Sort

Best practices in panel design to optimize the isolation of cells of interest

23-18846-00

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Cell sorting

Cell sorting: isolation of a population of interest for downstream analysis

- Cell enrichment
- Cell transplantation
- Downstream functional and genomic analysis

Goal: to obtain a pure population with maximum yield

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Increasing resolution for cell sorting

- Clear resolution of a population of interest is critical for an optimal sort.

- How to increase resolution in a sorting setting?
  - Eliminate the impact of unwanted cells
  - Increase the ability to visualize from population of interests.

Increasing resolution for cell sorting

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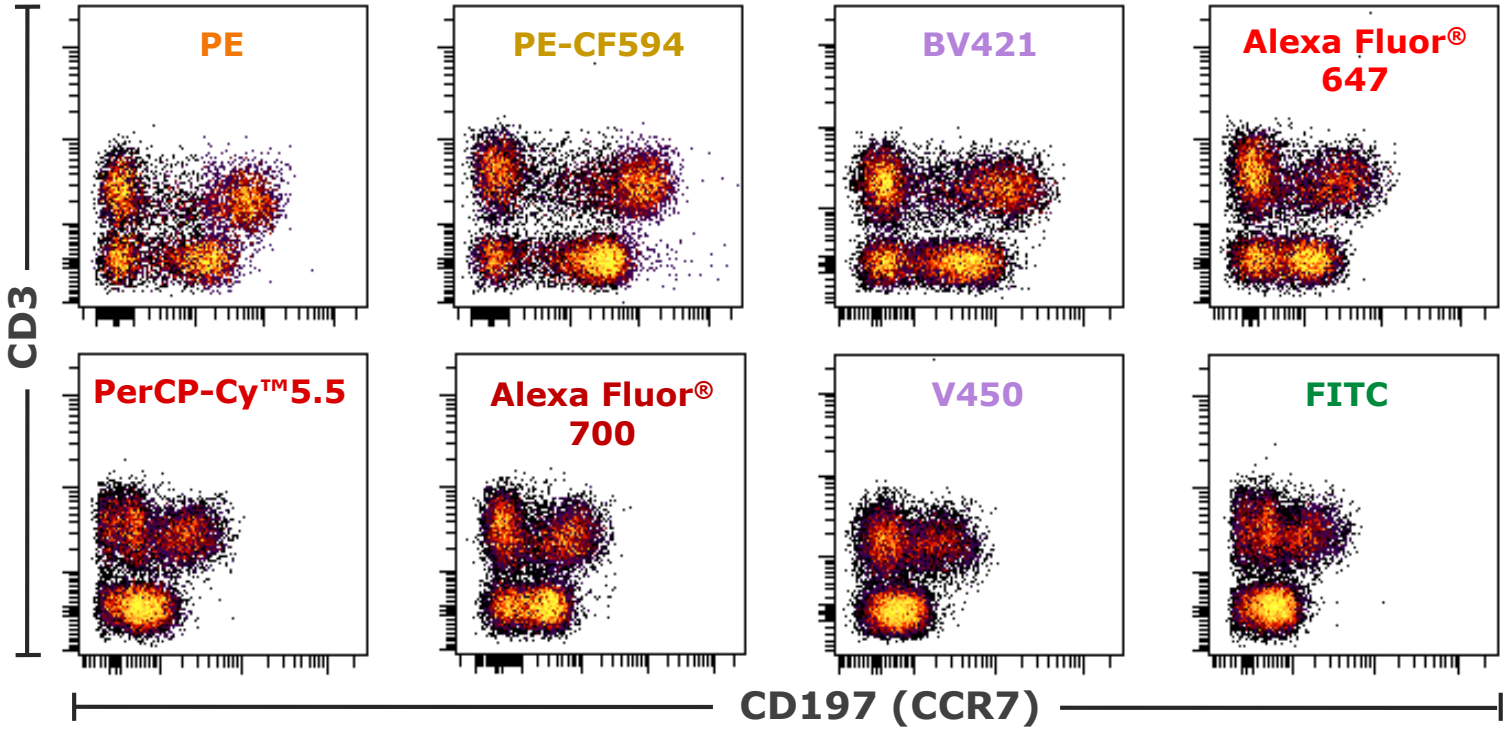

- How to increase resolution in a sorting setting?
  - Eliminate the impact of unwanted cells.
  - Increase the ability to visualize population of interests.

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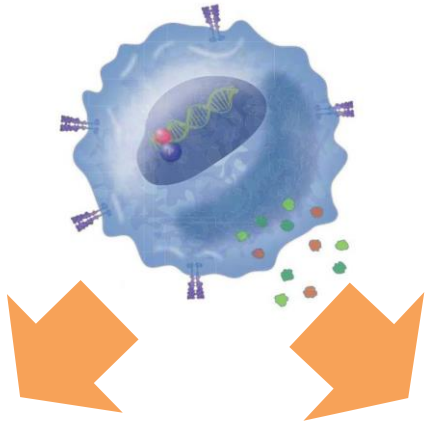
Considerations for cell sorting



Cell analysis

- Fluorochromes
- Biology
- Instrument setup

Cell sorting

- Fluorochromes
- Biology
- Instrument setup
- Sample preparation
- Gating strategy

Considerations for cell sorting: overview

- Experiment setup
  - Sample preparation
  - Instrument settings
- Gating strategy
  - Histogram vs plots
  - Biexponential scale
  - Doublet discrimination
  - Data display
- Panel design
  - Dead cell exclusion
  - Lineage exclusion/depletion
  - Antibody titration
  - Fluorochrome choice

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Considerations for cell sorting

Sample preparation, instrument setup

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Sample preparation

- Cell dissociation/detachment
- Cell resuspension buffer
- Staining volume and antibody concentration
- Cell sorting buffer and cell density
- Optional use of DNAse
- Temperature, pH

- Sample collection:
  - Cell collection buffer (cell culture, transplantation, genomic analysis)
  - Temperature, pH

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Instrument settings

- Nozzle size/sheath pressure
  - 70 µm/70 psi for lymphocytes
  - 100 µm/20 psi for larger and/or fragile cells
- Event rate (number of events/second)
  - Low speed for higher sorting efficiency
- Sort setup
  - Bulk sorting
  - Purity vs yield
  - Single cells
- Laser alignment
- Drop delay

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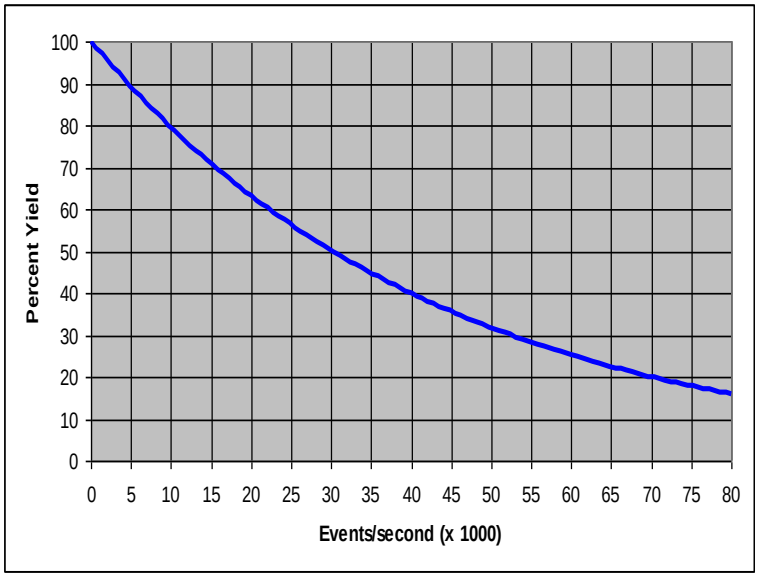
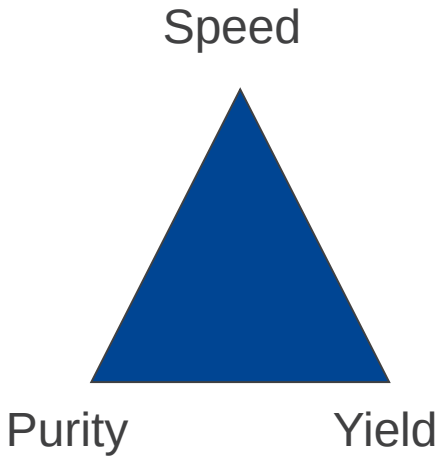
Instrument settings: speed vs yield vs purity

- Influenced by:
  - Drops per second, events per second, sort "mask" and target population frequency





- Good rule of thumb:
  - Maximum recommended event rate = drops per second / 5

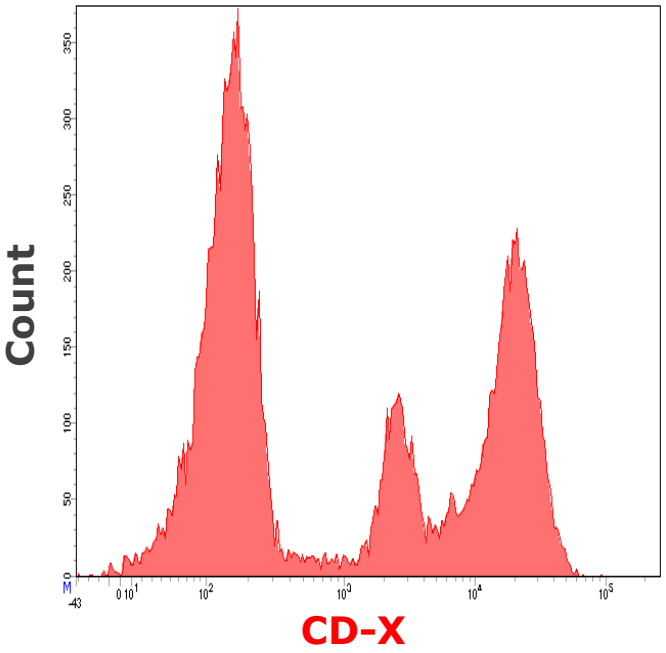
Considerations for cell sorting

Gating strategy

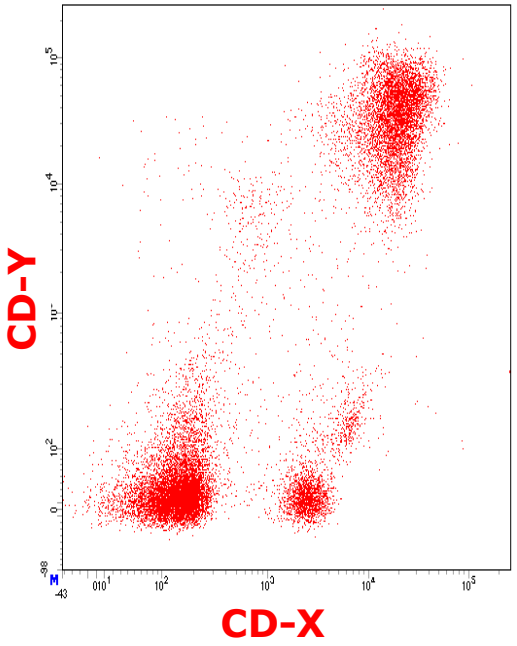
Histogram vs plots, biexponential scale, doublet discrimination, data display

