Flow cytometric analysis of EVs

How and why?

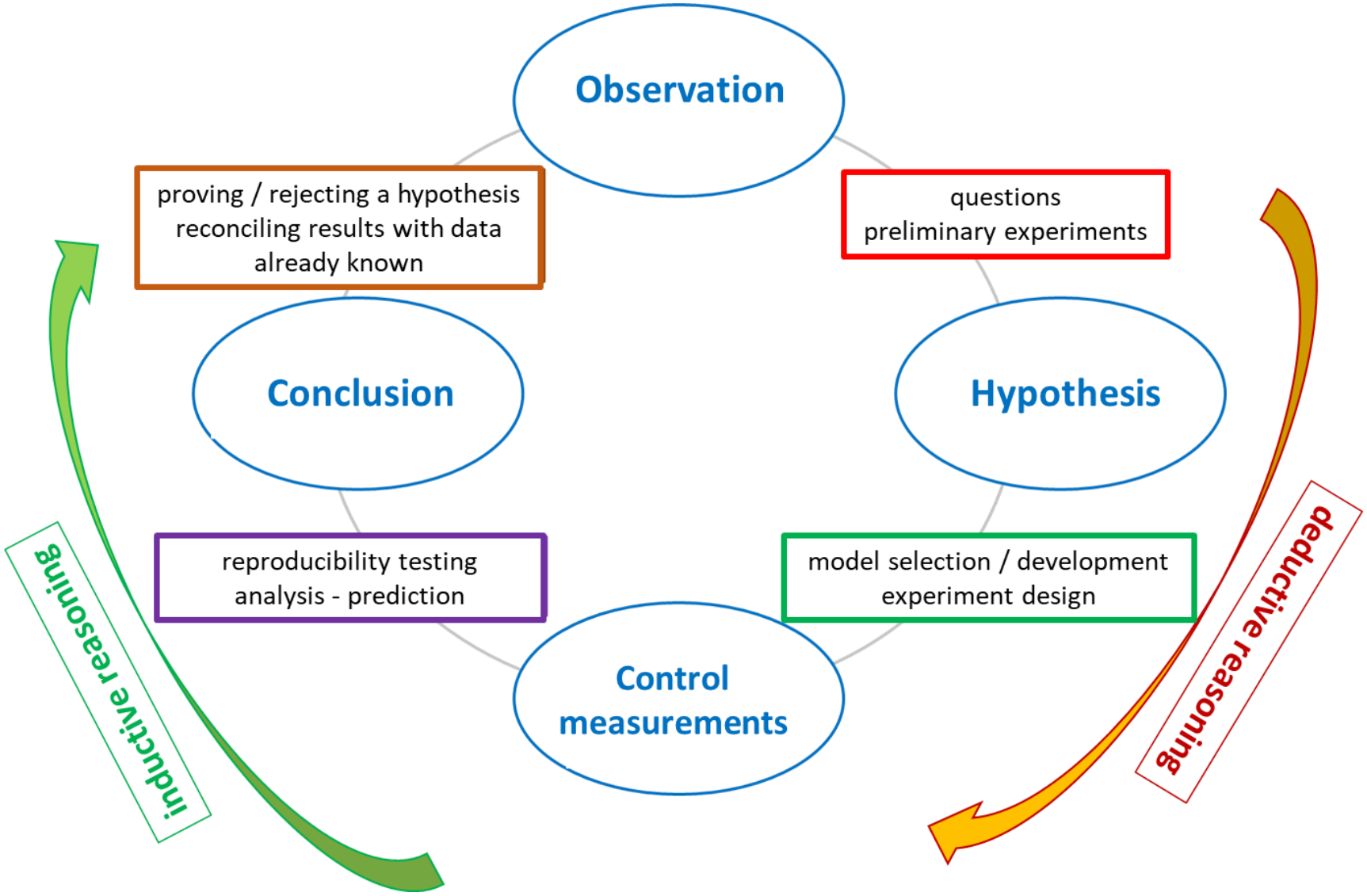
Definition of research

Scientific work is the solution of a scientific question. Achieving new scientific results by evaluating existing facts, observed data, experiences and drawing conclusions. *(Hungarian interpretive dictionary)*

