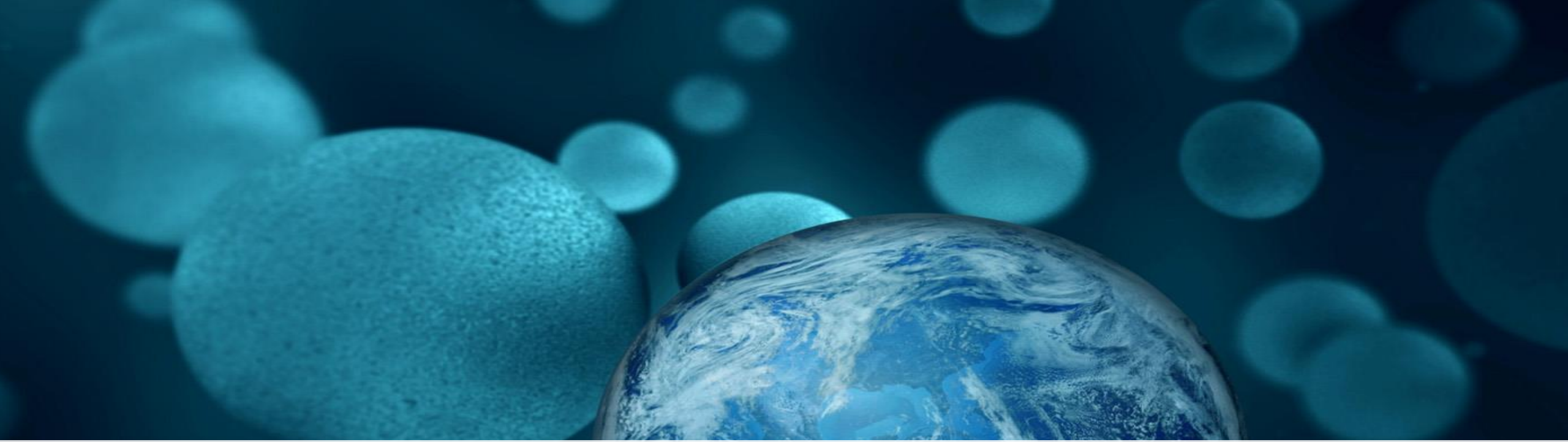


ThermoFisher SCIENTIFIC

Introduction to Small Particle Flow Cytometry

What are “small particles”?

- Microorganisms such as:
  - Bacteria
  - Yeast
  - Algae
- Microparticles such as:
  - Exosomes
  - Microvesicles
  - Apoptotic microvesicles

Flow Cytometry and Small Particles

Appropriate controls and instrument maintenance are required to perform analysis on small particles:

