronic hurdle that establishes a criteria whether events are recorded or not

Above threshold:
A pulse is recorded



Below threshold:
pulse Not recorded

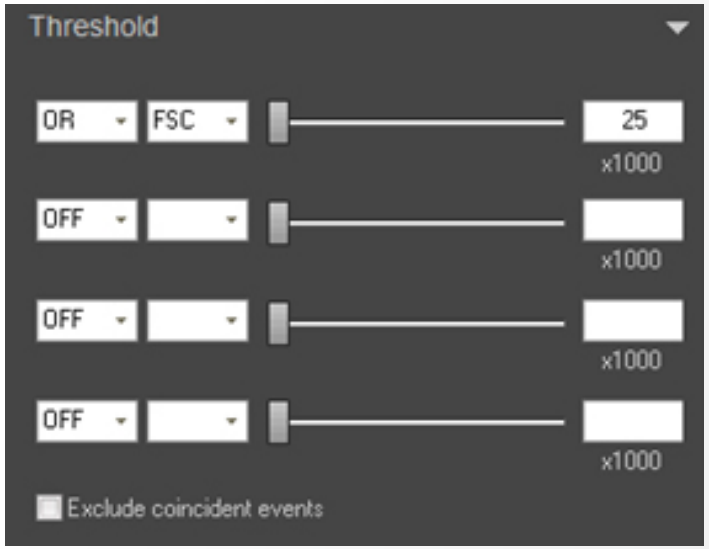


Threshold used in small particle analysis

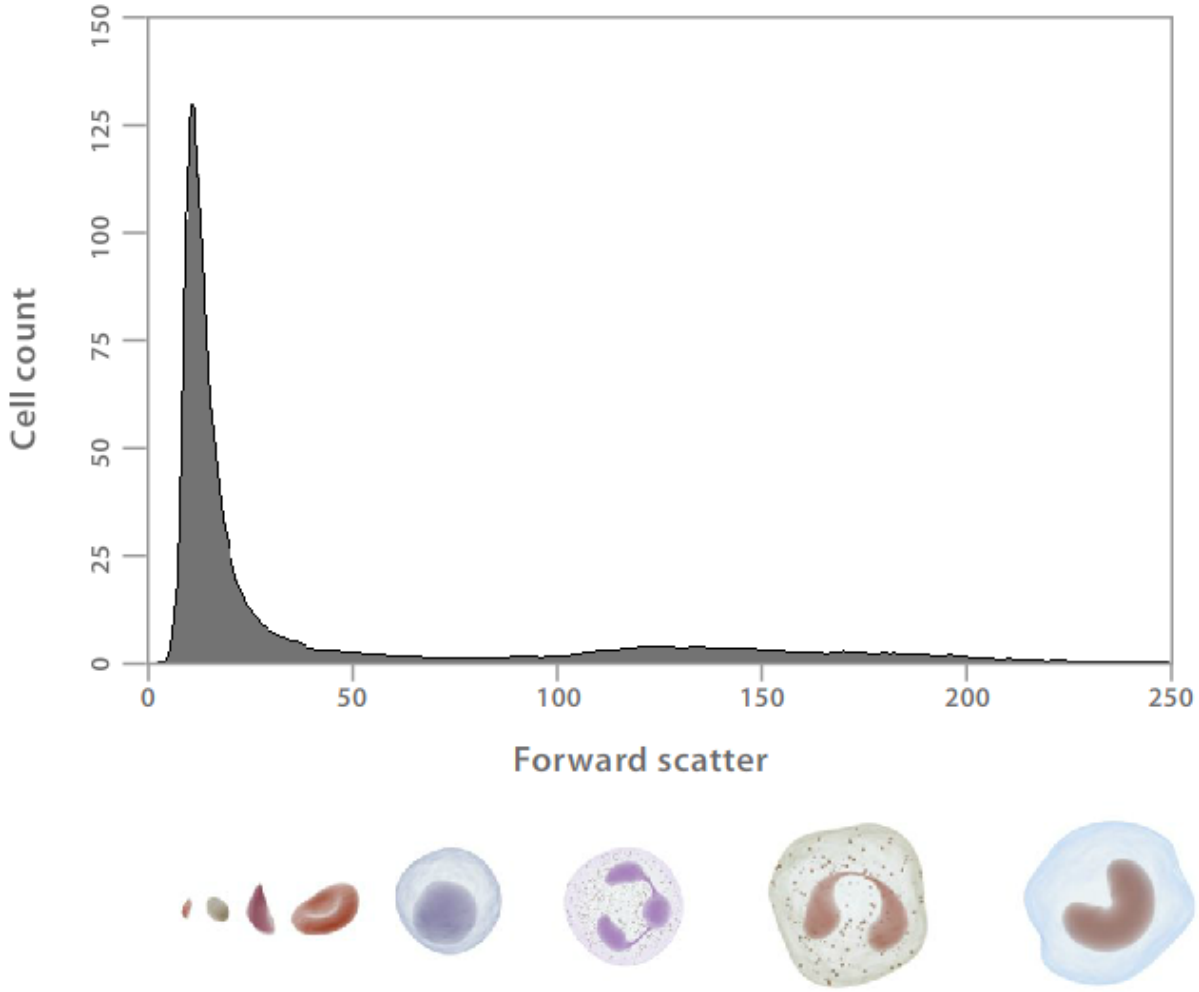
If every single particle passing through the laser caused the instrument to collect data, the data would be dominated by information coming from a large number of minute particles.

To prevent this, a threshold is set such that a certain size or fluorescent intensity must be exceeded for the instrument to collect data.