In the course of scientific research, after defining the research problem or question, the researcher collects data in a planned and regular manner, processes the data, analyzes it and publishes it for others according to the expected content elements and form of scientific publications. *(Gábor Héra, Methodology, 2005)*

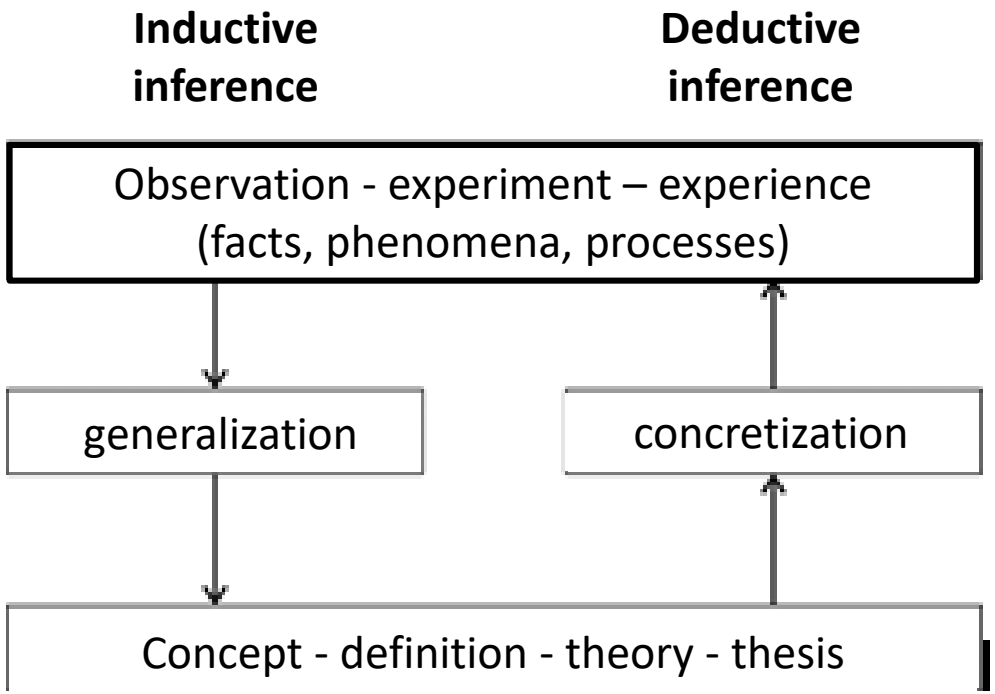
„Researching something is all about seeing what everyone sees and thinking about what no one else has.” *(Szent-Györgyi Albert)*

RESEARCH


Observation
questions
preliminary experiments
Hypothesis
model selection / development
experiment design
Control
measurements
reproducibility testing
analysis - prediction
Conclusion
proving / rejecting a hypothesis
reconciling results with data
already known
deductive reasoning
inductive reasoning

Strategies of cognition: induction and deduction

In deductive inferences we get from the general to the individual,
and in the case of inductive inferences we get from the individual to the general.



First step: set a goal

What's the question? What do I want to be answered?

How can we use flow cytometry in EV research?