1. Flow cytometer must be free from contaminations.

2. Appropriate to experiment controls needs to be used. That can include:

- Size-calibrated beads
- Filtered PBS
- Unstained and stained controls

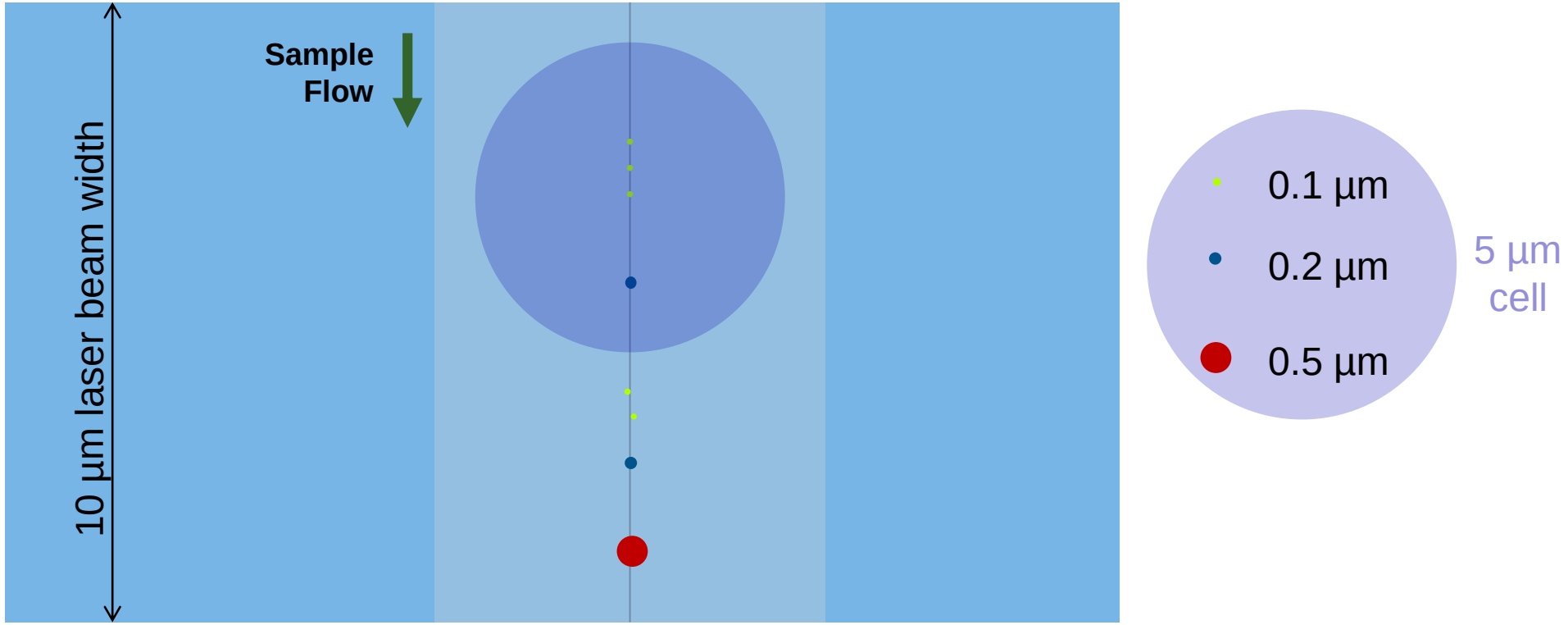
Important sample guidelines

Sample Flow Rate	Max. Sample Concentration	Particle Size	Particle Velocity
1000 µL/ min	2.1×10^6 cells/mL	- Particles > 4 µm - Predominantly acoustic focusing	8 m/sec*
500 µL/ min	4.2×10^6 cells/mL	- Particles > 2 µm - Predominantly acoustic focusing	8 m/sec*
200 µL/ min	6.7×10^6 cells/mL		4 m/sec
100 µL/ min	1.3×10^7 cells/mL		
25 µL/ min	2×10^7 cells/mL	- Small particles < 2 µm - Best resolution from background for dimly positives assays - Smallest sample core - Predominantly hydrodynamic focusing	4 m/sec
12.5 µL/ min	2×10^7 cells/mL		

*Higher flow rates may show some loss of sensitivity

Eliminate coincident events

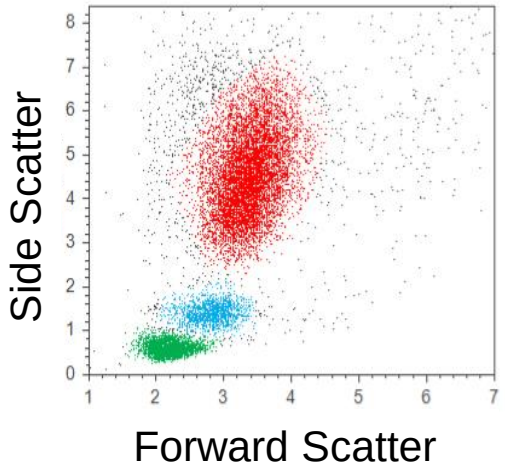
- Sample preparation
  - Ensure to have single cells using an appropriate method for your cells or particles (i.e. vortex, EDTA…), and avoid to much concentrated samples
  - **<u>Sample dilution is essential for smaller events!</u>**