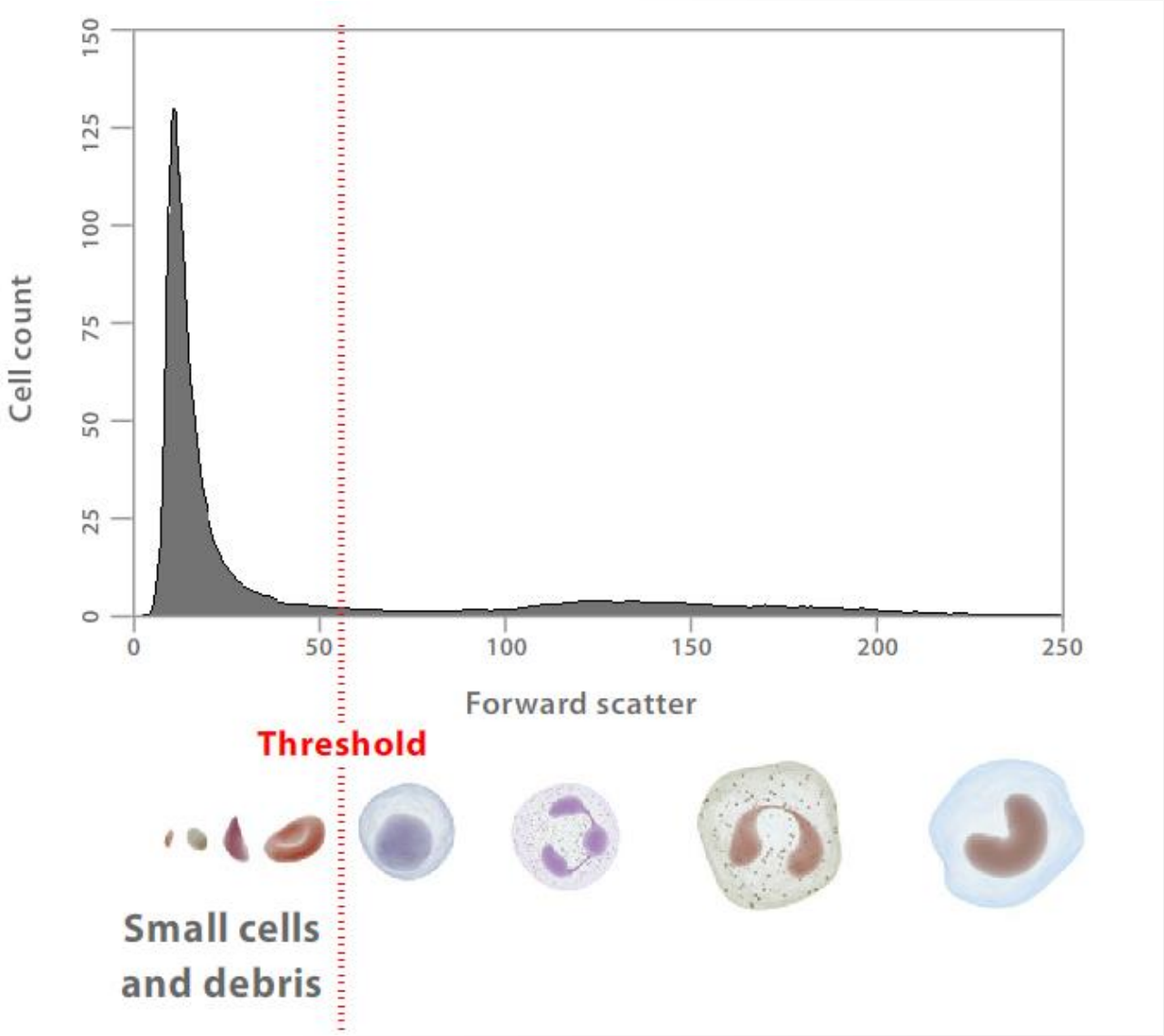
Thresholds may be set on a scatter parameter, or a fluorescence parameter, or a combination of parameters.