Different options for data display

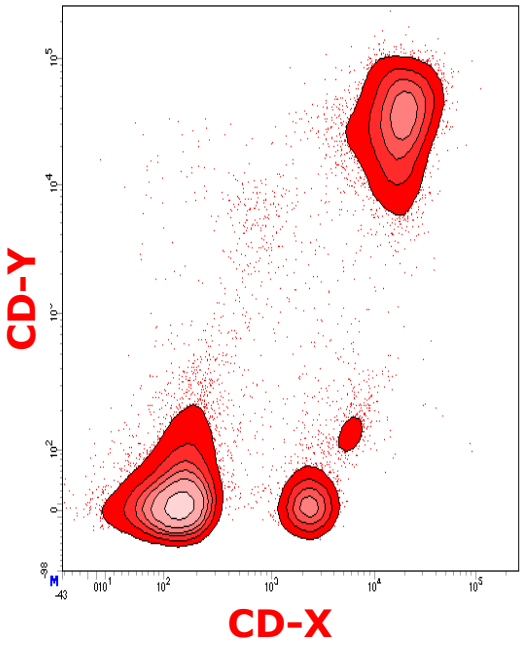
Histogram



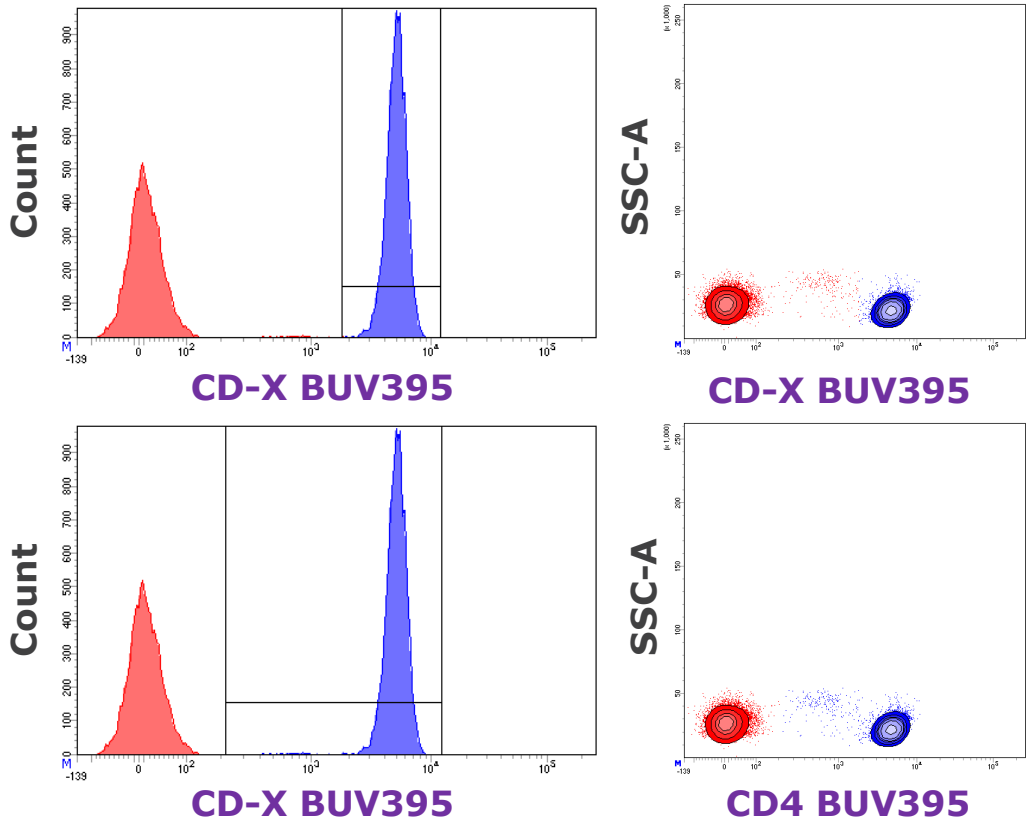
Dot plot



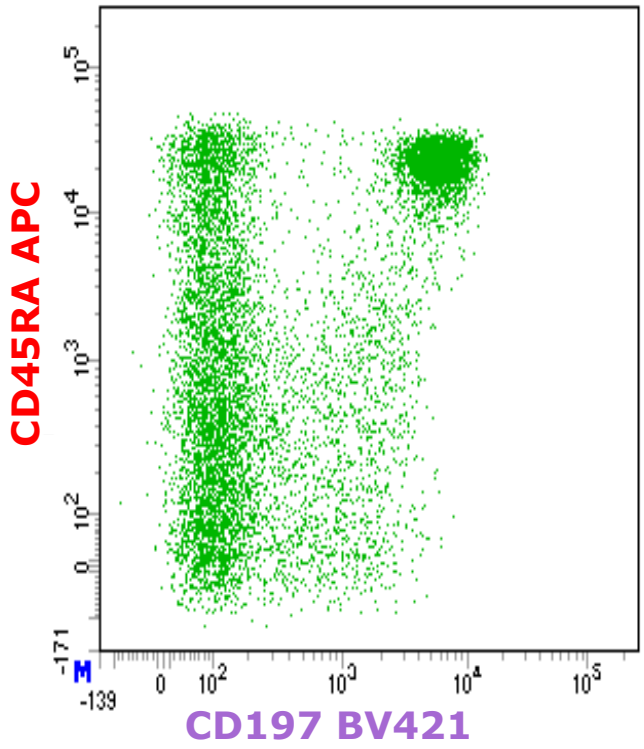
Contour plot

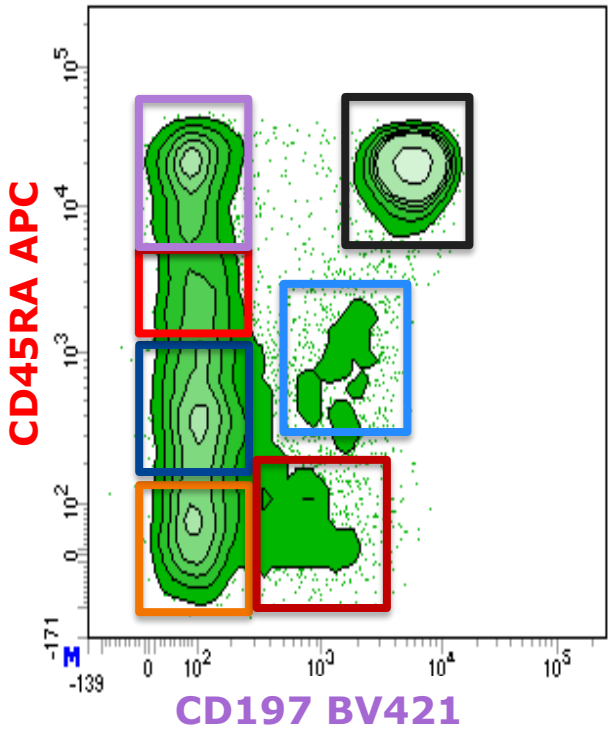


Histograms vs plots:
How many populations do you see?

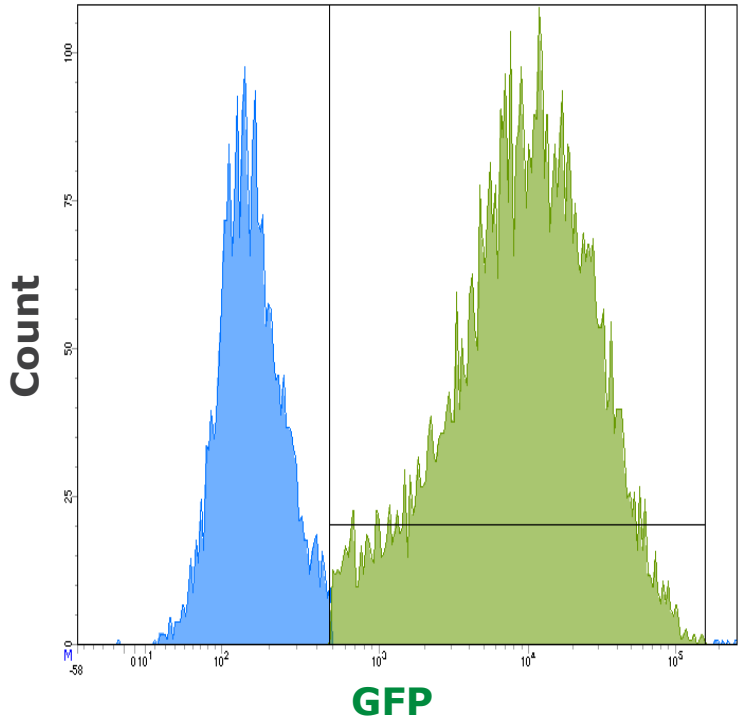


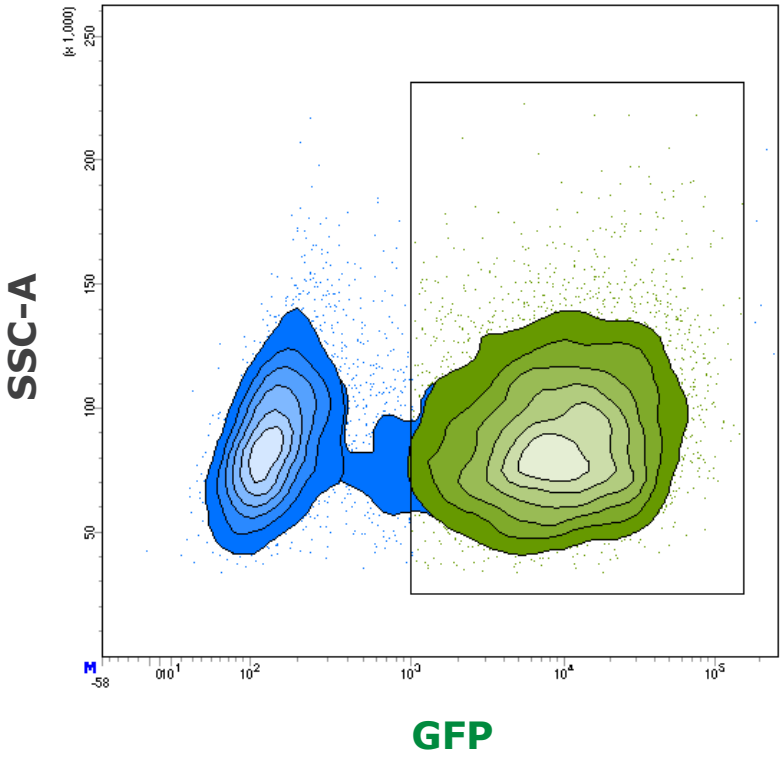
Dot plots vs contour plots: How many population do you see?





Histograms vs bivariate plots: where to draw the gate?

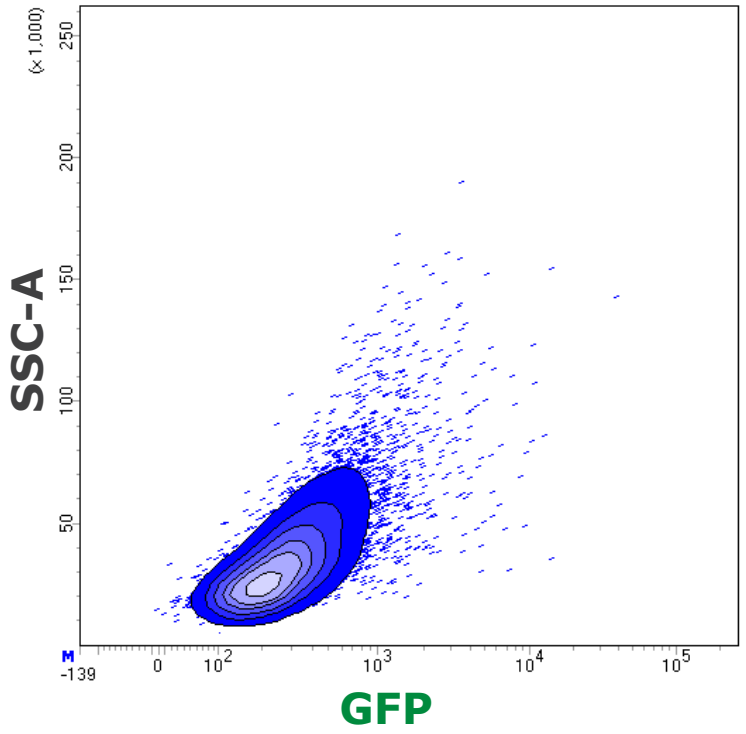




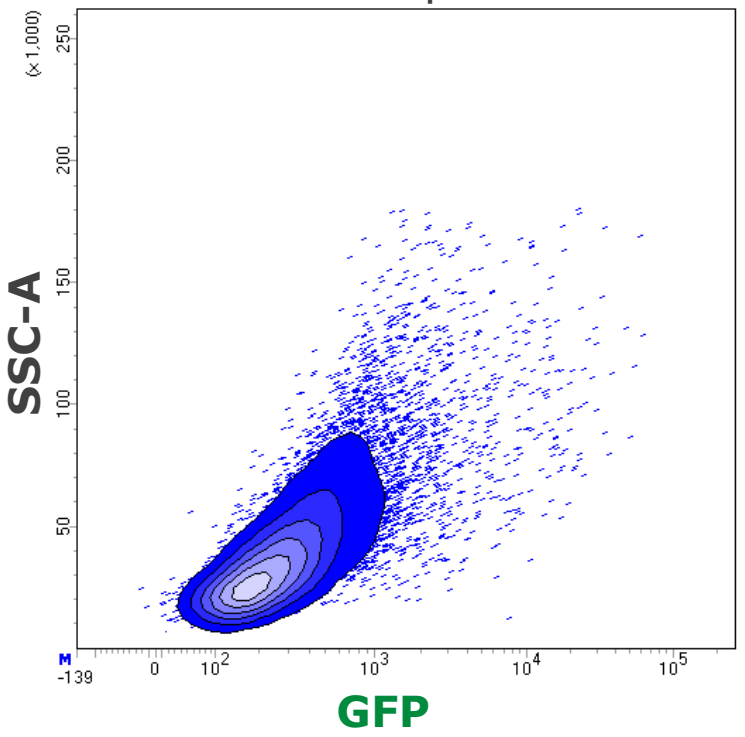
- Transfected cells express different levels of GFP.
- Bivariate plots better reveal the separation from negative/dim to positive cells.
- What if GFP is expressed at low levels?

Gating low GFP expressing cells: what is real and what is not?

Parental cells



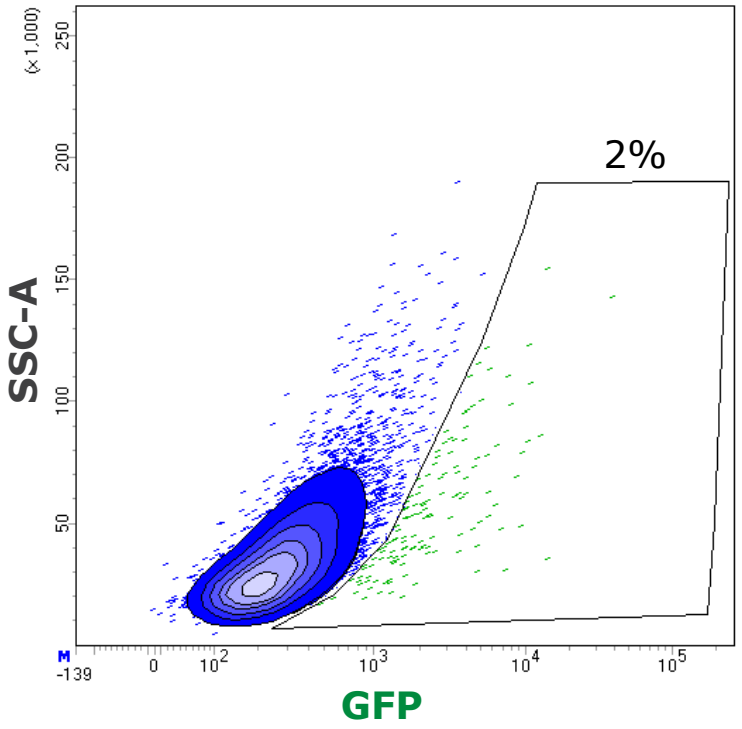
Transfected reporter cells



- High background in the GFP channel is usually due to autofluorescence.
- Negative controls are instrumental for proper gating.

Gating low GFP expressing cells: what is real and what is not?