Visualization of your population

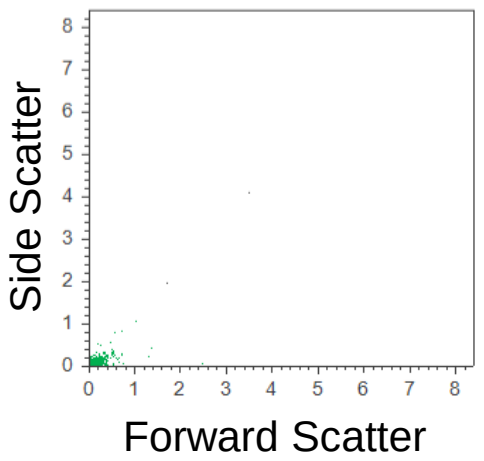
- A scatter plot is displayed with the axis on a linear scale by default
  - This display is ideal for most eukaryote cells analysis, such as human blood analysis

Whole human blood, lysed (~5-10µm +)



Linear scale

Bacterial cells (<1µm)

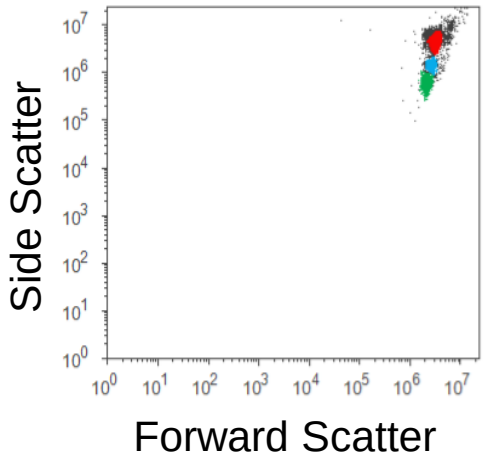


- But not adequate for small events

Visualization of your population

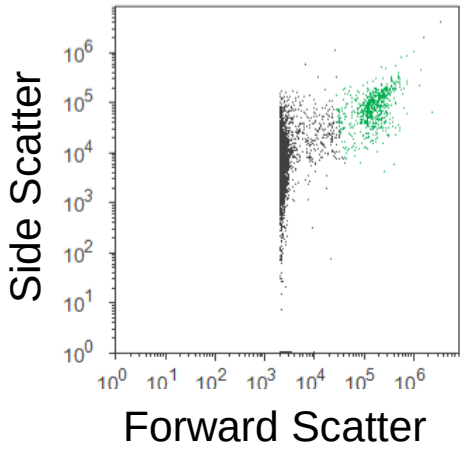
- In order to be able to distinguish small particles from the noise we need to change those axis to a logarithmic scale

Whole human blood, lysed (~5-10µm +)



Log scale

Bacterial cells (<1µm)



Visualization of your population

- It is usually recommended to use the height of the voltage pulse rather than the area
  - For small size events, the height of the pulse generally allow a better resolution of populations.
  - It is less sensitive to coincident events and allow to work with more concentrated samples

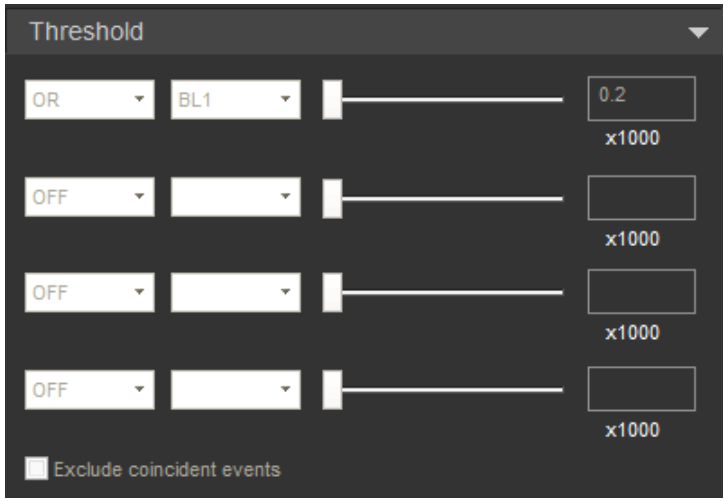
- Using different parameters to find your populations
  - For very small events (typically under 500nm) the separation on the forward scatter (FSC) is not possible on most standard flow cytometers including the Attune NxT
  - It is then recommended to use the side scatter (SSC) and a fluorescent channel

THE KEY OF A SUCCESSFUL EXPERIMENT IS TO SELECT PROPER PMT VOLTAGES AND THRESHOLD

Use threshold

- Setting the Attune™ instrument settings is key to correct identification of the bacterial population of interest. Optimal settings are found with a balance of threshold and PMT voltage.