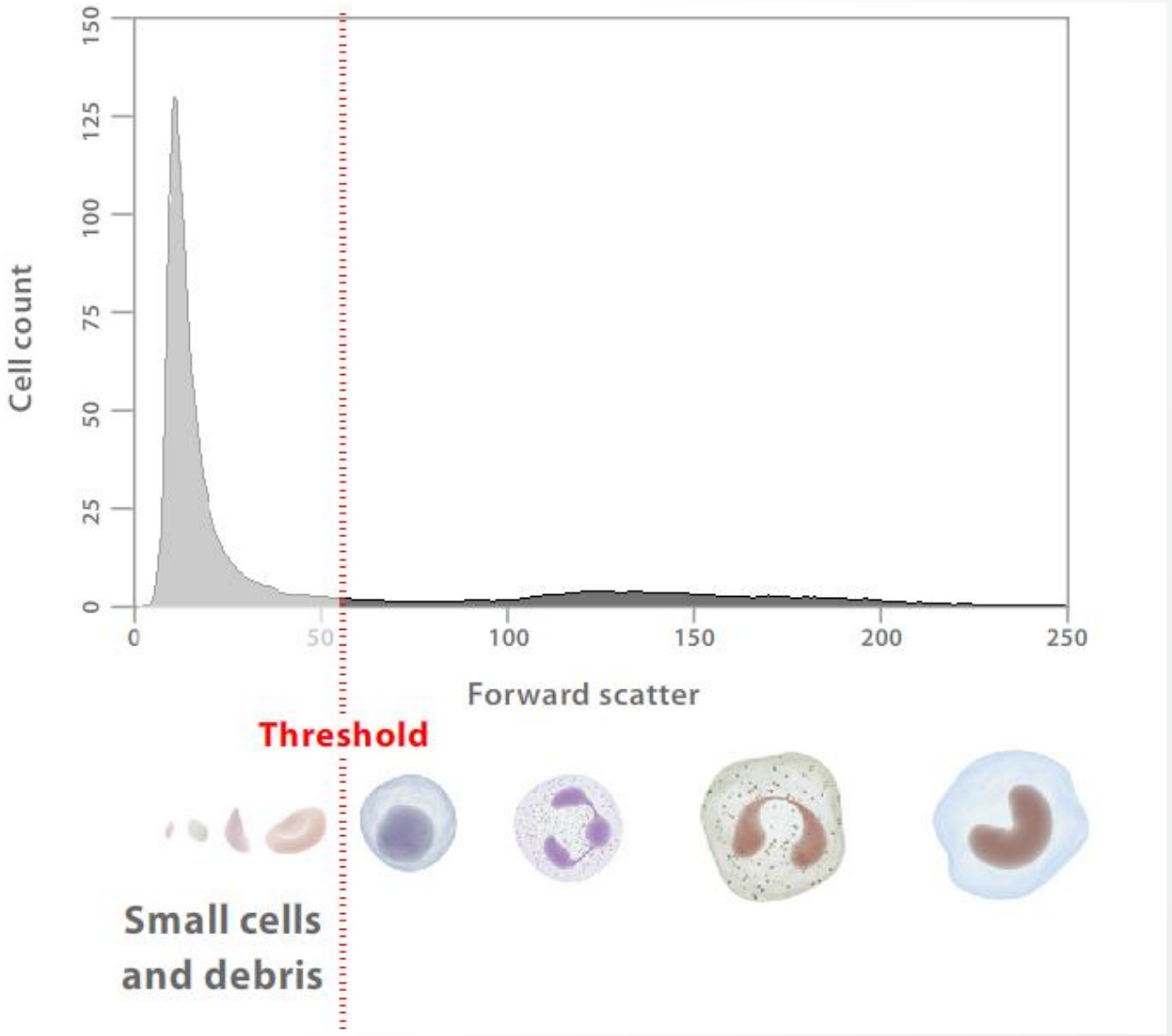
Forward scatter threshold



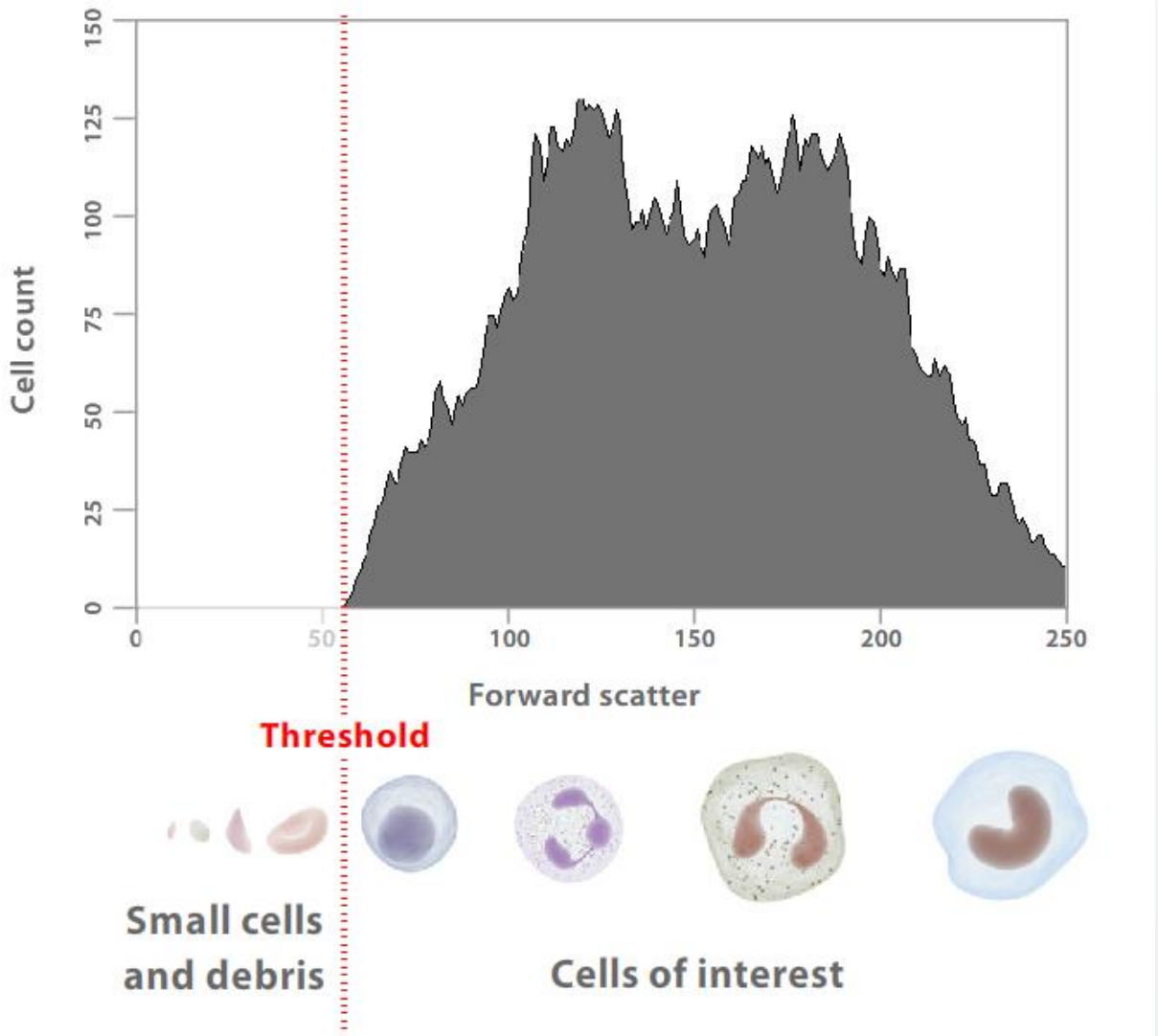
Forward scatter threshold



Forward scatter threshold

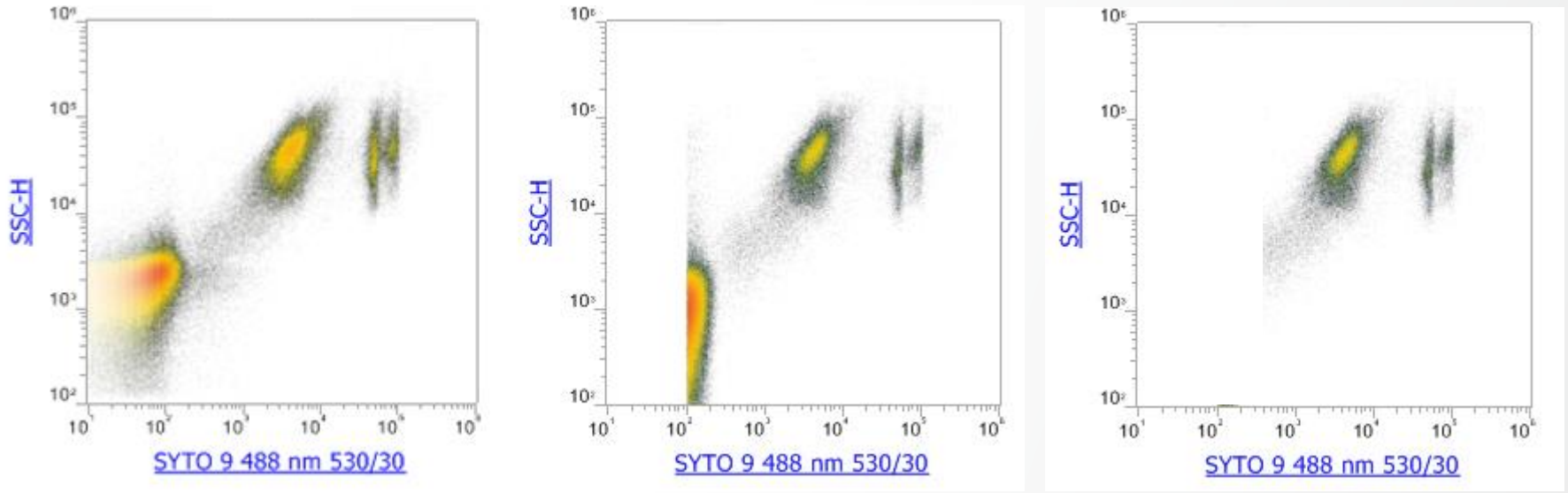


Forward scatter threshold



Fluorescence threshold

Increasing Fluorescence Threshold
Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid stain (BL1-H)

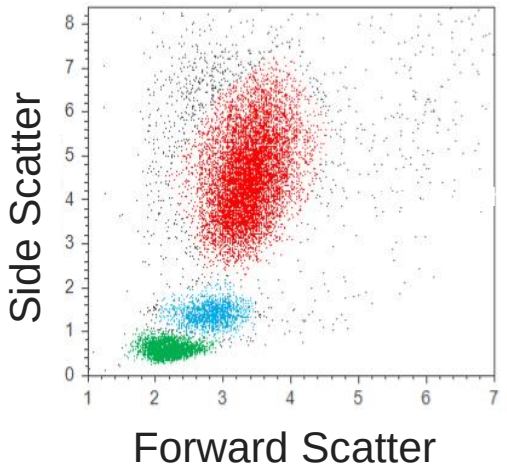


Adjustment of fluorescence threshold is done empirically

- While acquiring sample, and
- Before Recording sample

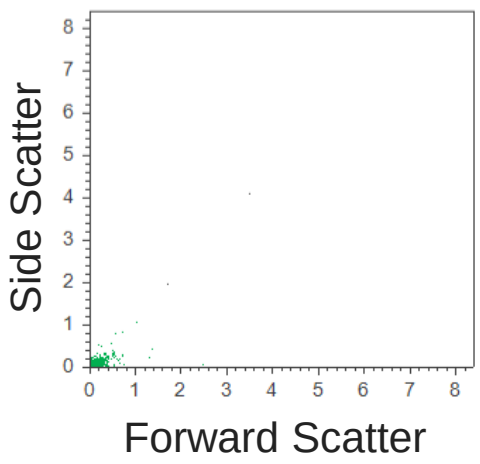
Linear vs Log Display for FSC and SSC

Whole human blood, lysed (~5-14 µm)