Parental cells



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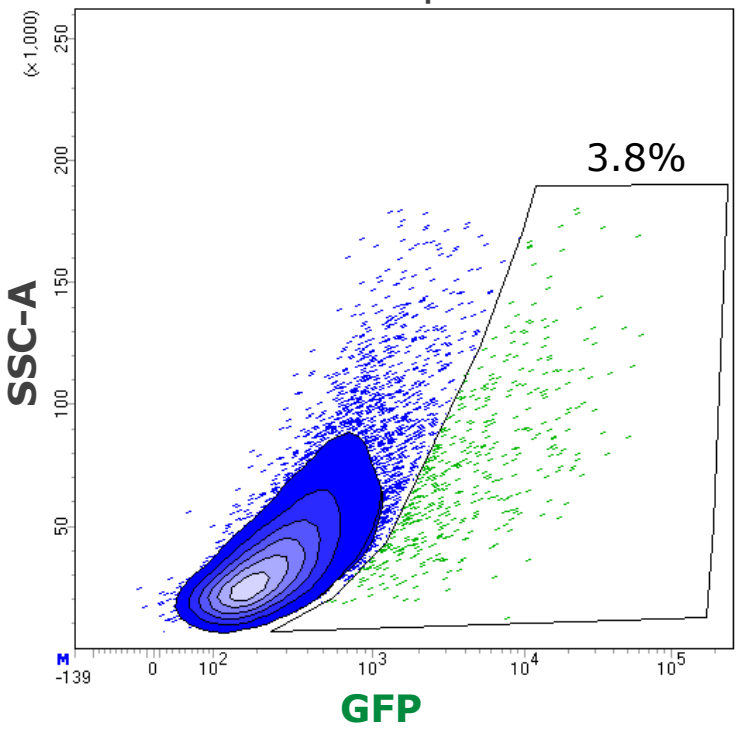

- High background in the GFP channel is usually due to autofluorescence.
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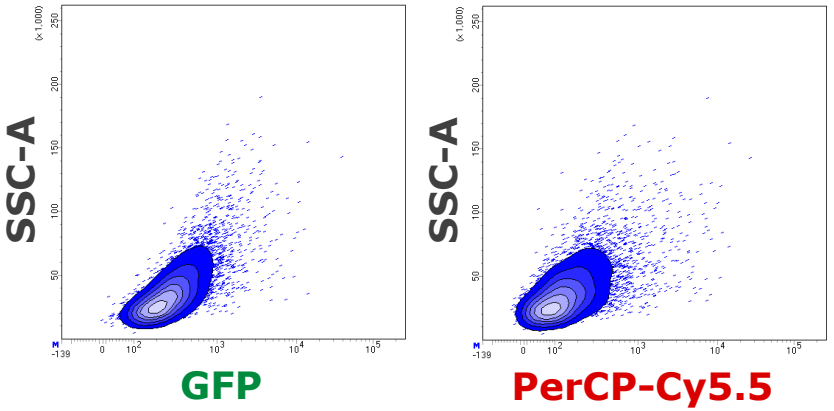
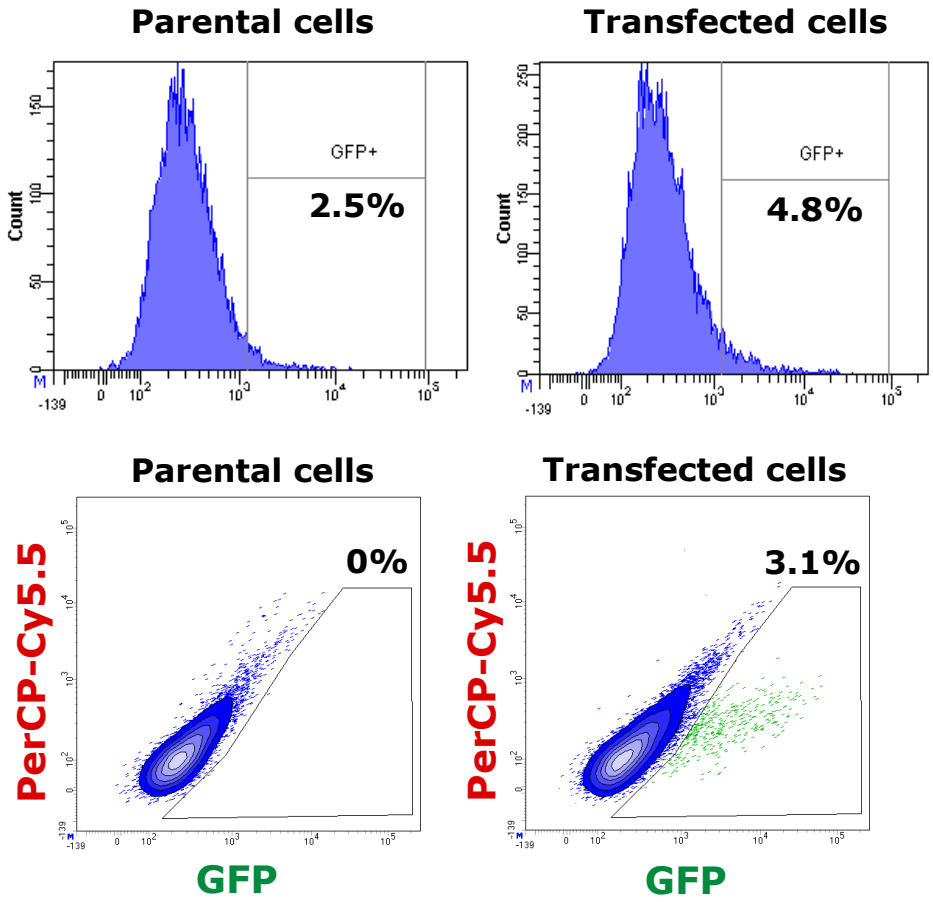
Gating low GFP expressing cells: leveraging autofluorescence





- Autofluorescence is detected in multiple channels.
- Plot GFP against another channel with autofluorescence.
- Autofluorescent cells will be "double positive" (diagonal), revealing true GFP single positives.

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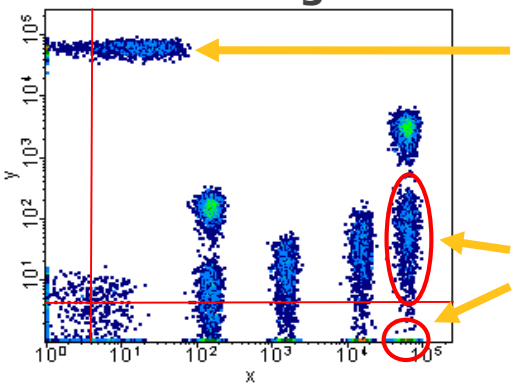
The biexponential scale:
the best way to look at compensated data

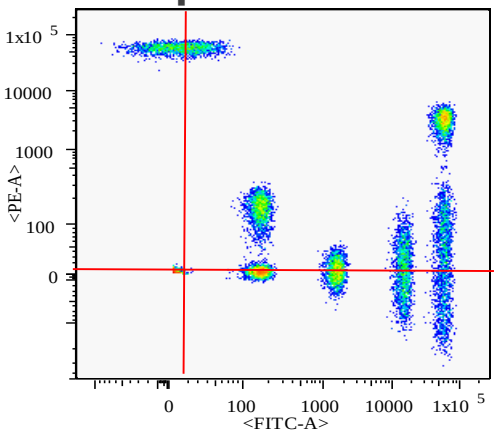
Standard log scale



This population "looks" under compensated.

These look like two separate populations.

Biexponential scale

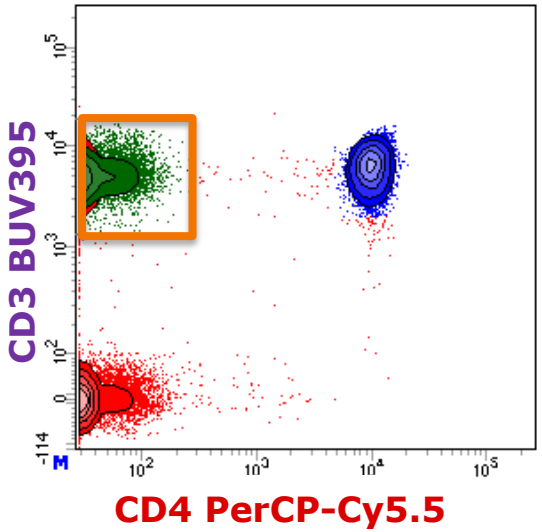


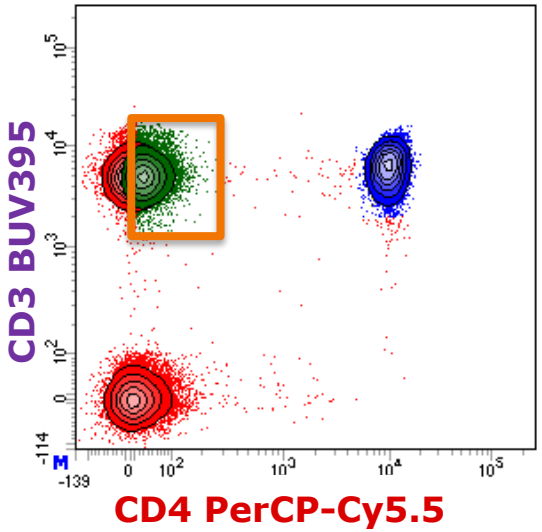
Visualization of compensated data is greatly improved using the biexponential scale.

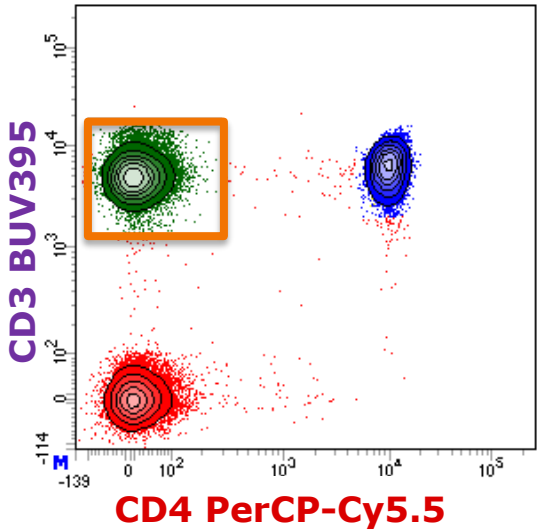
This example showing different displays of the same data shows the value of the biexponential scale, a mostly logarithmic scale on the upper end, linear at the low end and symmetrical about the negatives.

- Compensated single positives are continuous.
- All populations are visible.