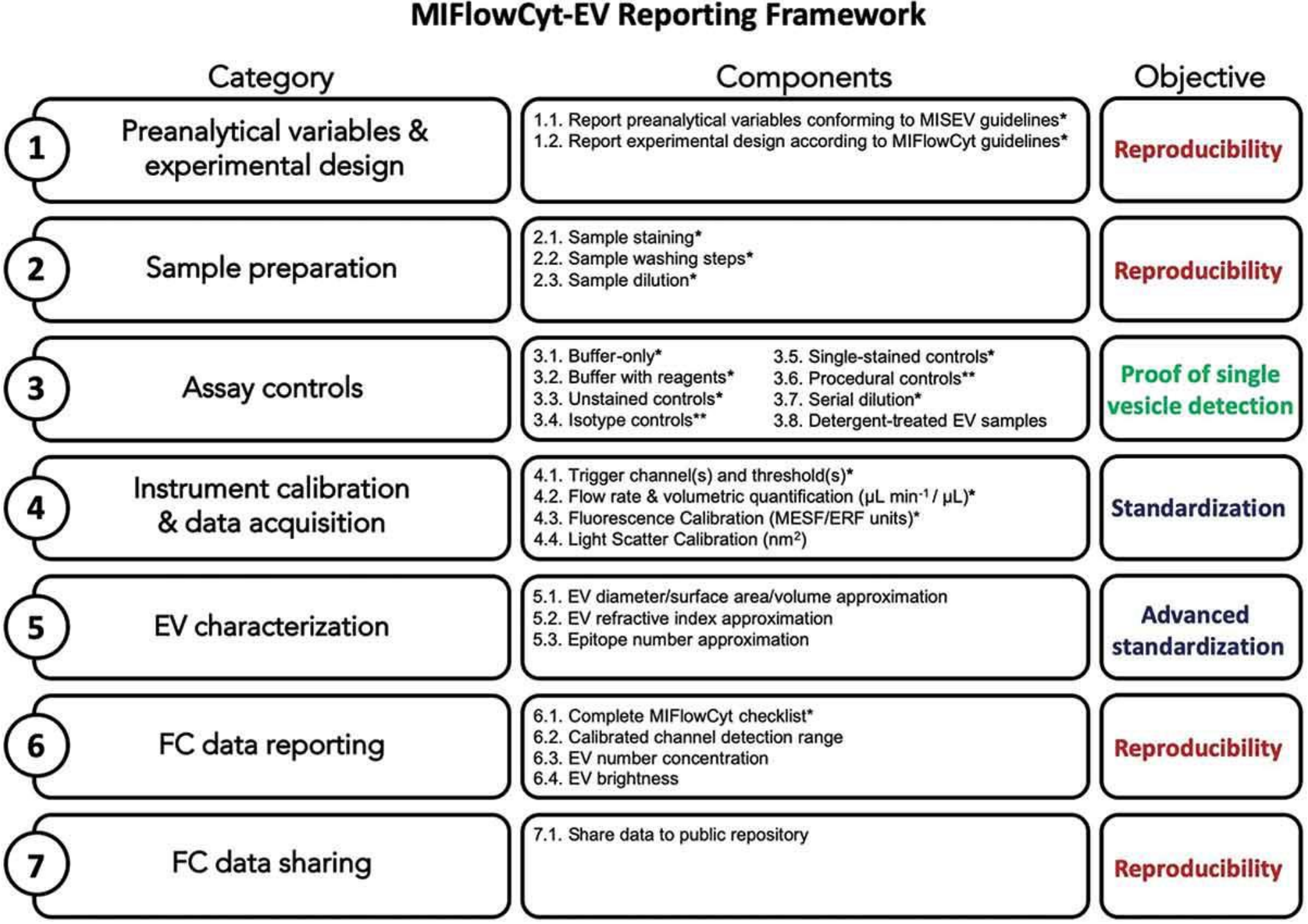
MIFlowCyt-EV

Journal of Extracellular Vesicles

ISSN: (Print) 2001-3078 (Online) Journal homepage: https://www.tandfonline.com/loi/zjev20

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

we need to pay attention to the followings:



checklist

Table 1. MIFlowCyt-EV framework.

Framework components	Complete for each component
1.1 Preanalytical variables conforming to MISEV guidelines*	
1.2 Experimental design according to MIFlowCyt guidelines*	
2.1 Sample staining details*	
2.2 Sample washing details*	
2.3 Sample dilution details*	
3.1 Buffer-only controls*	
3.2 Buffer with reagent controls*	
3.3 Unstained controls*	
3.4 Isotype controls**	
3.5 Single-stained controls*	
3.6 Procedural controls**	
3.7 Serial dilutions*	
3.8 Detergent-treated controls	
4.1 Trigger channel(s) and threshold(s)*	
4.2 Flow rate/volumetric quantification*	
4.3 Fluorescence calibration*	
4.4 Scatter calibration	
5.1 EV diameter/surface area/volume approximation	
5.2 EV refractive index approximation	
5.3 EV epitope number approximation	
6.1 Completion of MIFlowCyt checklist*	
6.2 Calibrated channel detection range	
6.3 EV number/concentration	
6.4 EV brightness	
7.1 Sharing of data to a public repository	

*Highlights the components that are broadly applicable to the majority of single-EV analysis experiments regardless of design or instrumentation. **Highlights the components that are only applicable in cases where certain reagents or protocols are used.