Linear scale

Small particles (~1µm)



Use Log Display for small particle detection

Pre-analytic considerations

Pre-analytic steps to consider:

- Source material
  - Collection
  - Transport
  - Storage
- Isolation/enrichment technique
- Exosome-depleted products
- Filter fluids
  - Sheath Fluid
  - Buffers
- Choice of Ab/fluorophore/dye
  - Size
  - Brightness
  - Specificity
- Controls

Sample Preparation

Journal of Immunological Methods

Volume 438, November 2016, Pages 11–20

Research paper

The impact of various preanalytical treatments on the phenotype of small extracellular vesicles in blood analyzed by protein microarray

Rikke Bæk, Evo K.L. Søndergaard, Kim Varming, Malene M. Jørgensen

Department of Clinical Immunology, Part of Extracellular Vesicle Research Center Denmark (EVsearch.dk), Aalborg University Hospital, Aalborg, Denmark

Received 5 April 2016, Revised 23 August 2016, Accepted 23 August 2016, Available online 24 August 2016

Gibco™ Fetal Bovine Serum, exosome-depleted

Sample Isolation methods

EV Isolation, aka Separation, Purification, Enrichment, Concentration

- Usually required for subsequent analysis
- Each method has its strengths and limitations

Primary Isolation Methods

- Ultra-centrifugation
- Combinations of Methods
- Density Gradient
- Filtration
- Size Exclusion Chromatography
- Precipitation
- Bead Capture

Total Exosome Isolation Reagent

Journal of Extracellular Vesicles

ORIGINAL RESEARCH ARTICLE

Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey

Chris Gardiner[1]*, Dolores Di Vizio[2], Susmita Sahoo[3], Clotilde Théry[4], Kenneth W. Witwer[5], Marca Wauben[6] and Andrew F. Hill[7]

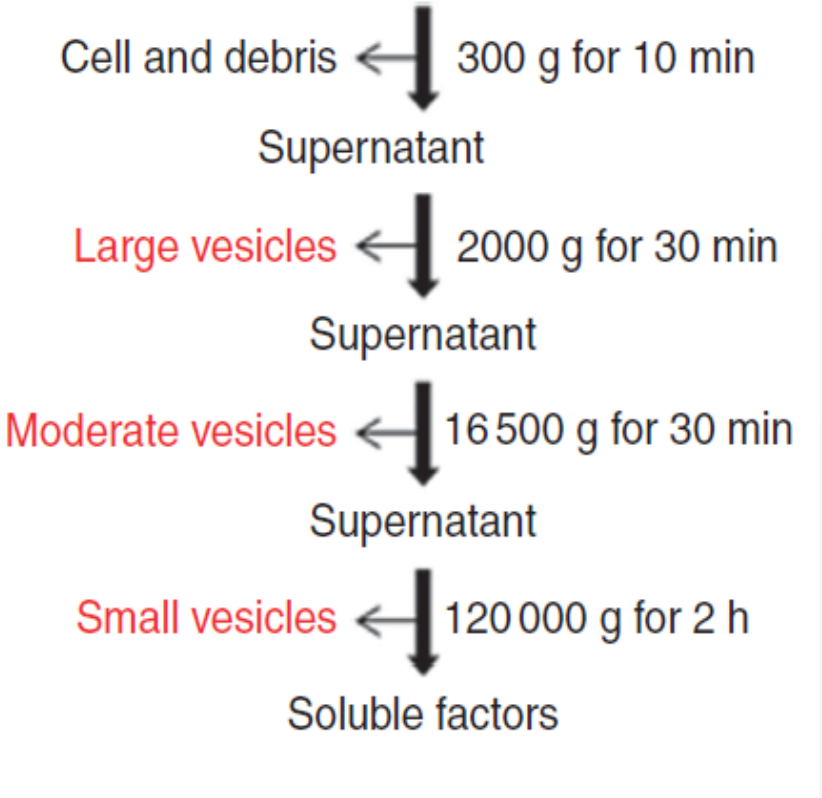
[1]Haemostasis Research Unit, Research Department of Haematology, University College London, London, UK; [2]Icahn School of Medicine, Cardiovascular Research Center, Cedars-Sinai, Los Angeles, CA, USA; [3]Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, NY, USA; [4]Institut Curie, PSL Research University, INSERM U932, Paris, France; [5]Johns Hopkins University School of Medicine, Baltimore, USA; [6]Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [7]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Australia

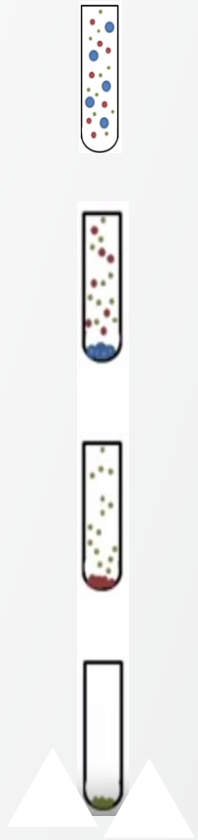
Chris Gardiner, et. al., Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey, Journal of Extracellular Vesicles (2016) 5:1, DOI: 10.3402/jev.v5.32945

OPEN ACCESS

Differential Ultra-Centrifugation

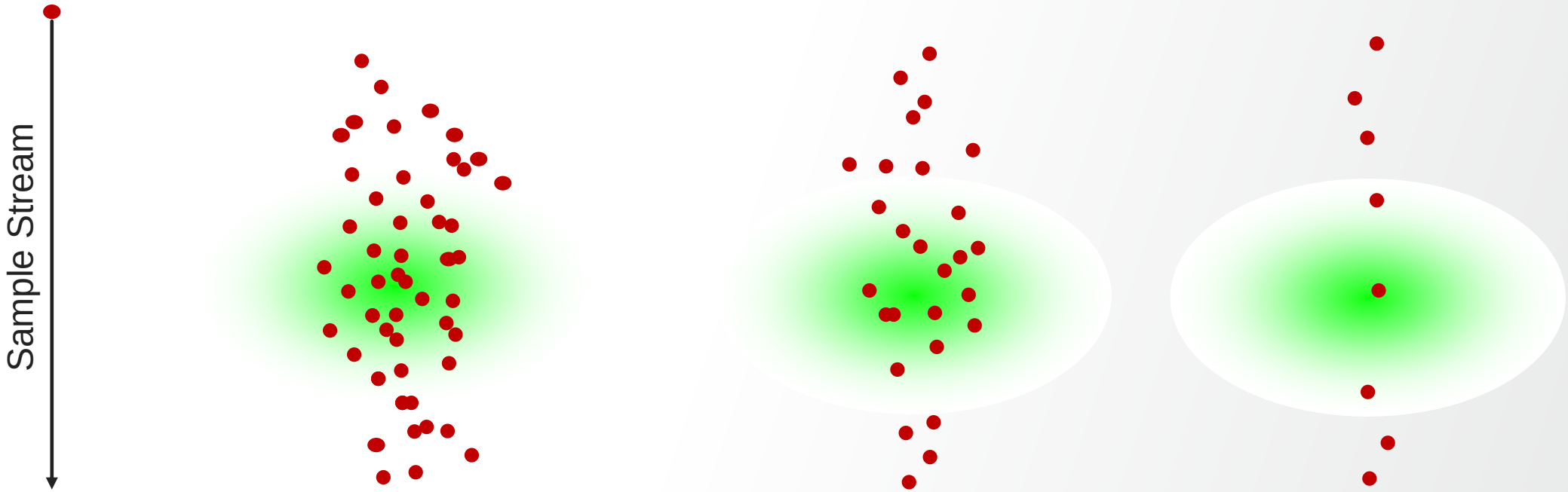
From cell culture supernatant or body fluids