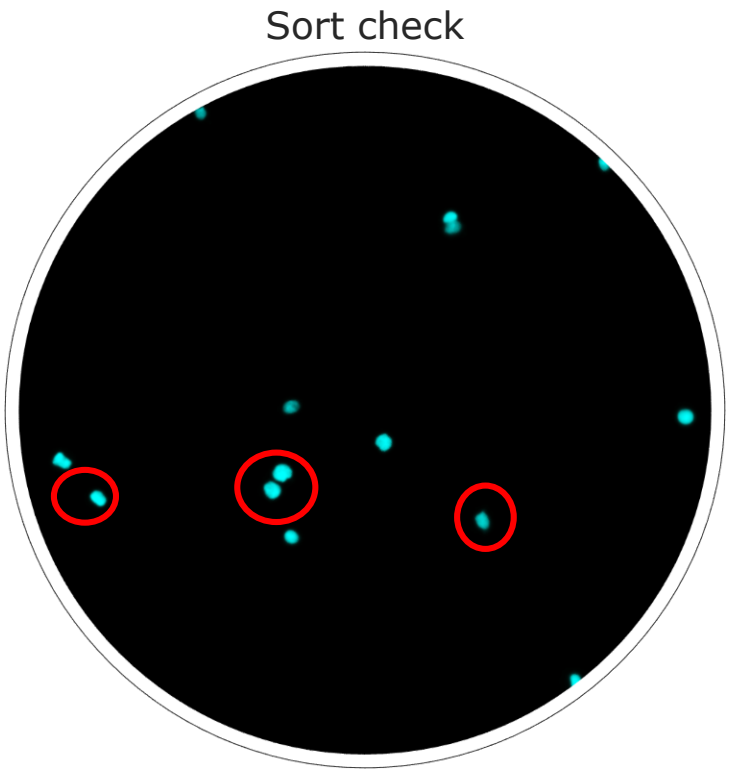
Gating strategy: biexponential scale



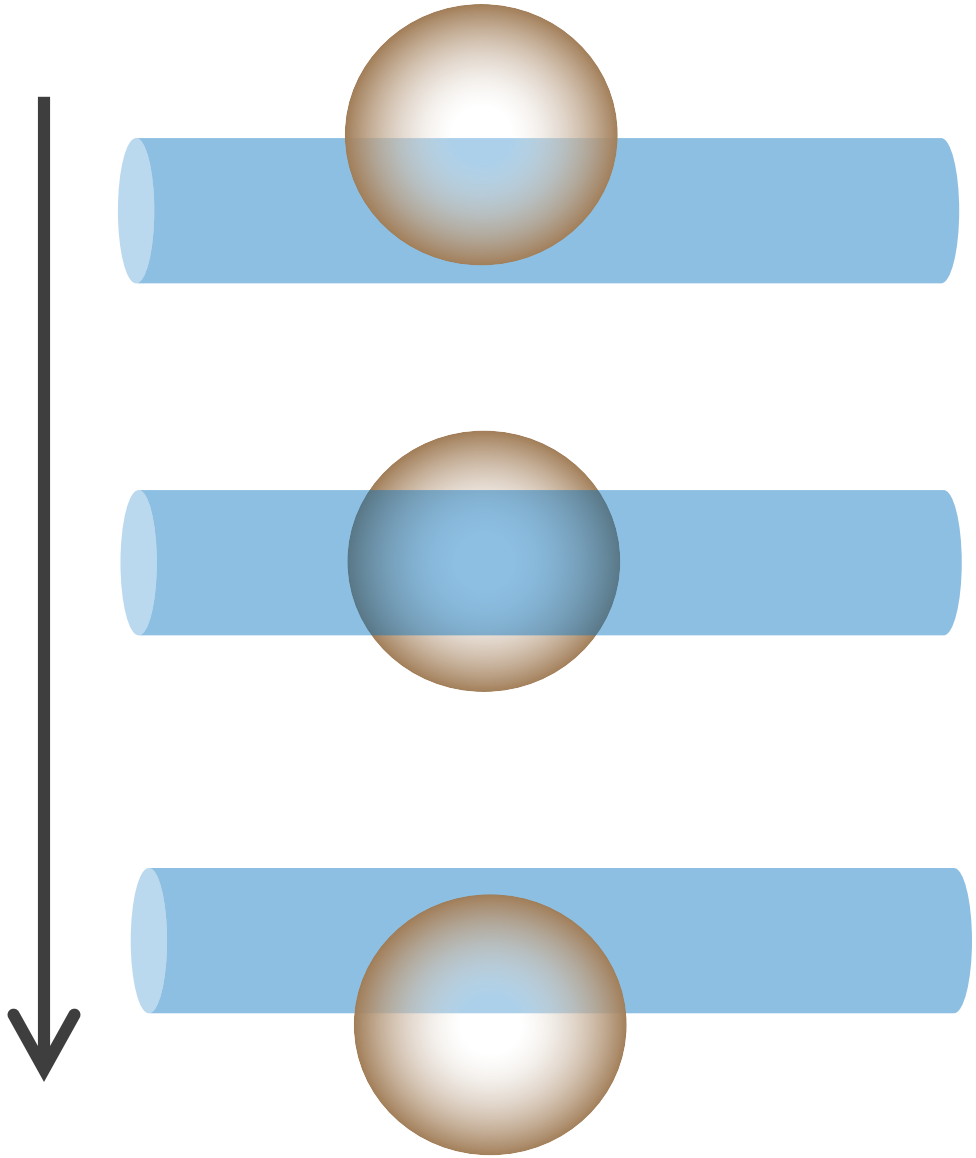


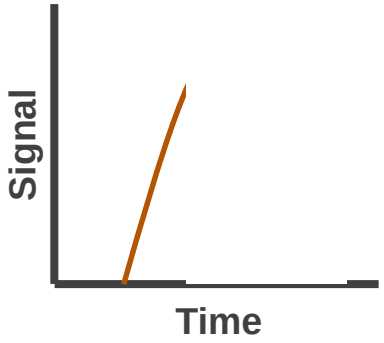


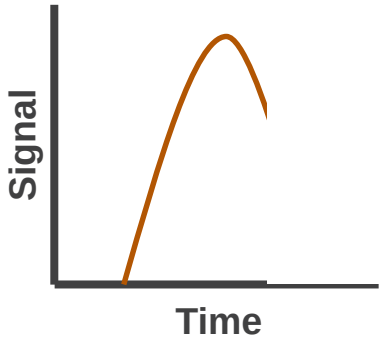
Doublet discrimination

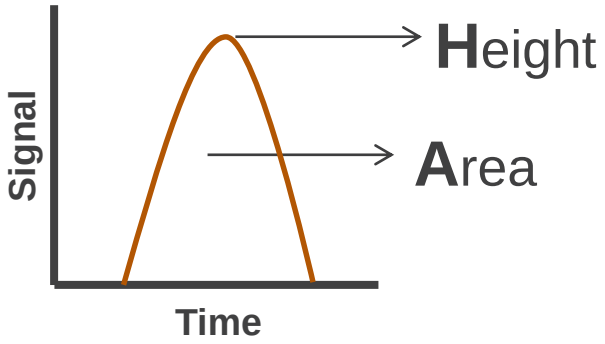


Electronic pulse









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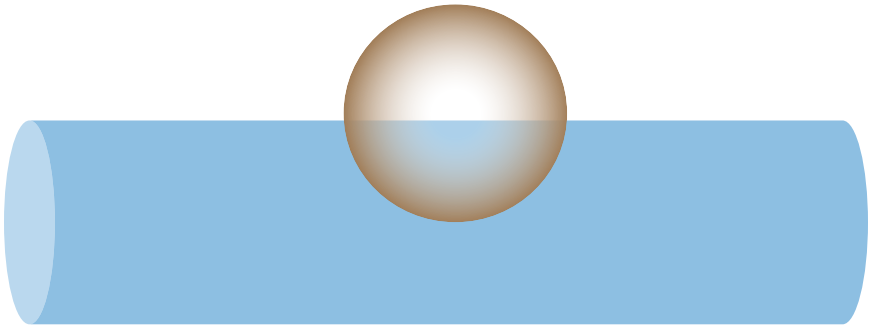
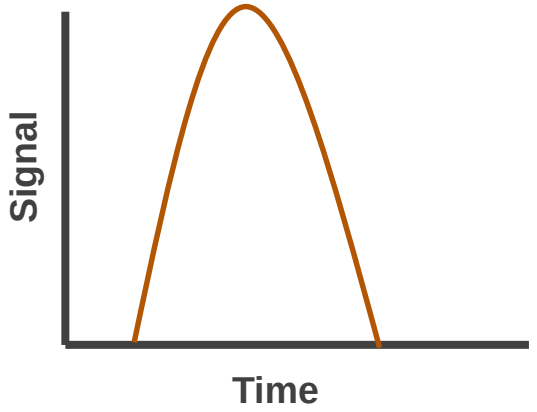
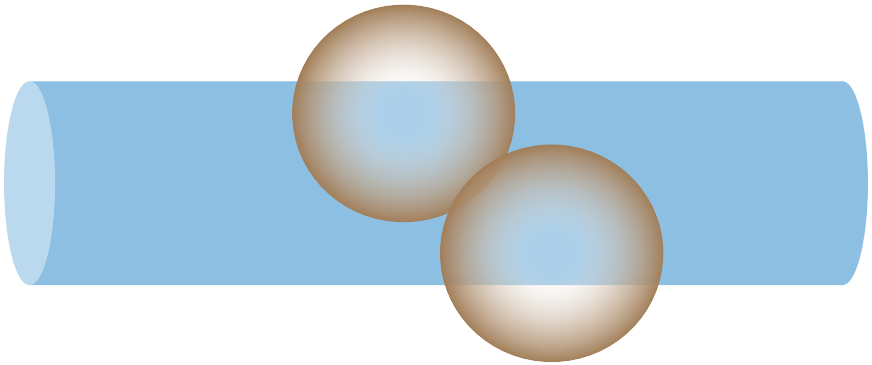
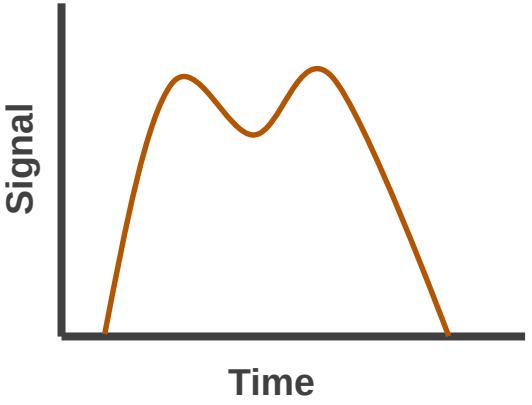