1.1. and 1.2. Preanalytical variables and experimental design

Pre-analytical factors:

method of sample collection,
isolation
storage before measuring

Recommendation: pre-analytical variables must be reported according to the MISEV guidelines

1. characteristics of the source
   - **cell culture conditioned media** (basic characterization of the releasing cells and culture and harvesting conditions including passage number or days in culture for suspension cells, density/confluence at harvest ; quantification of apoptotic and necrotic cells; contamination with Mycoplasma; culture medium compositions and preparation details)
   - **biological fluid** (plasma sample: donor age, biological sex, current or previous pregnancy, menopause, pre/postprandial status (fasting/nonfasting), time of day of collection (Circadian variations), exercise level and time of last exercise, diet, body mass index, specific infectious and noninfectious diseases, medications, and other factors which may affect circulating Evs)
   - **tissue** (tissue harvest, processing (e.g. mechanical disruption), storage (including freezing; method of EV preparations; cell distribution inside the tissue)
2. **how the source material is manipulated** (treatments and treatment conditions)
3. storage
4. experimental conditions

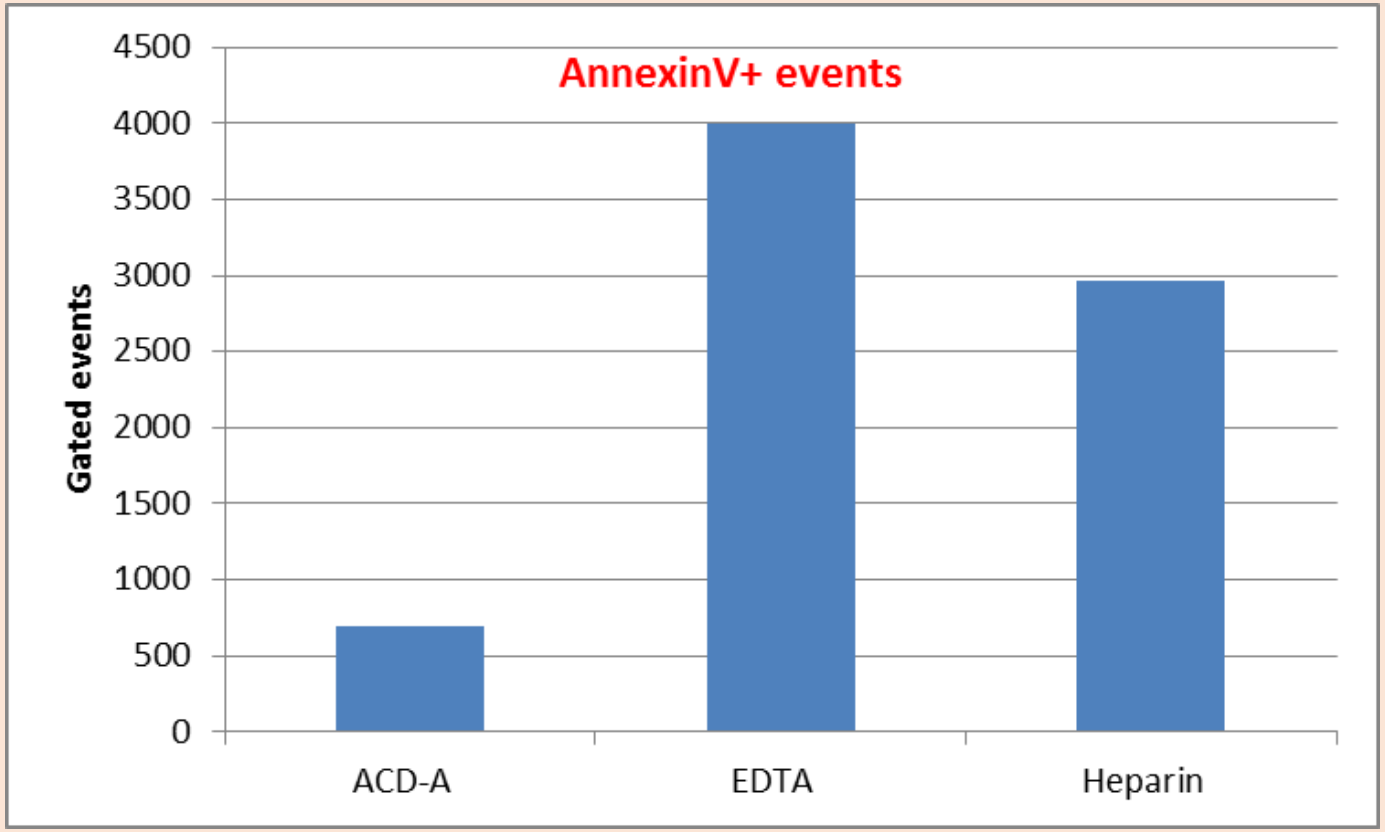
Cell type
Viability
cell number
Infections / contaminations