Dilution for single cell detection

- Higher particle concentrations result in coincidence effect (swarm)
- Significant sample dilution is required to ensure single particle detection

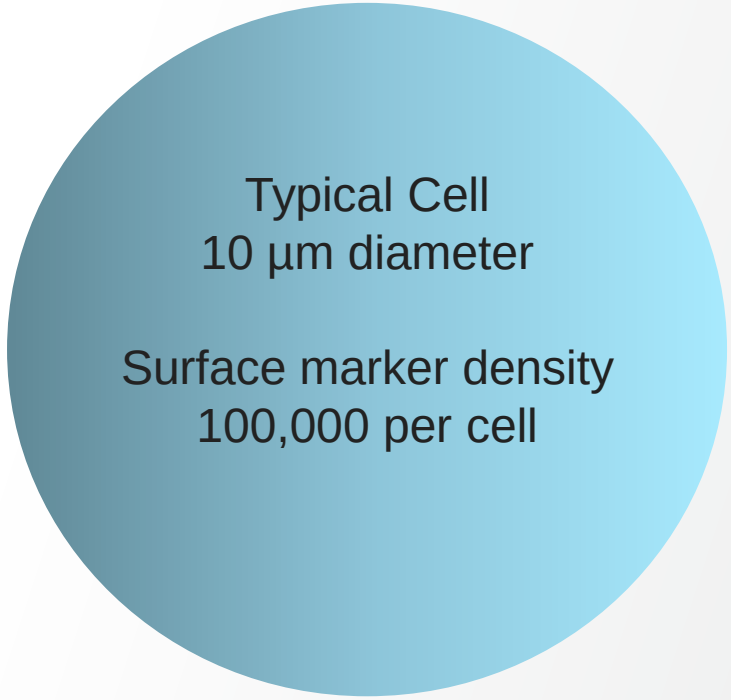


Flow cytometry challenges

EVs have much smaller surface area and volume compared to cells

Several approaches are needed

- Signal Detection
  - Bright Fluorophores
  - More sensitive SSC
- Background Reduction
  - Attention to system cleanliness
  - UltraFilter Instrument Fluids
  - UltraFilter Buffers
- Acquisition/Analysis
  - Coincidence
  - Thresholds



Extracellular Vesicle
100 nm diameter

Surface marker density
~100 per cell

One-millionth volume of
a 10 µm diameter cell

EV labeling

- Labeling Reagent Selection
  - Bright fluorophore
- Antibodies
  - Abundant targets
  - CD9, CD63, CD81 standard

Label all EVs (Fluorescence Threshold)

- Lipid specific Di-8-ANNEPS dye
- Lipophilic styryl compounds
  - FM™ 1-43 dye or FM™ 4-64 dye
- Carbocyanine lipophilic dyes
  - Vybrant™ DiO, DiI, DiD
- Invitrogen™ Bodipy™ dyes
  - Bodipy 493/503
  - Bodipy 581/591
- Invitrogen™ CellTrace™ dyes
  - Numerous ex/em

Additional tips for small particle success

- Background Reduction
  - Use a Fluorescence Threshold
  - Advanced Settings
    - Decrease Windows Extensions
    - Decrease Width Threshold
  - Focusing Fluid with 0.1 µm or smaller Filter
    - ideally filter to 0.03- 0.05 µm
  - Filter buffers with 0.1 µm or smaller Filter
  - Change Focusing Fluid Filters Monthly
- Signal Increase

Choice™ PES Syringe Filters

Attune™ NxT Focusing Fluid Filters

Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane

Guidelines for acquisition & Concentration/Dilution testing

	Sample type	
	0.2–3 µm	>3 µm
Example sample type	Bacteria Microspheres Extracellular Vesicles may require additional instrument prep	Jurkat cells Ramos cells Leukocytes Microspheres Cardiomyocytes
Flow rate	12.5–1,000 µL/min	100–1,000 µL/min
Sample concentration	500–10^6 particles/mL	
Event rate	<8,000 events/sec	
Sample volume	50–4,000 µL	

Recommendation for EVs:

- 12.5 or 25 µL/min flow rate
- Measure ≥ 100 µL Sample Volume
- Record consistent volume every sample
- Keep Event Rate < 8,000 events/sec
- Attention to cleaning: Instrument & between samples
- Ultra-Filter fluids used, rinse containers

Ensure Single Particle Analysis-Eliminate Coincidence

Dilution	Theoretical Events/sec	Observed Events/Sec
1:100		5711
1:200	2816	2881
1:400	1408	1568
1:800	704	750

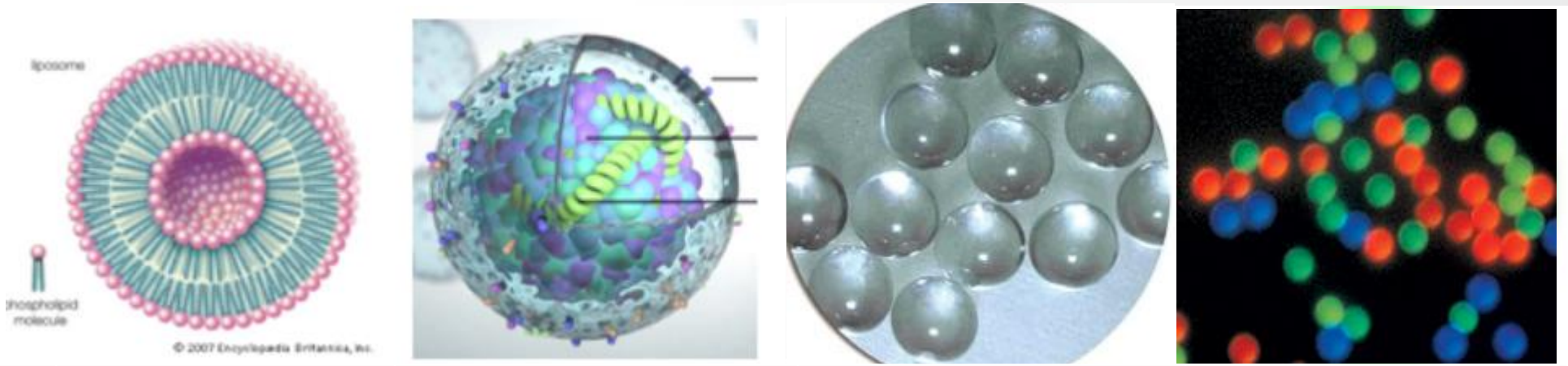
Event Rate decrease in proportion to dilution

Size standardization

- Scatter standardization for sizing of particles relies on theoretical modeling combined with analysis of beads of know diameter and refractive index.
  - Availability of reference particles with refractive index close to EVs for standardization would yield accurate EV diameter data directly, without the need for complex models.
    - Silica beads, hollow silica beads, fluorescent silica beads
    - FP labeled EVs
    - Liposomes of know diameter
    - Combination of fluorescent polystyrene beads with silica beads
  - Limited commercial availability of standards, but growing