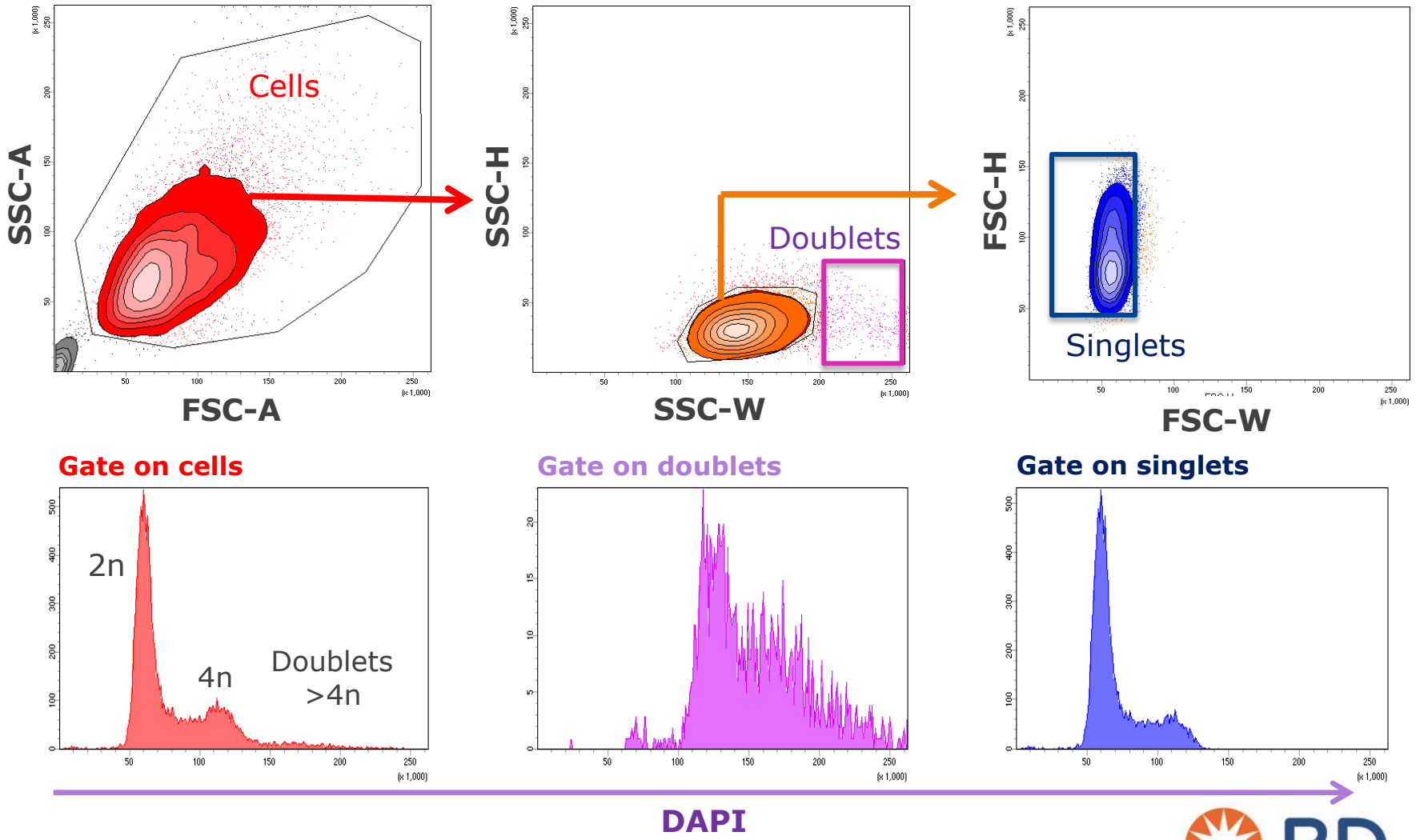
1x **W**idth



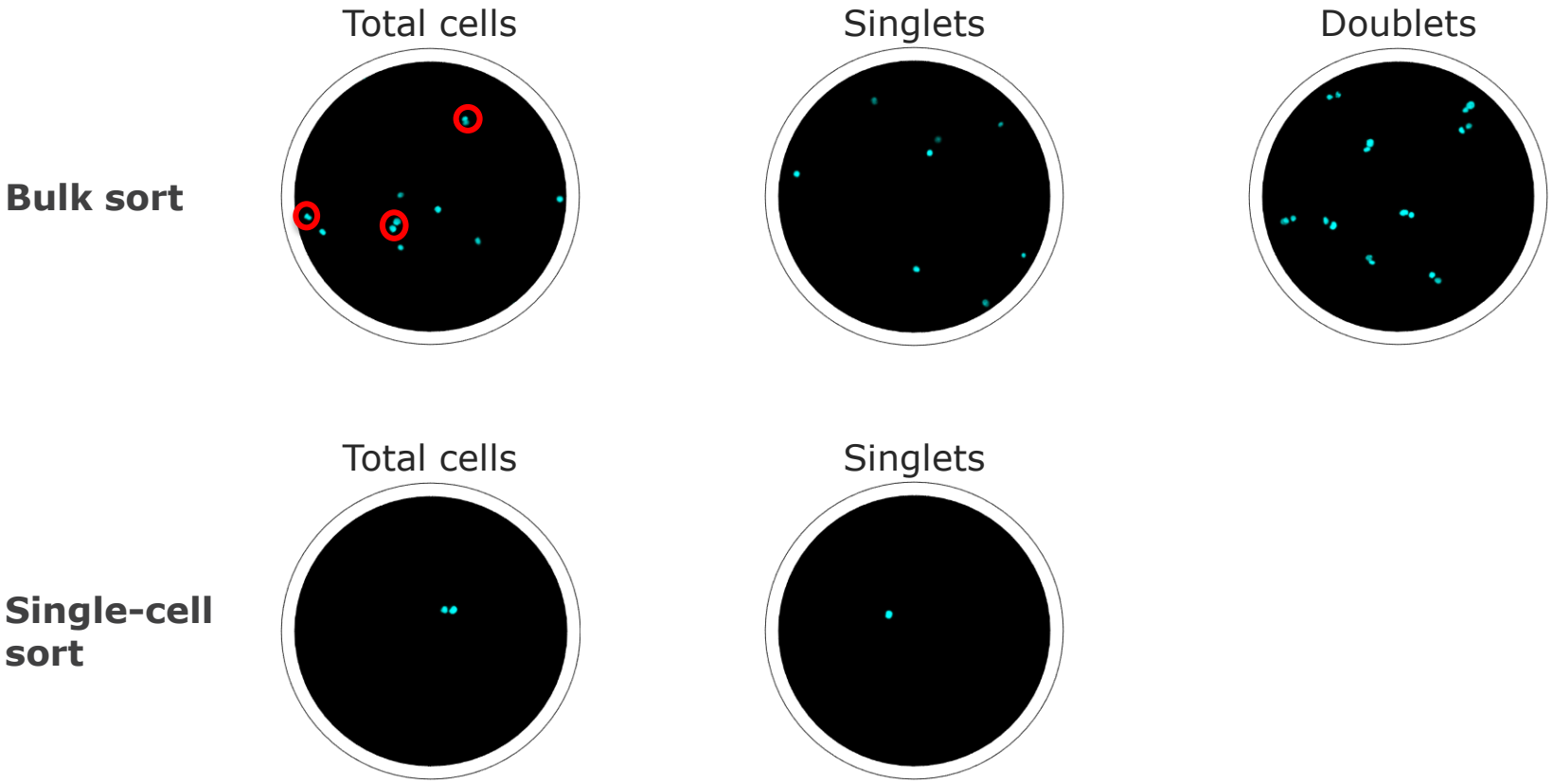


2x **W**idth

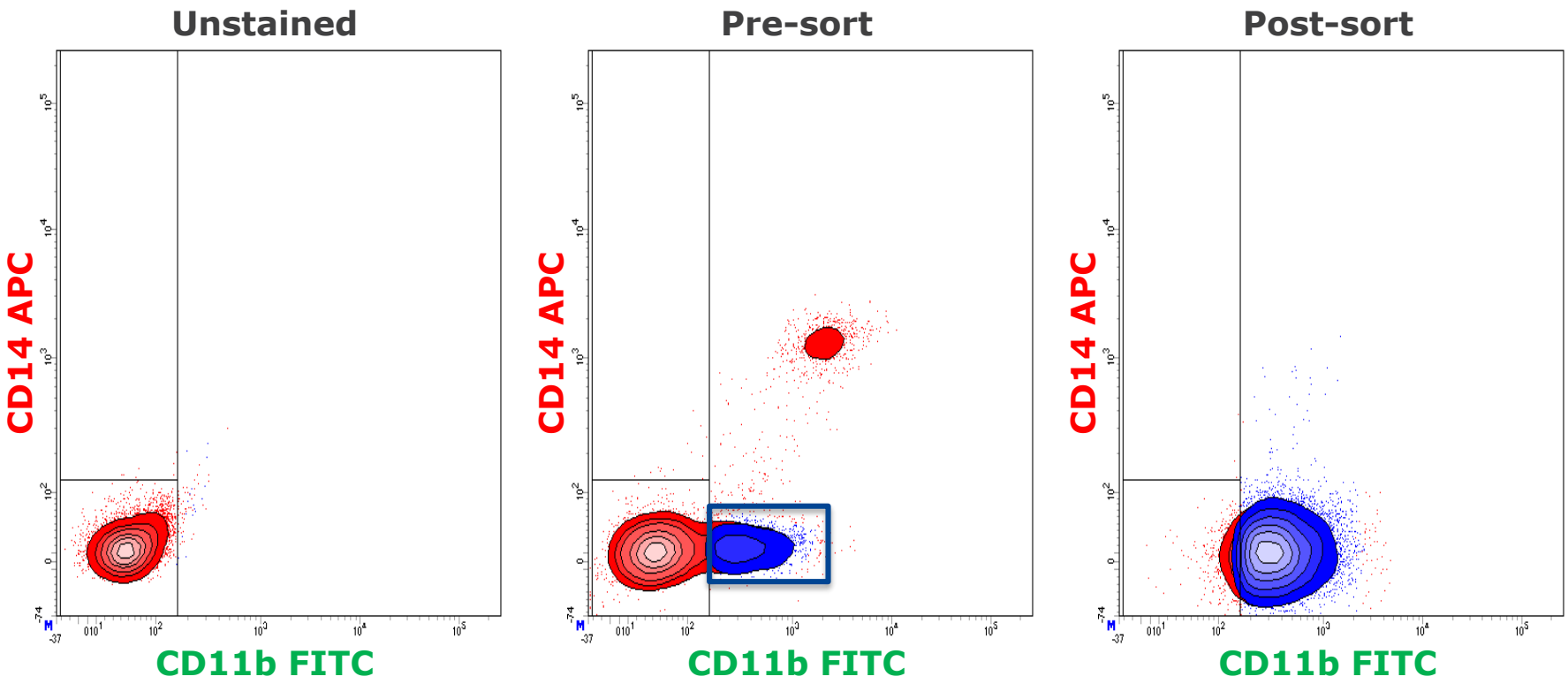
Doublet discrimination



Doublet discrimination



Gating strategy: low antigen density populations



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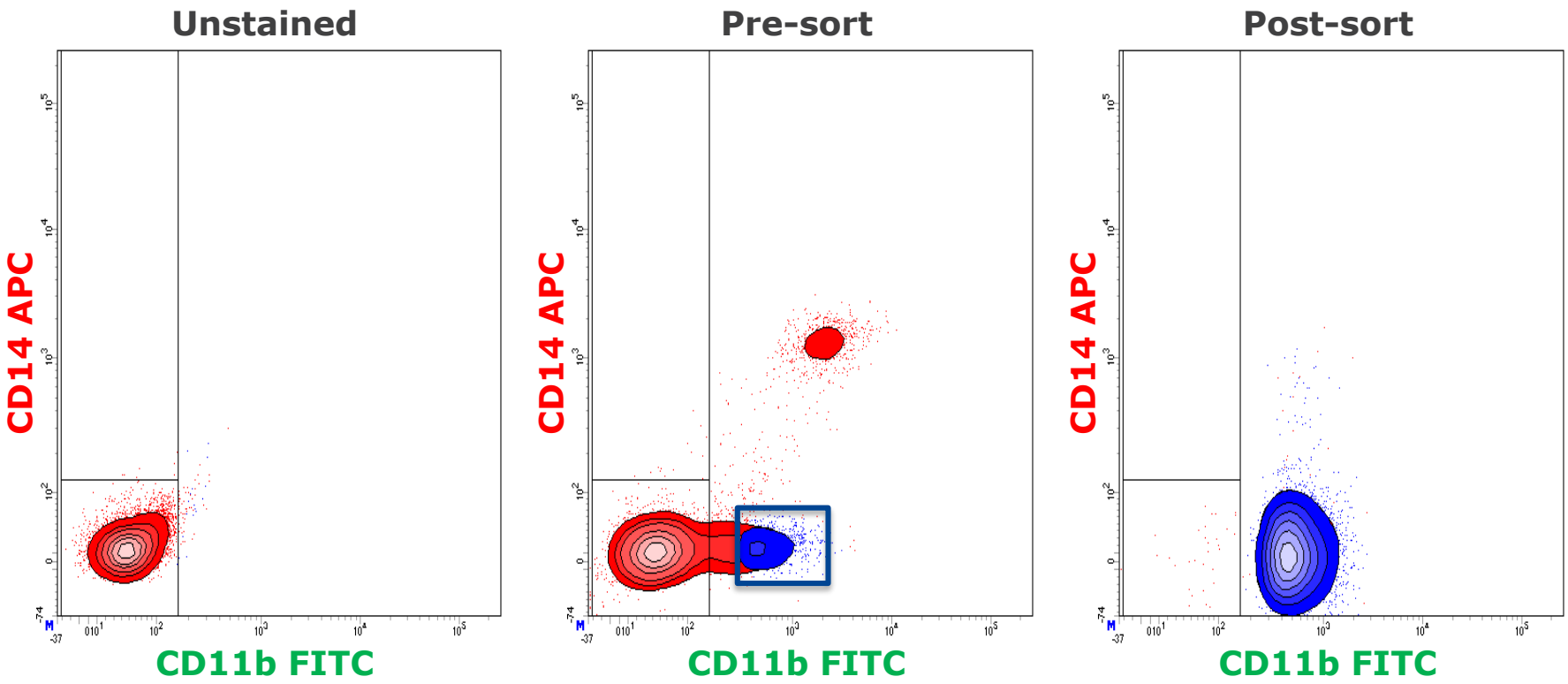
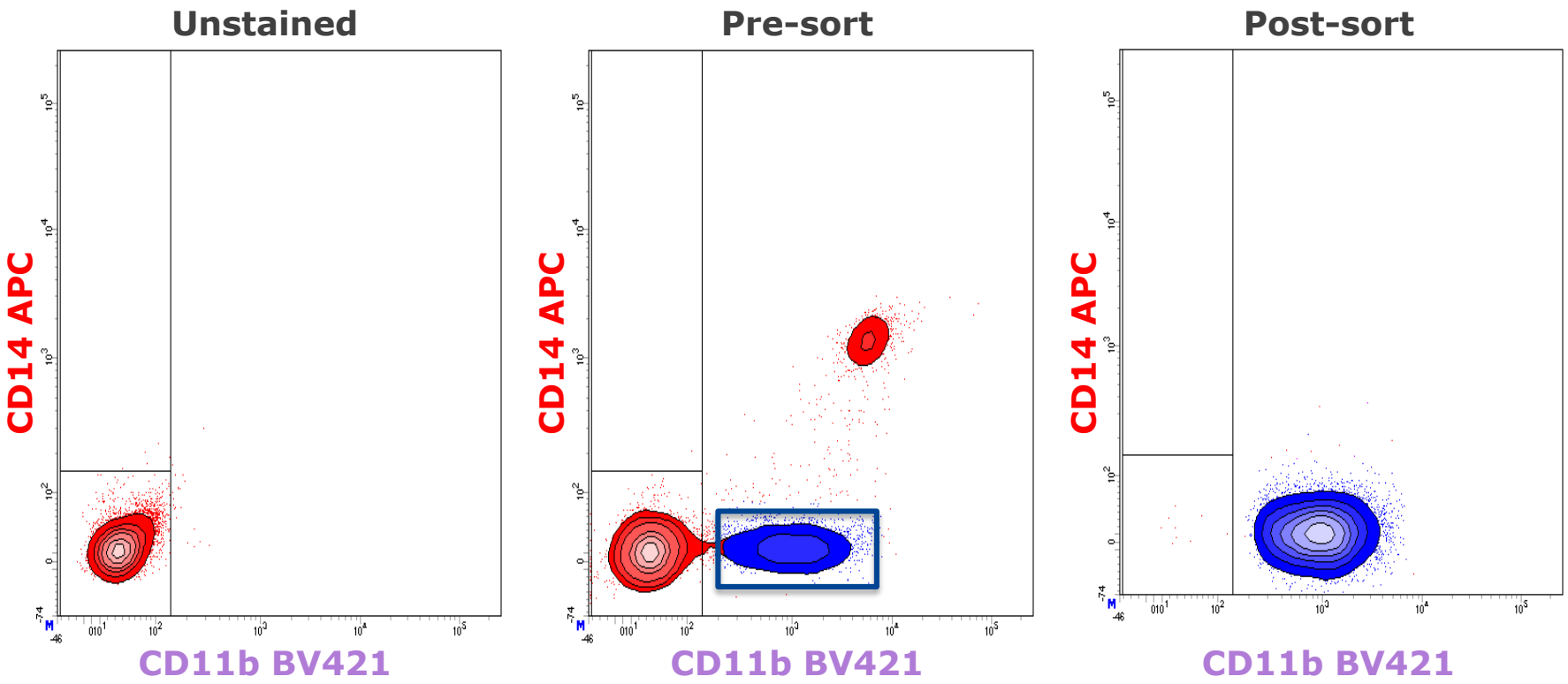


Gating strategy: low antigen density populations



Gating strategy: summary

- Use bivariate plots rather than histograms.
- Use contour plots for a clearer identification of populations of interest.
- Manually adjust the biexponential scale to gate all the cells of interest.
- Use proper controls to identify and eliminate background (autofluorescence).
- Use a doublet discrimination strategy for proper isolation of a single-cell suspension.

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Considerations for cell sorting

Panel design

Fluorochrome choice, dead cell exclusion, lineage exclusion/depletion

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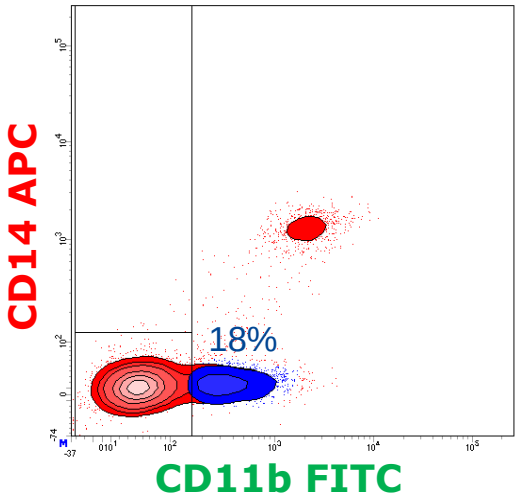
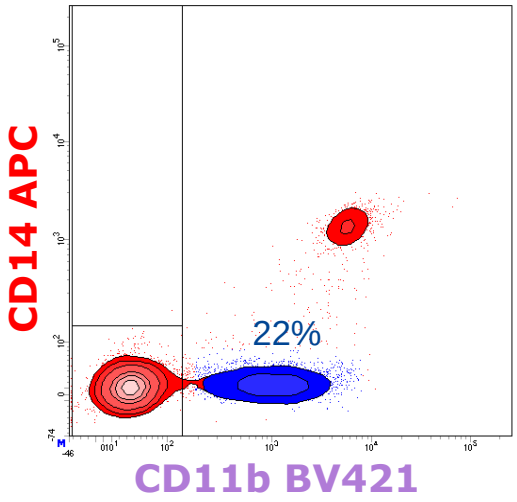
Why is it relevant to design an optimized panel for cell sorting?

- Best practices to build an optimized panel for analysis apply to the cell sort as well.
- Additional considerations may be taken in account when designing a panel for cell sorting to obtain:
  - Highest purity and yield
  - Clear resolution from unwanted cell populations





How to build a panel for cell sorting?