Donor / patient data including pathological states and drugs
Sample type
Collection

Pre-analytical factors

The effects of anticoagulation of plasma derived Evs

(after Triton lysis)



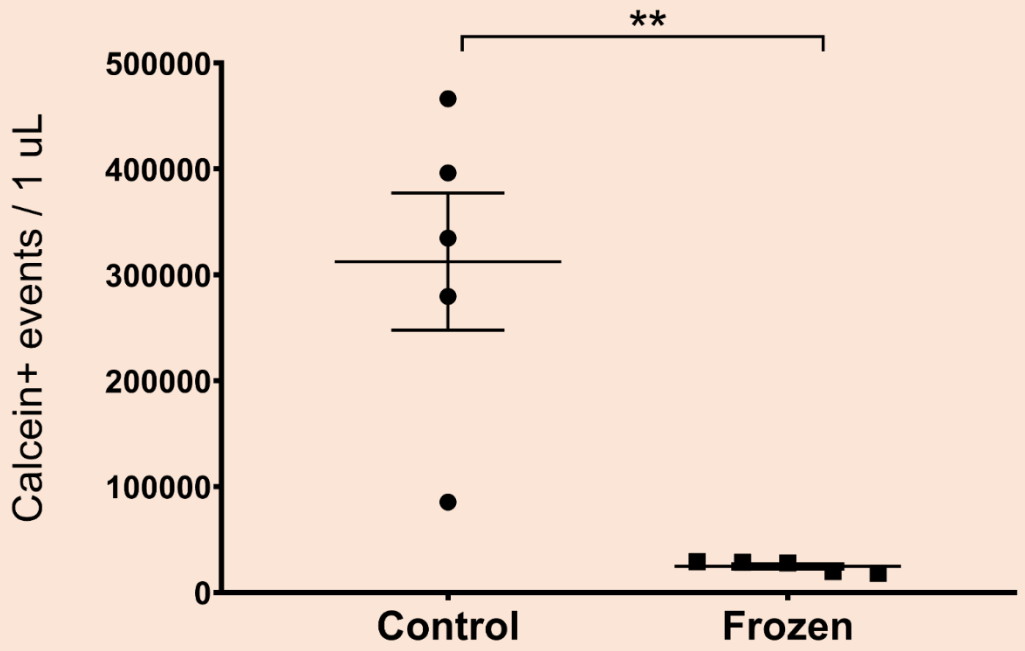
1. György et al: ACD anticoagulant is suggested in the case of peripheral blood collection
2. You can compare only those plasma samples which were anticoagulated by the same anticoagulant.
3. Never use serum, because the coagulation removes (traps) unknown amounts of EVs from the sample.