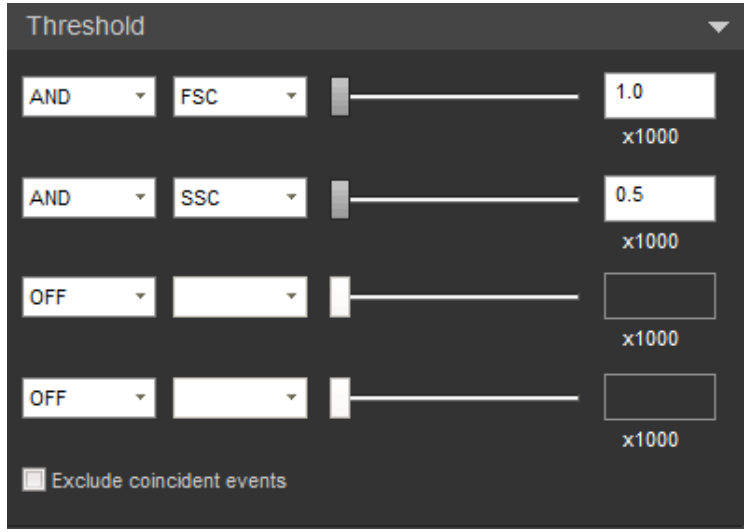
  - *If your cells/particles are labeled:* select a fluorescent threshold in the channel of interest by adding a fluorescence threshold of interest to “Or”. The only events recorded upon sample run are those events emitting fluorescence above this threshold.
  - *Upon sample run:* Increase the forward and side scatter PMT voltages until the population of interest is visualized in the SSC vs FSC density plot.

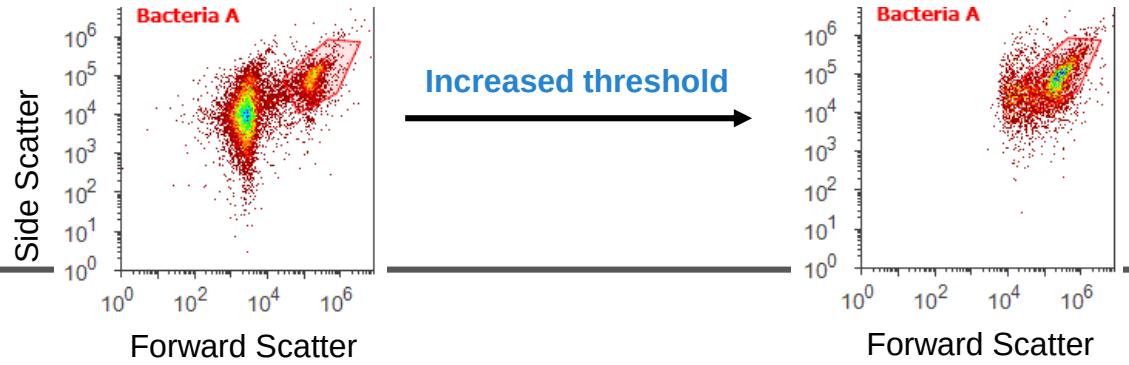


Use threshold

- *If your cells/particles are not labeled*: Select both a forward and side scatter threshold by selecting “And”. Once selected, lower the FSC and SSC thresholds from 10,000* to ~500.
- *Upon sample run:* Increase the forward and side scatter PMT voltages until you can differentiate the population of interest from electronic noise and/or debris. This will be easiest to visualize if the FSC and SSC threshold are set at 2,000 at the start.
- Gradually increase the FSC and SSC thresholds until electronic noise/debris is eliminated and only cells remain visible.