- A good panel for sorting relies on the use of negative as well as positive markers.
- Properly choose fluorochromes.
  - Antigen density
  - Spillover
  - Co-expression
- Know the biology.
  - Minimize spillover into the most critical markers to maximize the resolution of your population of interest.
- Exclude unwanted cells to increase the resolution of the target cells.
  - Dead cells
  - Lineage

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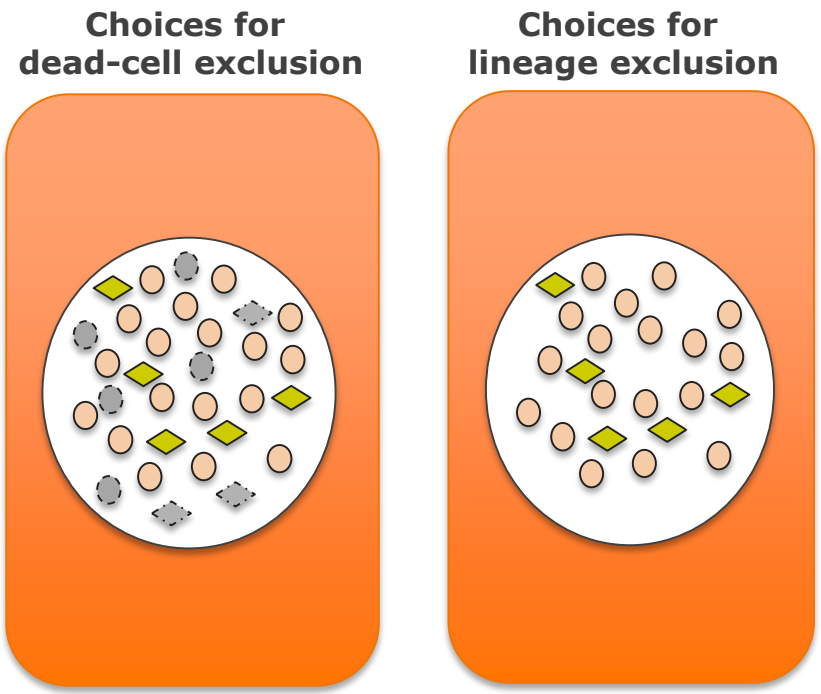
Choosing fluorochromes for a cell sorting panel: exclude unwanted cells

- Dead cells
- Lineage cells
- Cells of interest

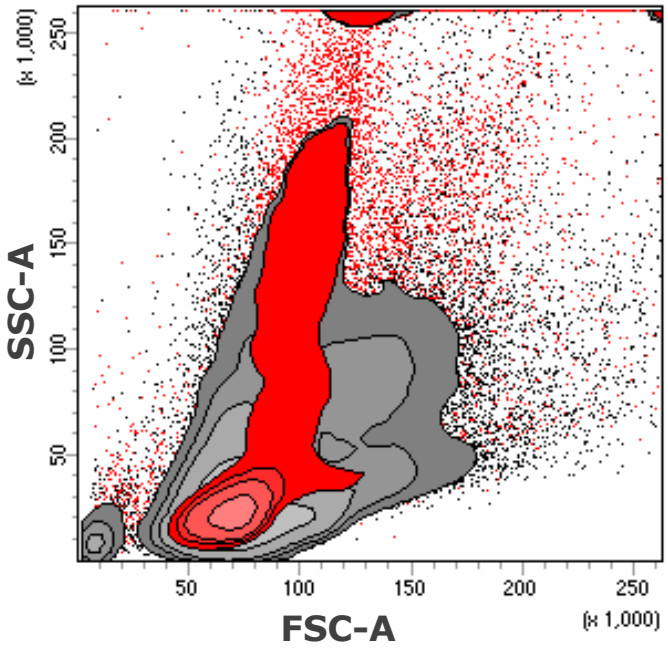


Dead-cell exclusion

The presence of dead cells impacts cell sorting.

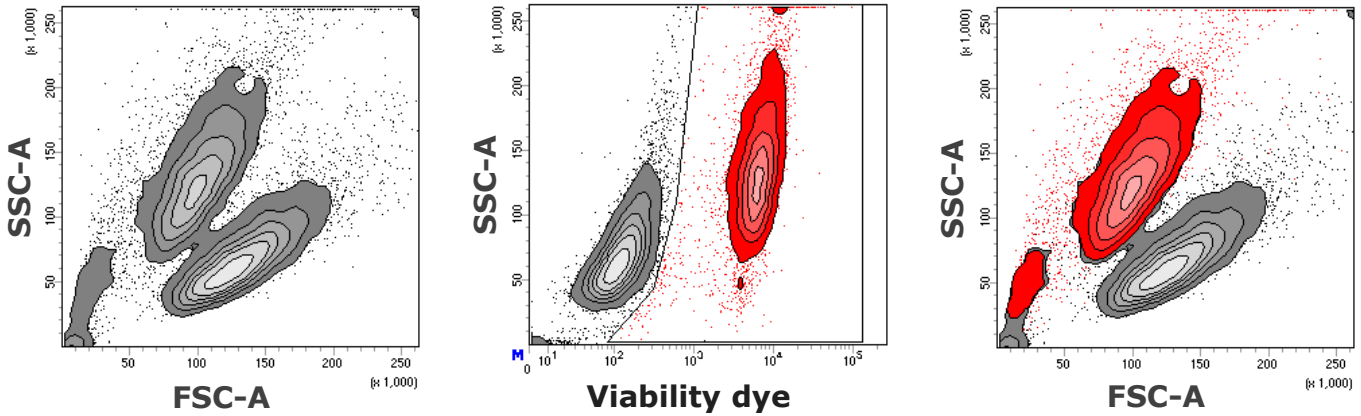
- Inaccurate quantification of the population of interest
- Reduced purity

- Dead cells can be excluded using:
  - Light scatter properties
  - Viability dyes

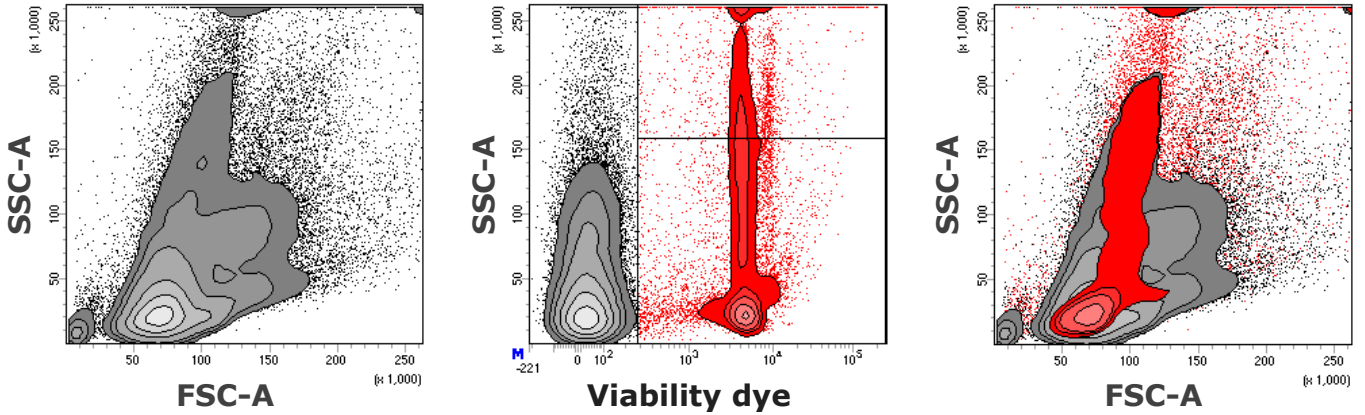


Dead-cell exclusion by light scatter

Live and heat-killed HeLa cells



Mouse bone marrow



- Scatter alone can be used to identify heat-killed HeLa cells.
- A viability dye is required to detect and gate out dead cells.

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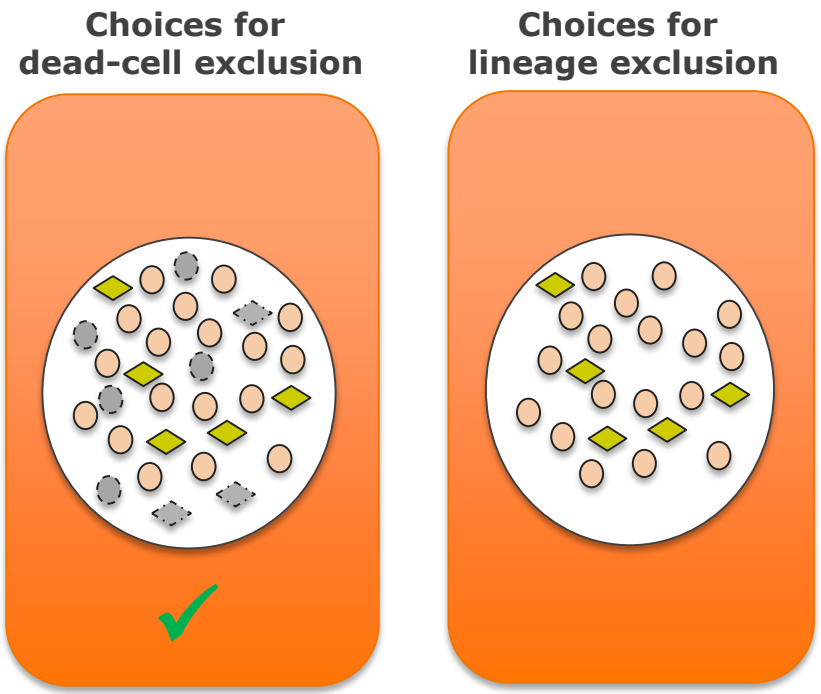
Dead-cell exclusion using viability dyes

Dye	Unfixed cells	Fixed cells	Detector	Laser
DAPI	√	X	BV421	UV/Violet
Via-Probe Green	√	X	FITC	Blue
PI	√	X	PE	Blue/YG
7-AAD	√	X	PerCP-Cy™5.5	Blue/YG
DRAQ7™	√	X	APC	Red
Via-Probe Red	√	X	APC	Red
FVS450	√	√	BV421	Violet
FVS510	√	√	BV510	Violet
FVS575V	√	√	BV605	Violet
FVS520	√	√	FITC	Blue
FVS570	√	√	PE	Blue/YG
FVS620	√	√	PE-CF594	Blue/YG
FVS660	√	√	APC	Red
FVS700	√	√	AF700	Red
FVS780	√	√	APC-H7	Red

- Nucleic acid dyes bind nucleic acids non-covalently
- No-wash stain procedure
- Recommended for sort of unfixed samples
- Fixable Viability Stains bind amine moieties covalently
- Wash is required after stain
- Recommended for sort of fixed samples

Choosing fluorochromes for a cell sorting panel: exclude unwanted cells

- Dead cells
- Lineage cells
- Cells of interest



Lineage-cell exclusion

The presence of lineage cells impacts cell sorting.

- Inaccurate quantification of the population of interest
- Reduced purity
- Increased time necessary to sort a rare population

- Lineage cells can be excluded using:
  - Light scatter properties
  - Lineage cocktails

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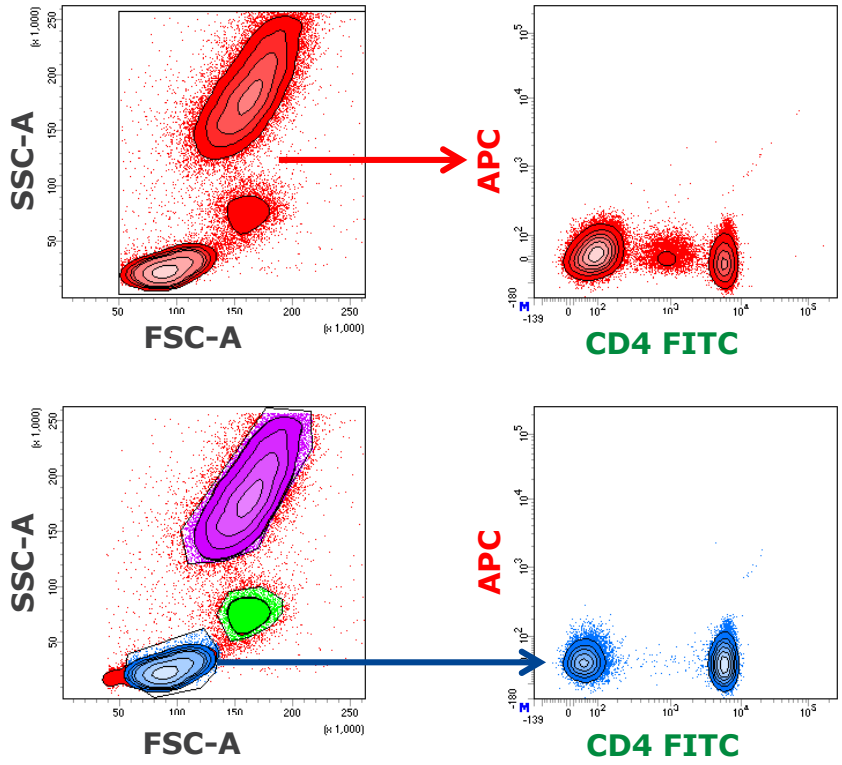
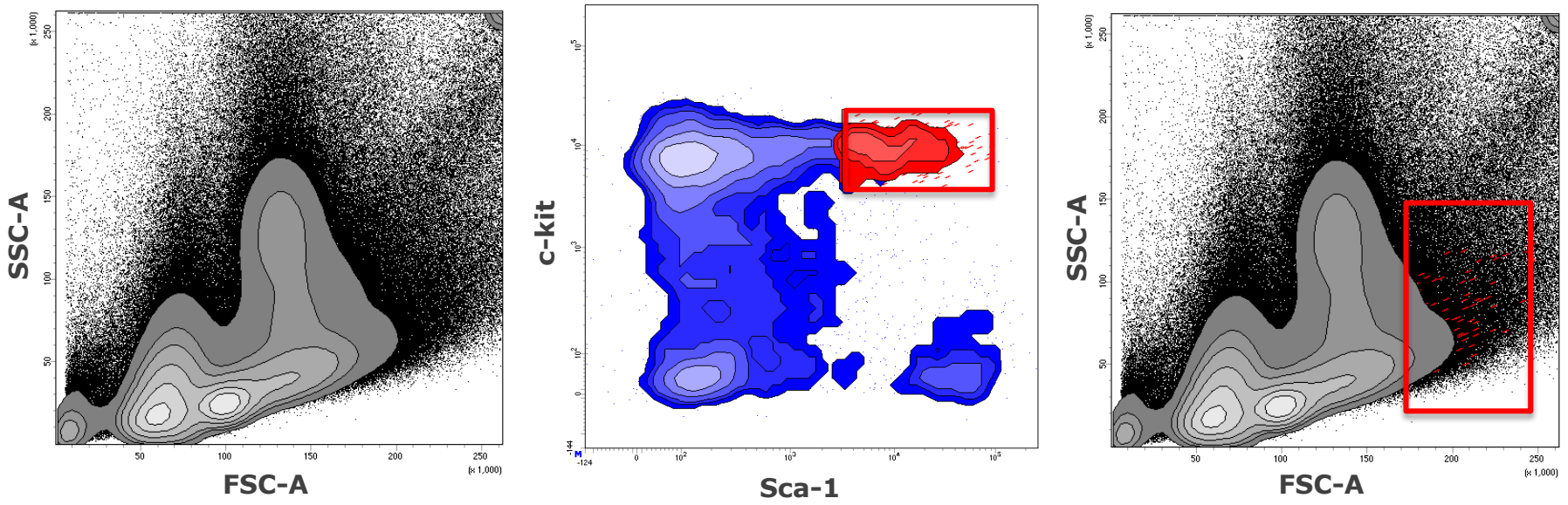
Lineage exclusion by light scatter



- In peripheral blood, different cell lineages can be easily discriminated based on light scatter.