Refractive Index and scatter

Scatter Intensity



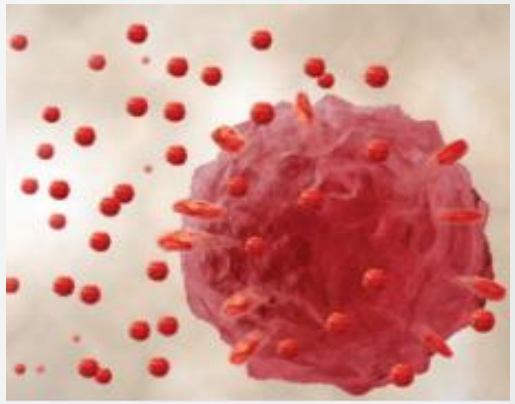
	Liposome	Extracellular Vesicle	Silica Bead	Polystyrene Bead
Refractive Index	1.39	1.37-1.42	1.4	1.59-1.63

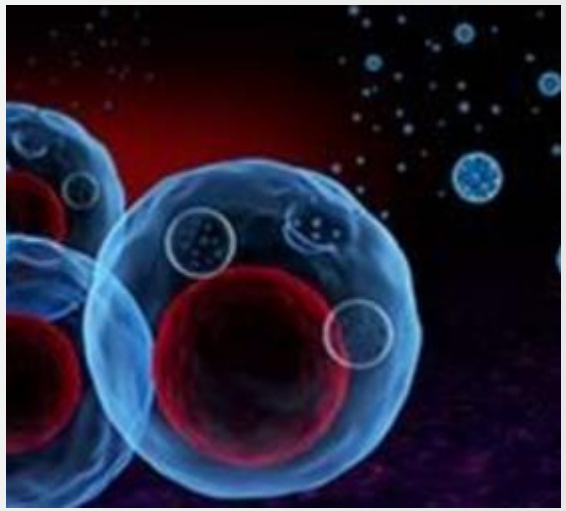
NOTE: Use of Polystyrene beads for Scatter ‘size calibration’ is not recommended due to differences in RI
Instead use a particle closer in RI to the EV; helpful to characterize your instrument too.

Controls

Suggested Controls

- Buffer only
- Buffer + all reagents (no EVs)
- Buffer + EVs only (no stain)
- Sample dilutions of EVs to ensure single particle detection
- Particle for instrument characterization
- Optional: isotype controls
- FMO, single color controls
- Detergent treated for negative control (such as Triton™ X-100 or NP-40)
- Particles for size and fluorescence standardization





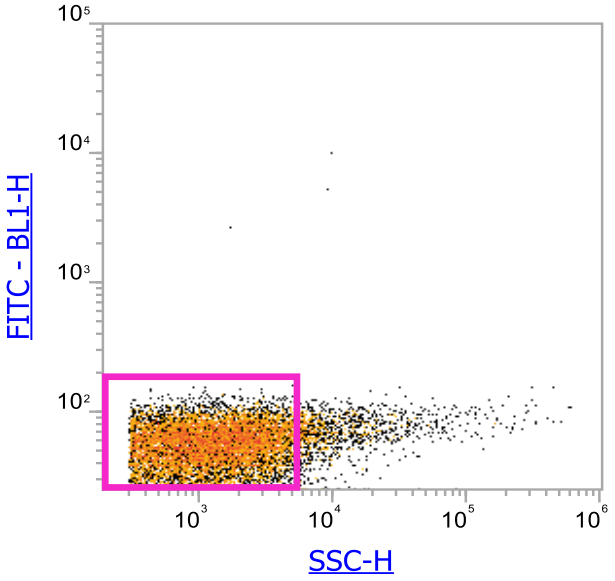
Attune NxT Small Particle Side-Scatter Filter

- Designed to increase the dynamic range of the SSC without sacrificing resolution
- The use of this 488/10 side scatter filter combined with ultra-filtering of the focusing fluid has been found to dramatically decrease background noise and enable discrimination of 100-nm particles
- Do not use the sp-BSSC filter for PT, or for cells
- ID the sp-BSSC in the SSC label
- Signal increase approx. 100-fold

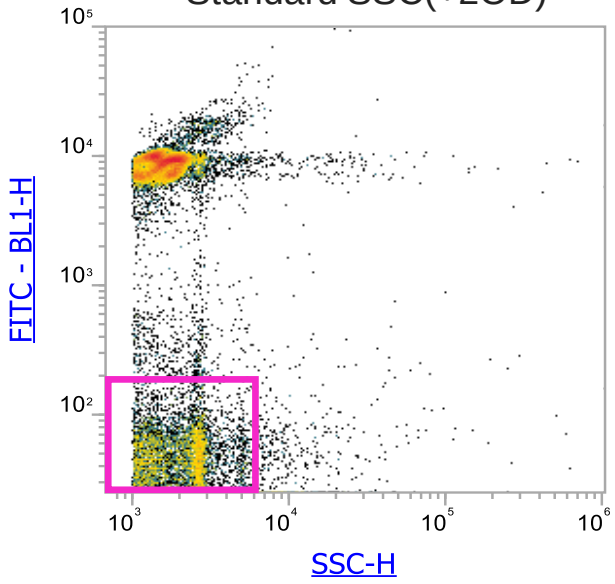
Attune NxT Small Particle Side-Scatter Filter testing

Ultra-Filtration of Focusing Fluid, running ApogeeMix particles:
- Silica beads sizes: 180, 240, 300, 590, 880, 1300 nm (non-fluorescent)
- ApogeeMix centrifuged and suspended in Ultra-Filtered PBS

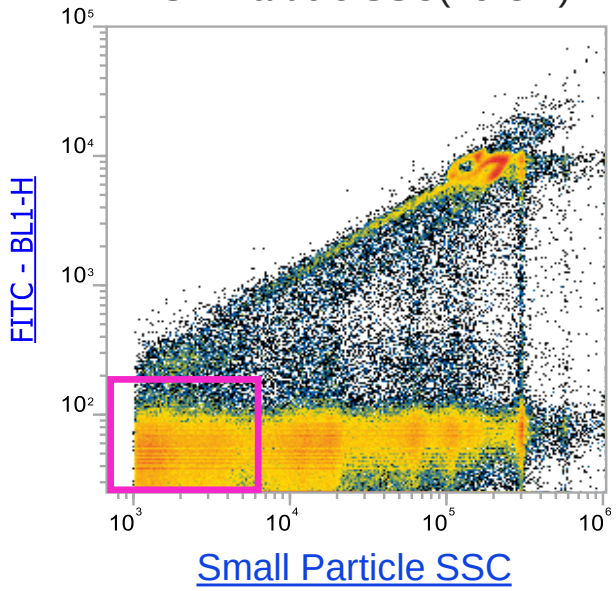
Collecting PBS
Standard SSC+2OD



Collecting ApogeeMix
Standard SSC(+2OD)