*Note: the default value is 25,000





Minimize the noise

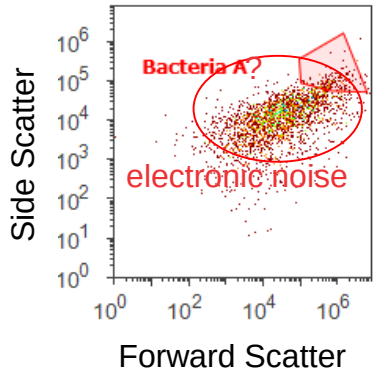
Noise can make population identification difficult and can come from different sources:

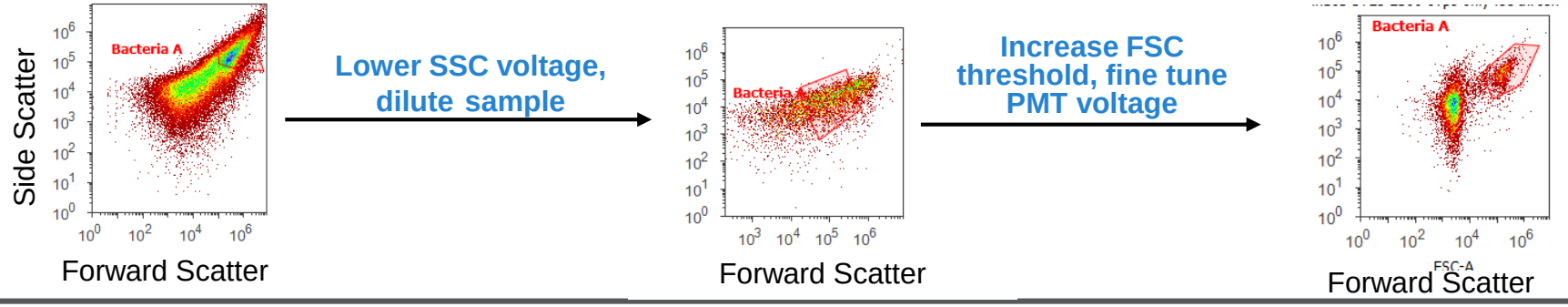
- Instrument
  - Source of noise:
    - Stray light
    - Low-level electronic signals
  - How to reduce it:
    - Serviced and well aligned / performances tested
    - Decontaminate regularly, at least quarterly or as needed
    - Focusing fluid filtered at 0.1 µm for events of a size < 500nm
    - Match your focusing fluid salt concentration with your sample

If I can’t find my cells or particles?

If your cells are unlabeled, differentiating the bacterial population from electronic noise and debris will take practice.

- Get comfortable with electronic noise:
  - You can see events on your plots when there is nothing in your sample (e.g.: 0.2µm filtered water). **This is normal.**
  - Expect to observe very high event rates. Event rate will be higher the lower the scatter thresholds are.
- Learn how to adjust instrument settings to differentiate your population of interest.
  - Incorrectly set PMT voltages and threshold settings can lead to frustration.





Tips for sample preparation

- Use a fluorescent marker
  - *Helps define microbial population*
- Understand the fluorescent marker
  - *Organisms stained? Appropriate controls?*
- Take care to reduce “noise”
  - *Filter buffers through a 0.1 µm filter or smaller*
  - *Use buffers with similar refractive index as “sheath”*
- Take care to avoid aggregation
  - *Use appropriate cell concentrations*
  - *Employ doublet discrimination*

Controls

- Control sample are needed to validate the quality of your data
- You will always need:
  - **Negative control: stained buffer/medium only**
  - **Negative control: non stained cells**
  - **Appropriate positive controls**

Carryover and Instrument Background

- Carryover is likely to be high for smaller particles.

- If carryover is detected or feared, there are some ways to minimize it:

  - Run an extra rinse between samples
  - For concentrated samples: Sanitize Attune SIP between sample
  - Using the AAS: increase the number of rinses between wells
  - Clean your instrument more often (using Deep Clean function)
  - Always run a thorough shutdown
  - Always use freshly prepared bleach

Key learning messages

- Be familiar with the noise
- Play with voltage and threshold
- Prepare the right controls (negative, positive, buffer only…)
- Setup plot as logarithm and using height
- Use fluorescence to discriminate from background
- Have a clean and properly maintained instrument
- Setup threshold on fluoresccence
- There is no standards for microvesicles
- Use filtered solutions
- Dilute sample to avoid several events at the same time in front of the laser
- Use low flow rate for very small particles