Lineage exclusion by light scatter is not sufficient for rare population detection



- In samples such as mouse bone marrow, detection of rare stem cells is confounded by the overwhelming presence of lineage cells.
- The use of lineage markers is needed to clearly detect rare populations of interest.

Examples of lineage marker cocktails

Lineage cocktail	Marker	Fluorochrome
T cells	CD3	FITC
B cells	CD19	APC
NK cells	CD56	PerCP-Cy5.5
Monocytes/macrophages	CD14	BV421
Erythrocytes	CD235a	BV786

- Take into consideration instrument configuration and available detectors.

Examples of lineage marker cocktails

Lineage cocktail	Marker	Fluorochrome
T cells	CD3	PerCP-Cy5.5
B cells	CD19	PerCP-Cy5.5
NK cells	CD56	PerCP-Cy5.5
Monocytes/macrophages	CD14	PerCP-Cy5.5
Erythrocytes	CD235a	PerCP-Cy5.5

- Take into consideration instrument configuration and available detectors.
- Combine all lineage markers in the same format to overcome configuration limitations and to increase panel design flexibility.

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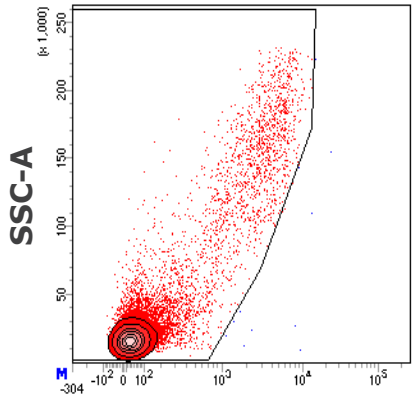
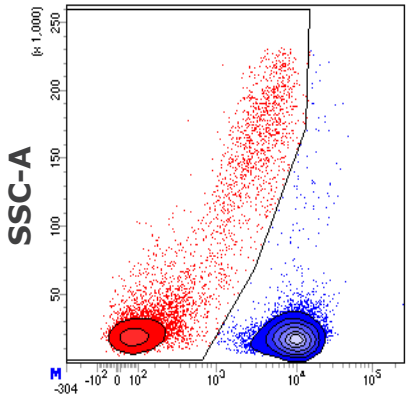
Choosing a fluorochrome for a lineage cocktail

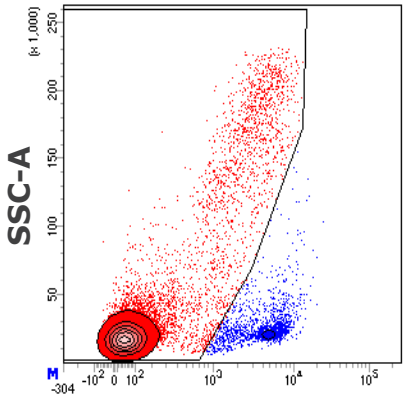
Fluorochrome	Viability dye
FITC	BD Via-Probe Green, FVS520
PerCP-Cy5.5	7-AAD, FVS620
APC	BD Via-Probe Red, FVS660
Alexa Fluor® 700	DRAQ7, FVS700
BV421	DAPI, FVS450

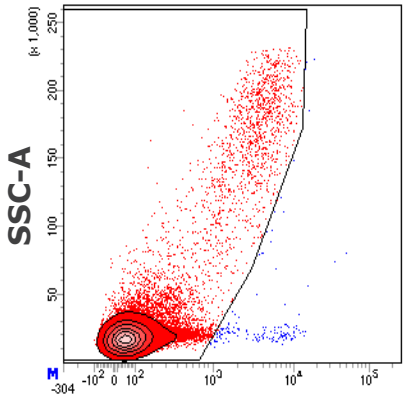
- Match the fluorochrome for the lineage cocktail with a viability dye detected in the same channel.
- In a single channel (dump channel), lineage and dead cells can now be excluded.
- Choose moderate dyes with high spillover into other detectors for the dump channel.
- Reserve dyes with bright signal and low spillover for the population of interest.

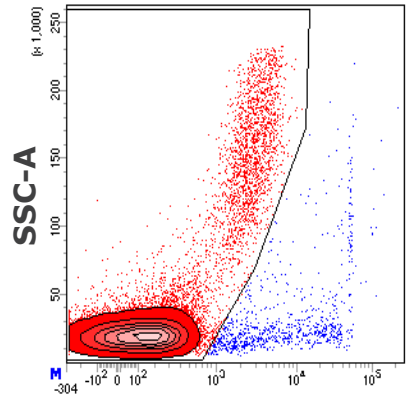
Building a lineage dump channel

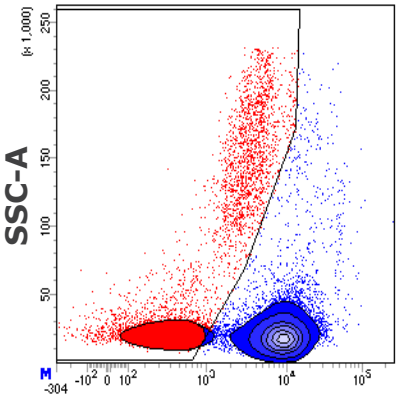




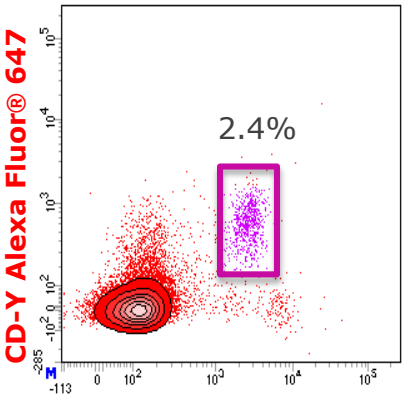




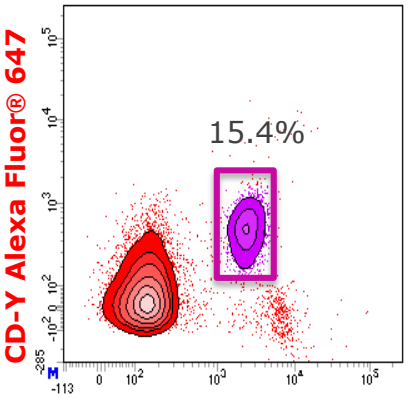




No lineage exclusion



Lineage exclusion



Advantages of depleting lineage cells prior to cell sort

- Lineage cells can be removed from the sample prior to sort using multiple rounds of magnetic selection or cell sorting
  - Cell enrichment
  - Cell depletion

- Lineage depletion can improve cell sorts of rare population of cells
  - Increased sort efficiency
  - Increased purity
  - Reduced sort time

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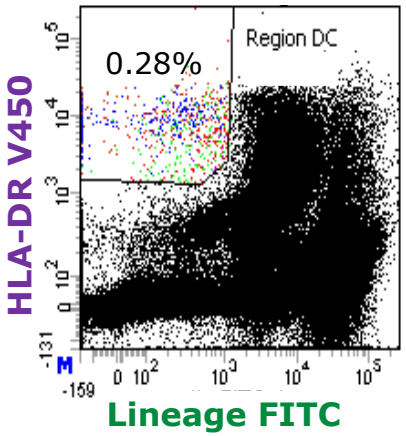
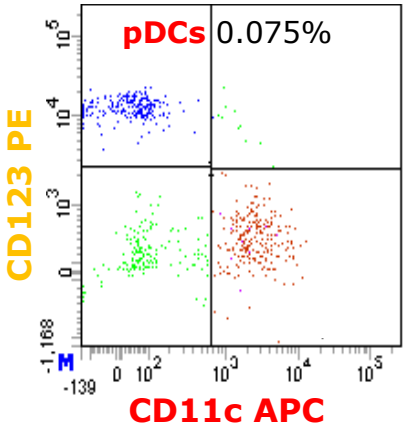
Magnetic depletion of lineage cells reduces sorting time…

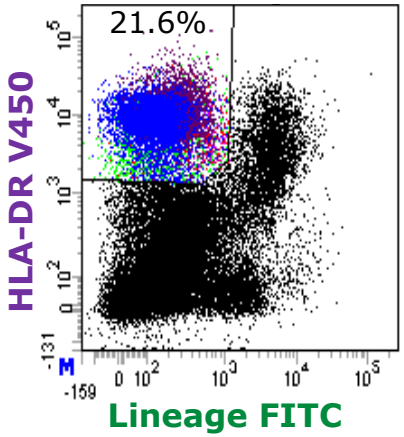
No lineage depletion

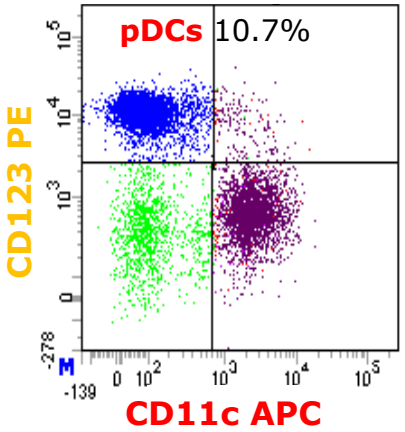




Theoretical time to sort 10^5 pDCs
➡ 6.5 hours

1st round of lineage depletion



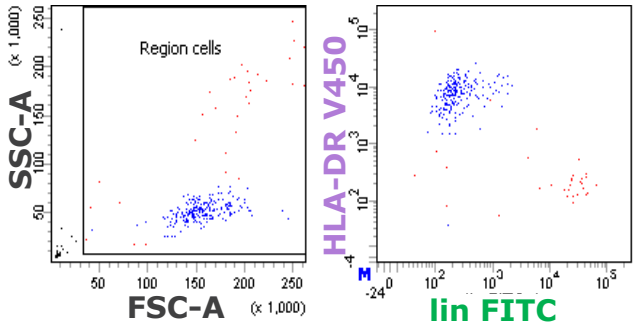


Theoretical time to sort 10^5 pDCs
➡ 2.5 minutes

…and increases purity

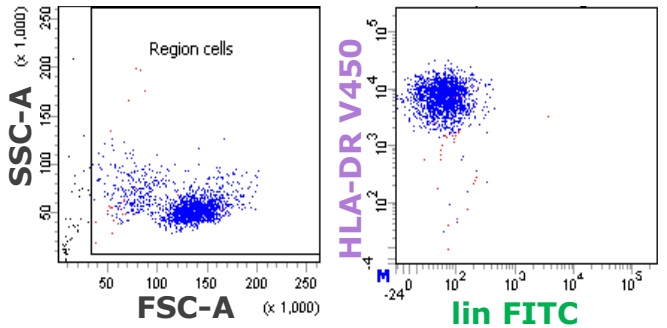
No lineage depletion

Population	#Events	%Total
All Events	293	100.0
Region cells	249	85.0
pDC	218	74.4



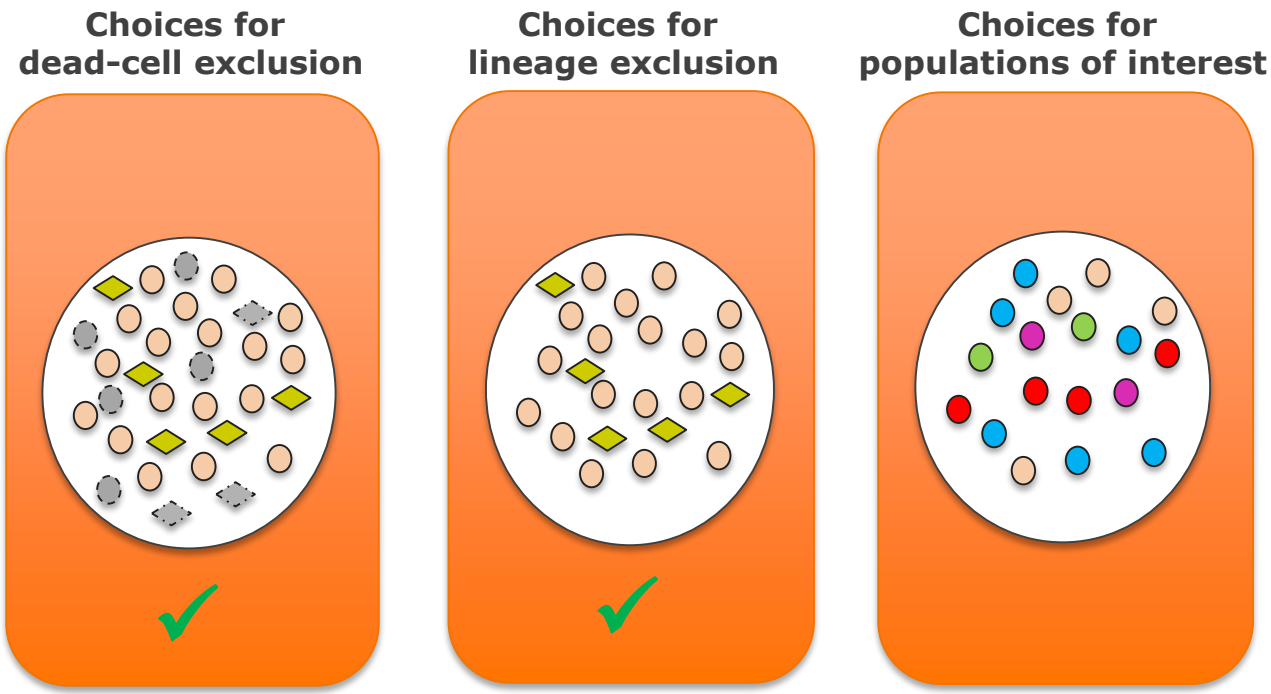
1st round magnetic lineage depletion

Population	#Events	%Total
All Events	1,908	100.0
Region cells	1,856	97.3
pDC	1,818	95.3