Freeze-thaw-damaged EVs' calcein staining is significantly reduced

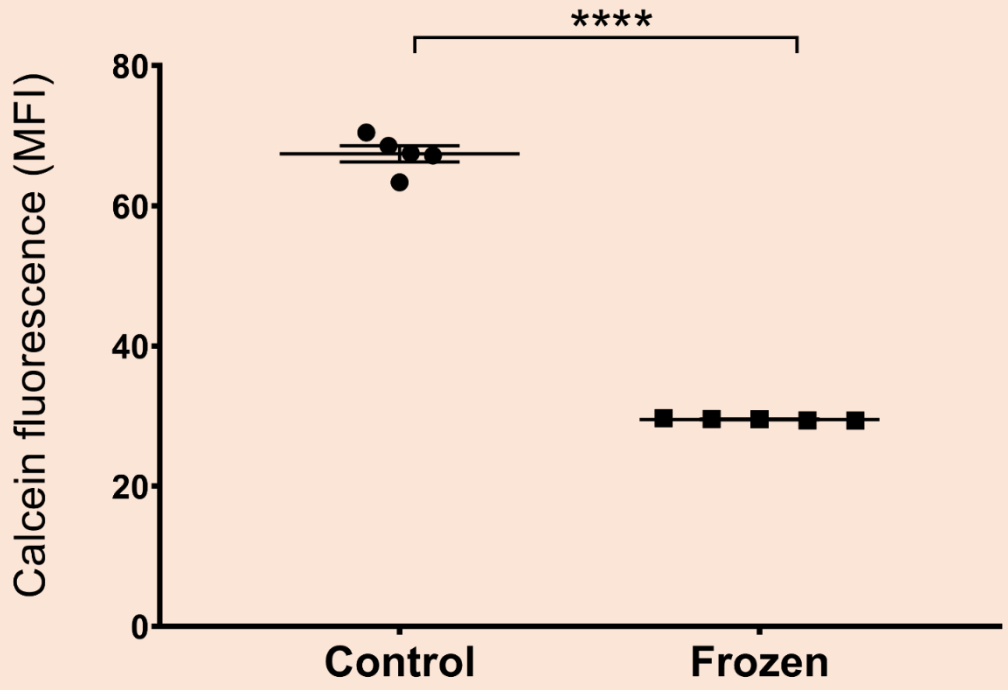
(Experiments of Márk Langer)

Pre-analytical factors

Calcein+ mEV number



MFI



1.2. Report experimental design according to MIFlowCyt guidelines

Providing a clear purpose of the performed experiments and a concise and detailed description of the variables of an FC experiment(s) allows the reader to understand and interpret the subsequent data of the performed experiment(s).

brief description of the experimental **aim**, **keywords** and **variables** for the performed FC experiment(s)

What is the question?

What questions do I want to be answered?

without question there is no answer

2.1., 2.2., 2.3. Sample preparation

Staining

EV concentration (**MISEV 2018 4-a**: light scattering technologies, NTA; FACS, resistive pulse sensing (RPS), cryo-EM platforms combining surface plasmon resonance (SPR) with AFM)
label concentration,
incubation time,
temperature,
conjugation efficiency,
label-type (and clone if monoclonal antibodies are used)
light exposure

The standardization of the experimental system may be longer than the experiment itself. **BUT** without standardization the results cannot be interpreted.

Washing (decreasing the concentration of excess label, thereby increasing the signal-to-noise ratio)

Dilution

Freshly filtered solutions!

Staining methods are in Sharepoint

3.1. – 3.8. Controls

3.1 Buffer-only controls

- detects background noise
- Helps to define the FS / SS gate

3.2 Buffer with reagent controls

- if you use multiple buffers during your experiment, you need to create multiple buffer controls
- if you use more than one dye in your experiment, you will prepare a control for each one
- if you make a multicolor labelling, the dye control should include all the dyes used at the same time

3.3 Unstained controls

- detects autofluorescence

3.4 Isotype controls

- detects aspecific labelling (labeling with antibodies)

3.5 Single-stained controls

- required for fluorescence compensation

3.6 Procedural controls

- Biological controls
- Optimization of staining (e.g. time, temperature and concentration dependence)