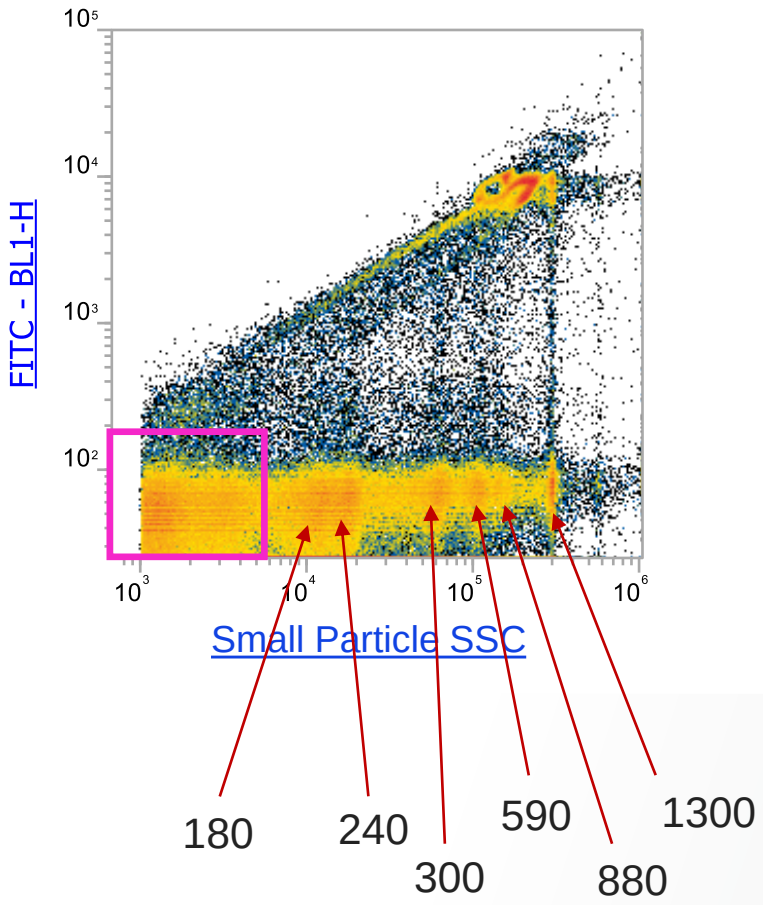


Collecting ApogeeMix
Sm Particle SSC(no OD)



Data from Steve McClellan, Mitchell Cancer Institute, University of South Alabama

ApogeeMix particles with SP Side-Scatter Filter



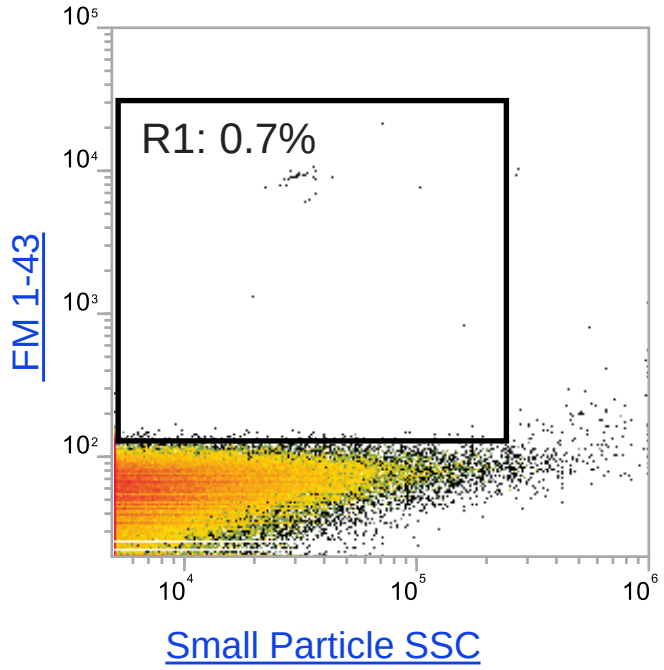
Silica Particle Sizes (nm)
180
240
300
590
880
1300

RI Silica: 1.43
RI polystyrene: 1.59

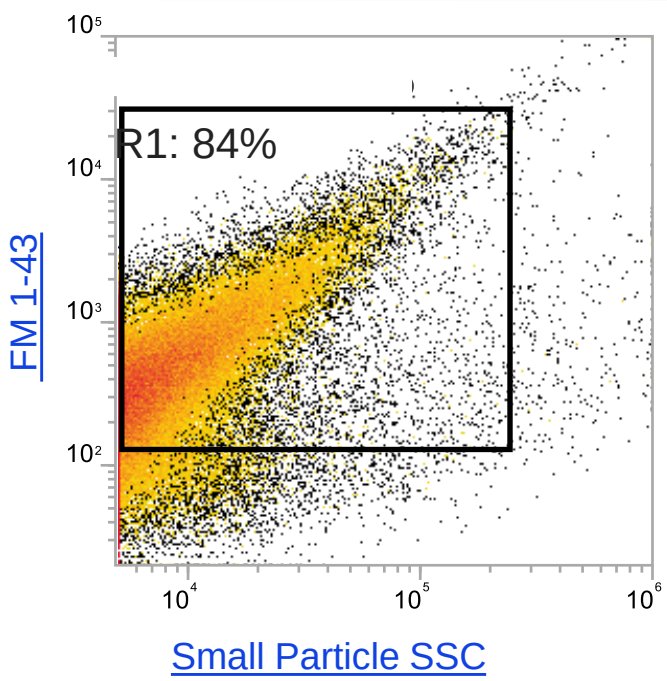
Data from Steve McClellan, Mitchell Cancer Institute, University of South Alabama