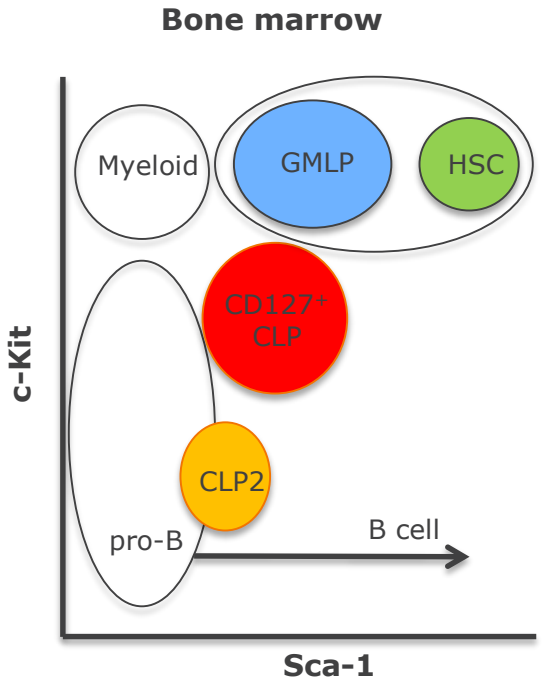


Choosing fluorochromes for a cell sorting panel: resolve the population of interest

- Dead cells
- Lineage cells
- Cells of interest



Detection of murine hematopoietic stem and progenitor cells



- Hematopoietic stem cells (HSCs) and common lymphoid progenitors (CLPs) are rare cell populations (<1%) in mouse bone marrow.
- HSCs: bright expression of c-kit and Sca-1
- CLPs: dim expression of c-kit and Sca-1
- Clear resolution of dim and bright c-kit and Sca-1 populations is critical for the isolation of HSCs and CLPs.

Adapted from Iwasaki H, Akashi K. Hematopoietic developmental pathways: on cellular basis. ***Oncogene.*** **2007; 26**:6687-6696.

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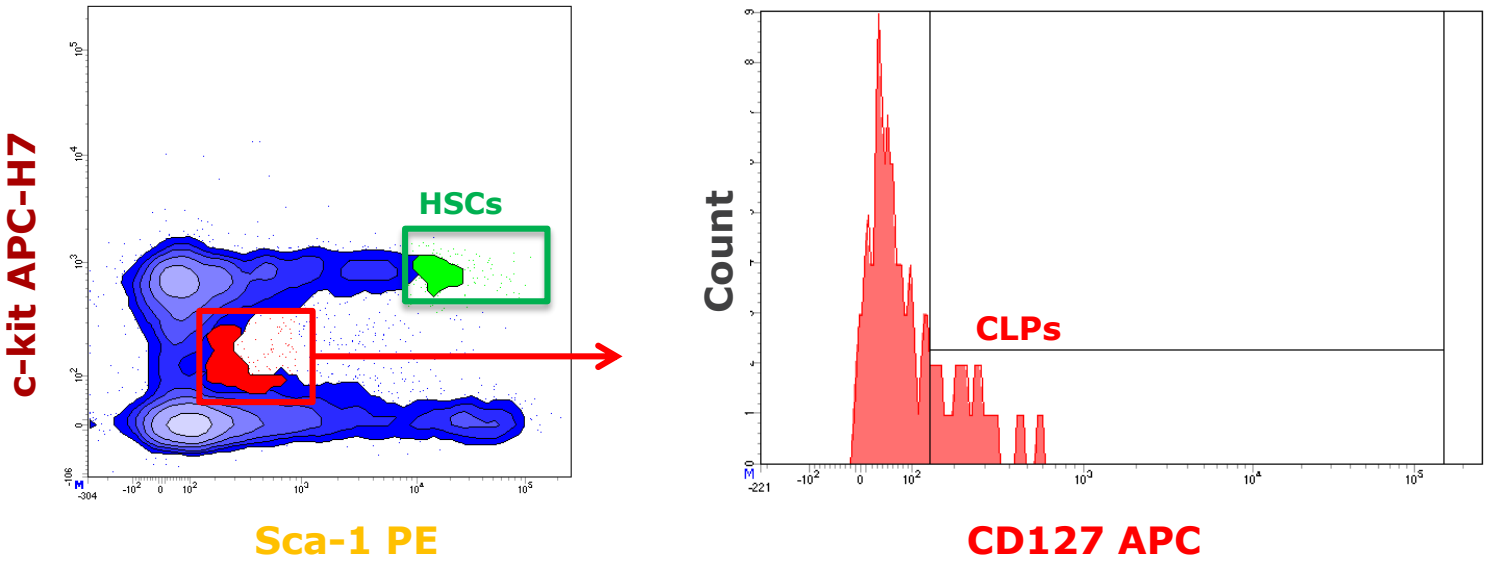
Impact of fluorochrome choice on HSC and CLP resolution

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
Lineage/7-AAD	**BV421**	
Sca-1	**BV650**	
c-kit	**PE**	●
CD127	**PE-CF594**	
	BB515	
	APC/Alexa Fluor® 647	●
	BV605	
	BV786	
	BV510	
	FITC/Alexa Fluor® 488	
	PerCP-Cy5.5	●
	V450	
	V500	
	Alexa Fluor® 700	
	APC-H7	●

Fluorochrome choice: panel 1



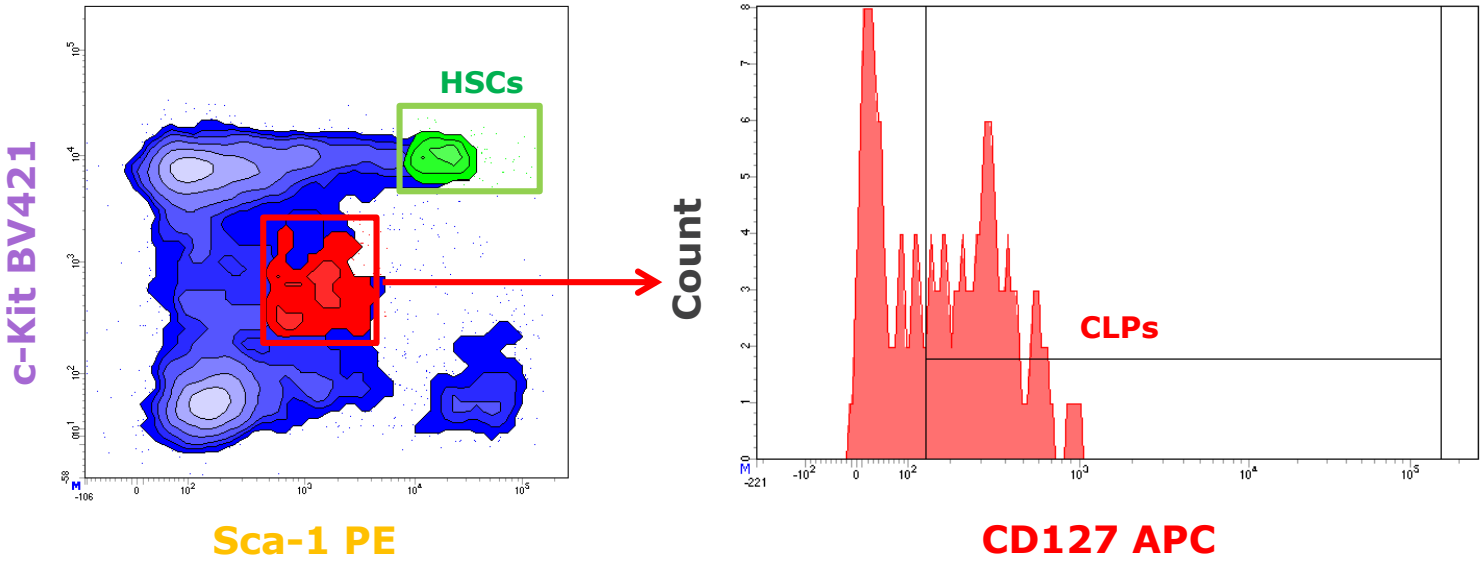
Antigen density: APC-H7 is not bright enough to clearly separate dim CLPs.

Adjacent spillover: Co-expression of c-kit and CD127 was not taken into consideration.

Impact of fluorochrome choice on HSC and CLP resolution

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
Lineage/ 7-AAD	**BV421**	violet
Sca-1	**BV650**	
c-kit	**PE**	blue
CD127	**PE-CF594**	
	BB515	
	APC/Alexa Fluor® 647	red
	BV605	
	BV786	
	BV510	
	FITC/Alexa Fluor® 488	
	PerCP-Cy5.5	blue
	V450	
	V500	
	Alexa Fluor® 700	
	APC-H7	

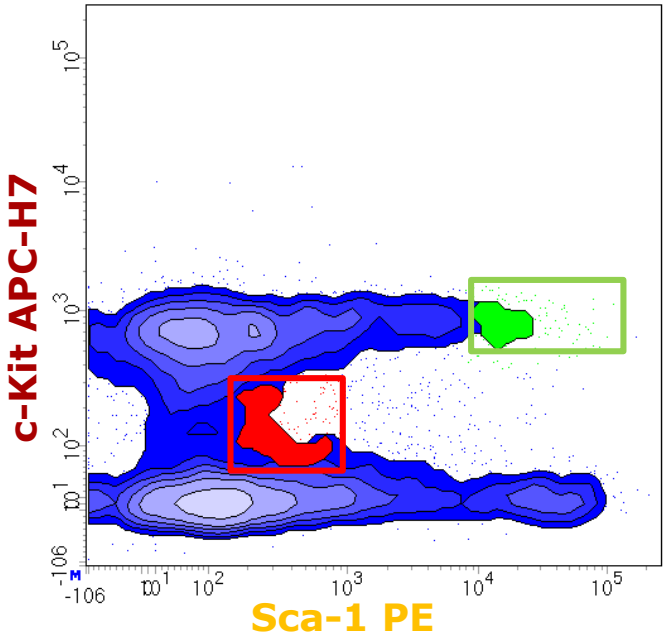
Fluorochrome choice: panel 2

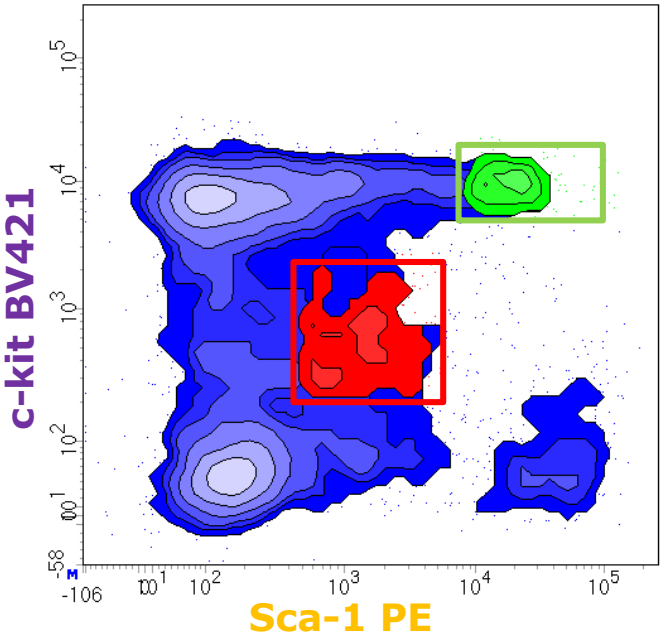


Antigen density: BV421 clearly separates dim and bright c-kit positive cells.

Adjacent spillover: Fluorochromes were spread across different lasers, for minimal spectral overlap, maximum resolution.

Fluorochrome choice: panel comparison





Tube: 16 Panel

Population	#Events	%Parent	%Total
All Events	1,400,000	####	100.0
Cells	811,405	58.0	58.0
Singlets	728,789	89.8	52.1
Lineage/7-AAD neg	4,777	0.7	0.3
HSCs	128	2.7	0.0
CLPs	214	4.5	0.0
CD127 CLPs	95	44.4	0.0

Tube: c-kit BV421 panel

Population	#Events	%Parent	%Total
All Events	1,400,000	####	100.0
Cells	814,960	58.2	58.2
Singlets	731,515	89.8	52.3
Lineage/7-AAD n	4,623	0.6	0.3
HSCs	156	3.4	0.0
CLPs	312	6.7	0.0
127 CLP	184	59.0	0.0

Recipe for best panel for sorting

- Use a dump channel to exclude dead and lineage cells.
- Magnetic depletion of lineage cells further improves the cell sort of rare populations.
- Take into consideration co-expression and spillover.
- Match the brightest fluorochromes with the antigens with lower antigen density.

Conclusion

- Dead cells
- Lineage cells
- Cells of interest