EV testing FM 1-43 dye

Unlabeled



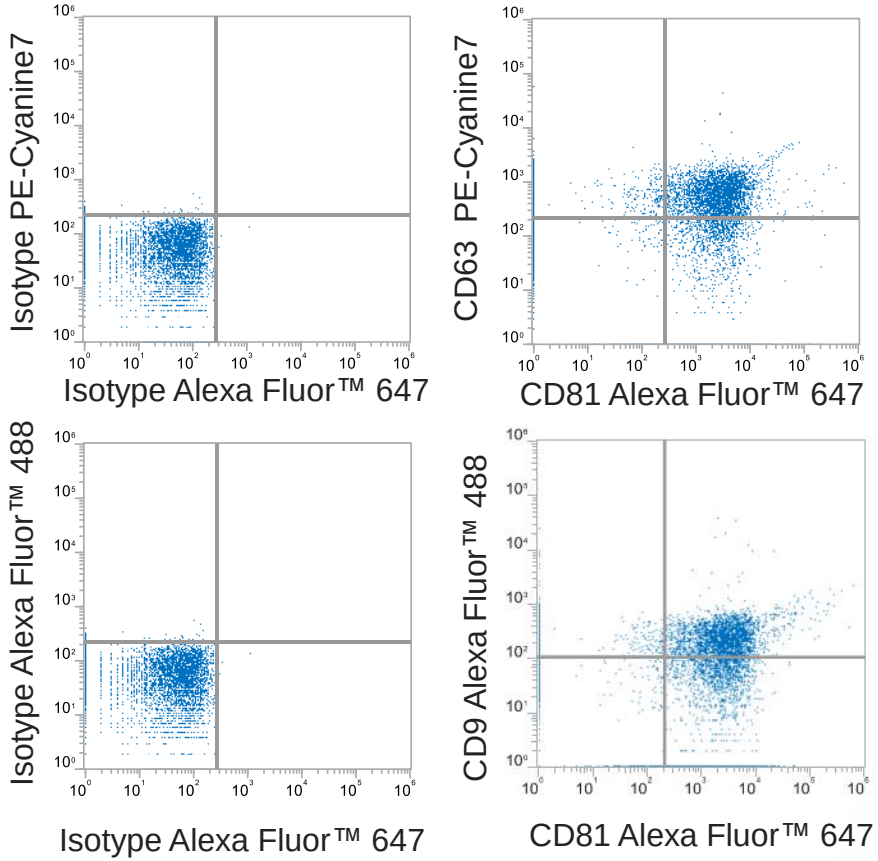
Labeled with FM 1-43 dye



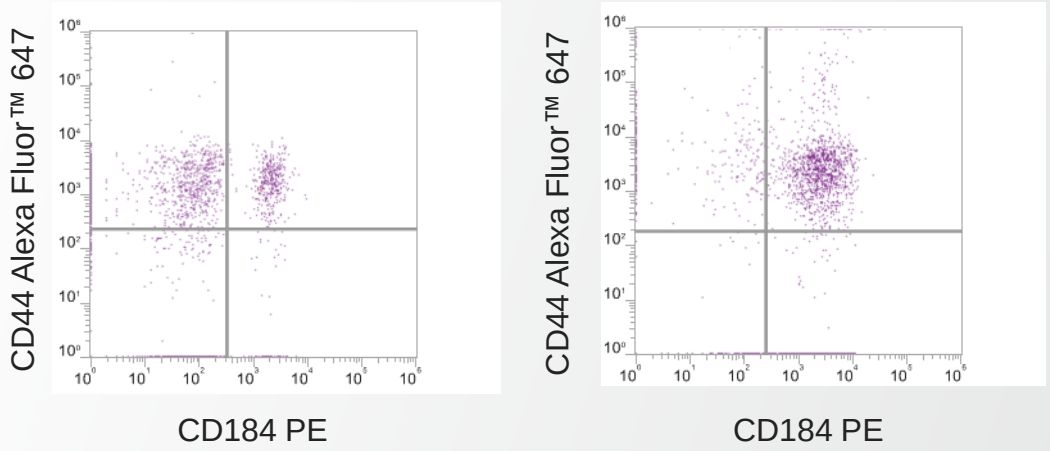
Data from Steve McClellan, Mitchell Cancer Institute, University of South Alabama

EV multi-color testing

Gated on FM 1-43 Positive



Sequential Gate from FM 1-43 Positive, then CD63 PE-Cynanine7 Positive



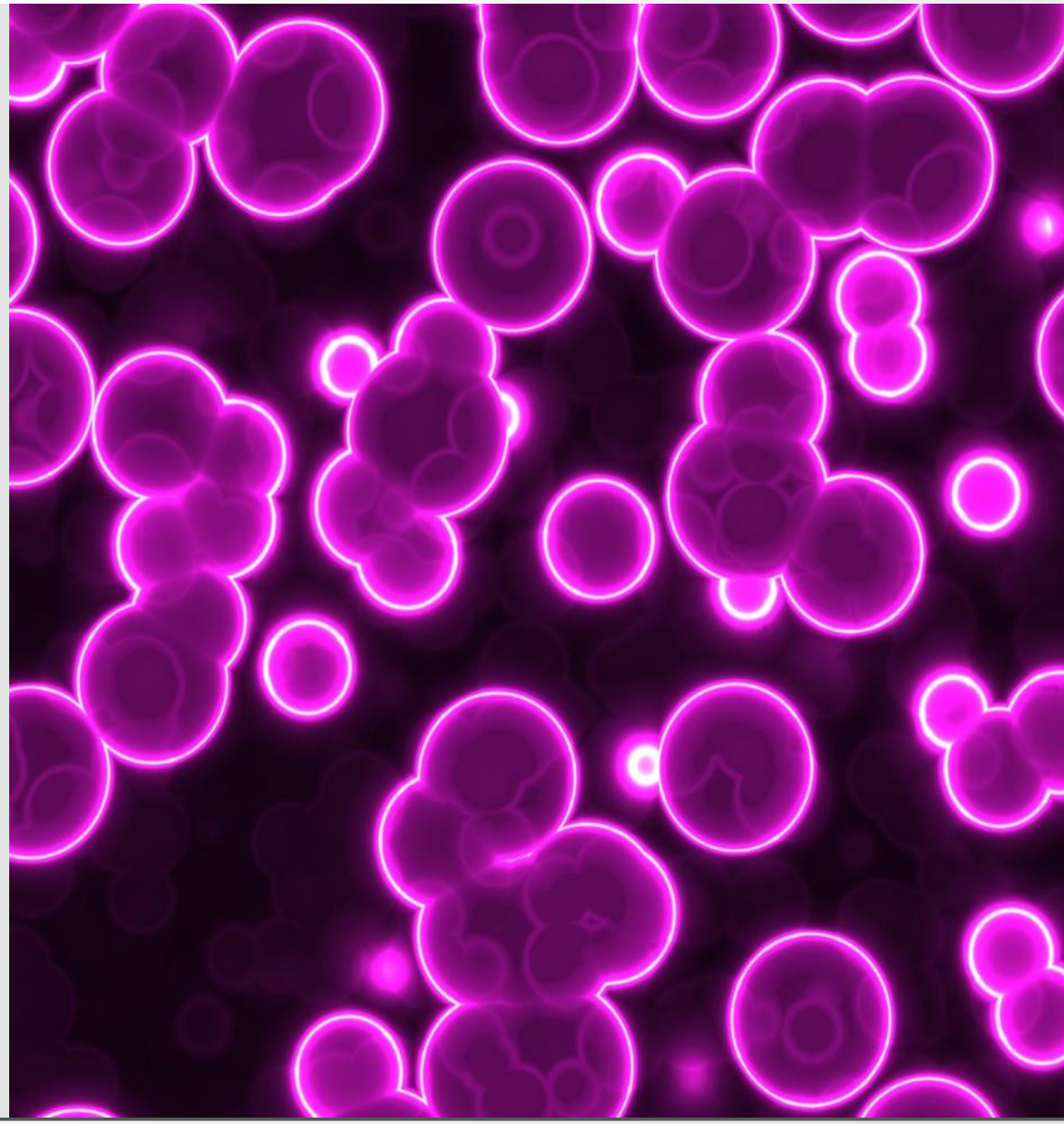
Normoxic Conditions

Hypoxic Conditions

Data from Steve McClellan, Mitchell Cancer Institute, University of South Alabama

Best practices to detect EVs using flow cytometry

- Attention to pre-analytical steps
- Maintain clean instrument
- Attention to cleaning between samples
- Particles for instrument characterization that have a Refractive Index close to EVs
- Sample flow rate set at low speed
- Fluorescence threshold for EV identification, OR
- Filter all fluids including buffer & sheath fluids
- Sample dilution to minimize coincidence
- Treat samples with detergent to confirm EVs
- Bright fluorophore selection
- Use Attune NxT Small Particle Side-Scatter Filter



Thank you

support.tw@thermofisher.com

Rescue Lens App for remote support