3.7 Serial dilutions

3.8 Detergent-treated controls

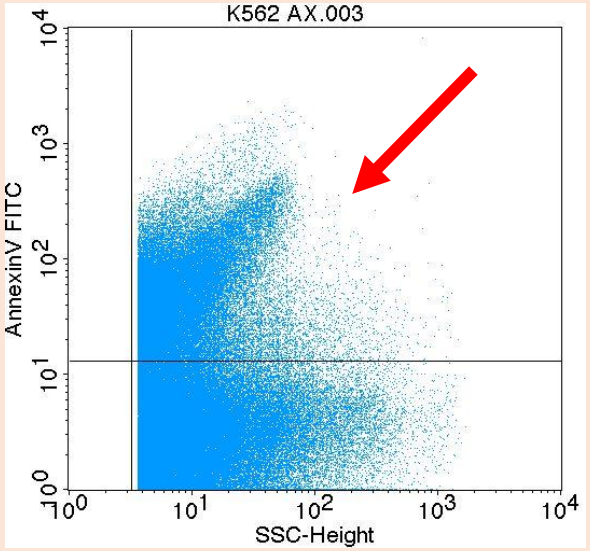
- TritonX lysis

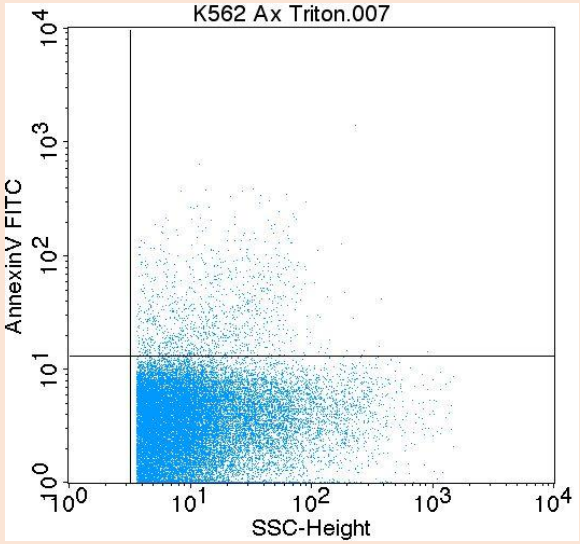
Annexin V staining of mEVs

stained

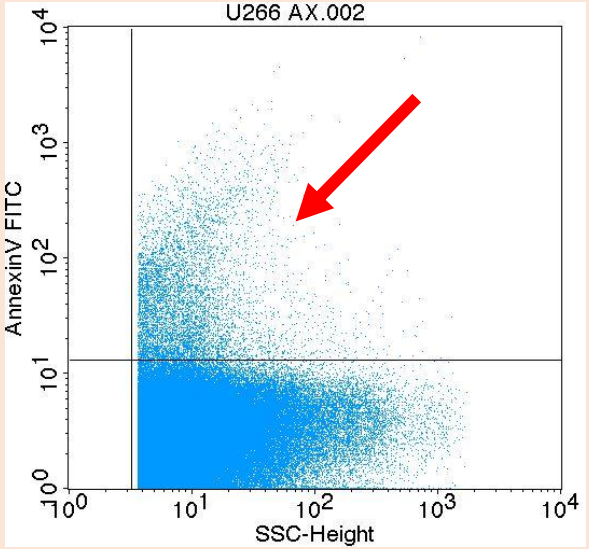
TritonX-100

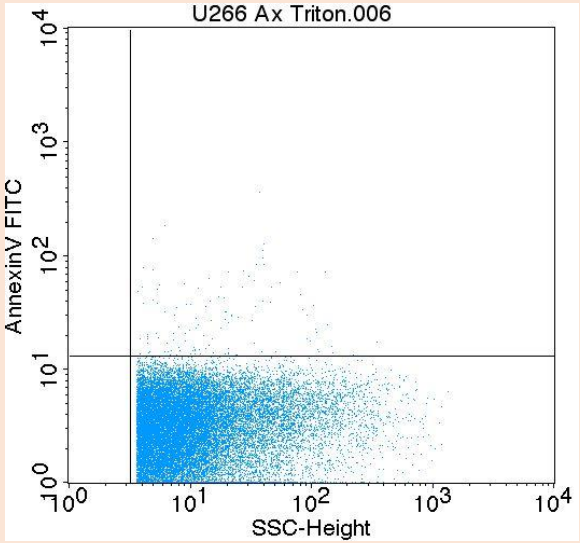
K562





U266





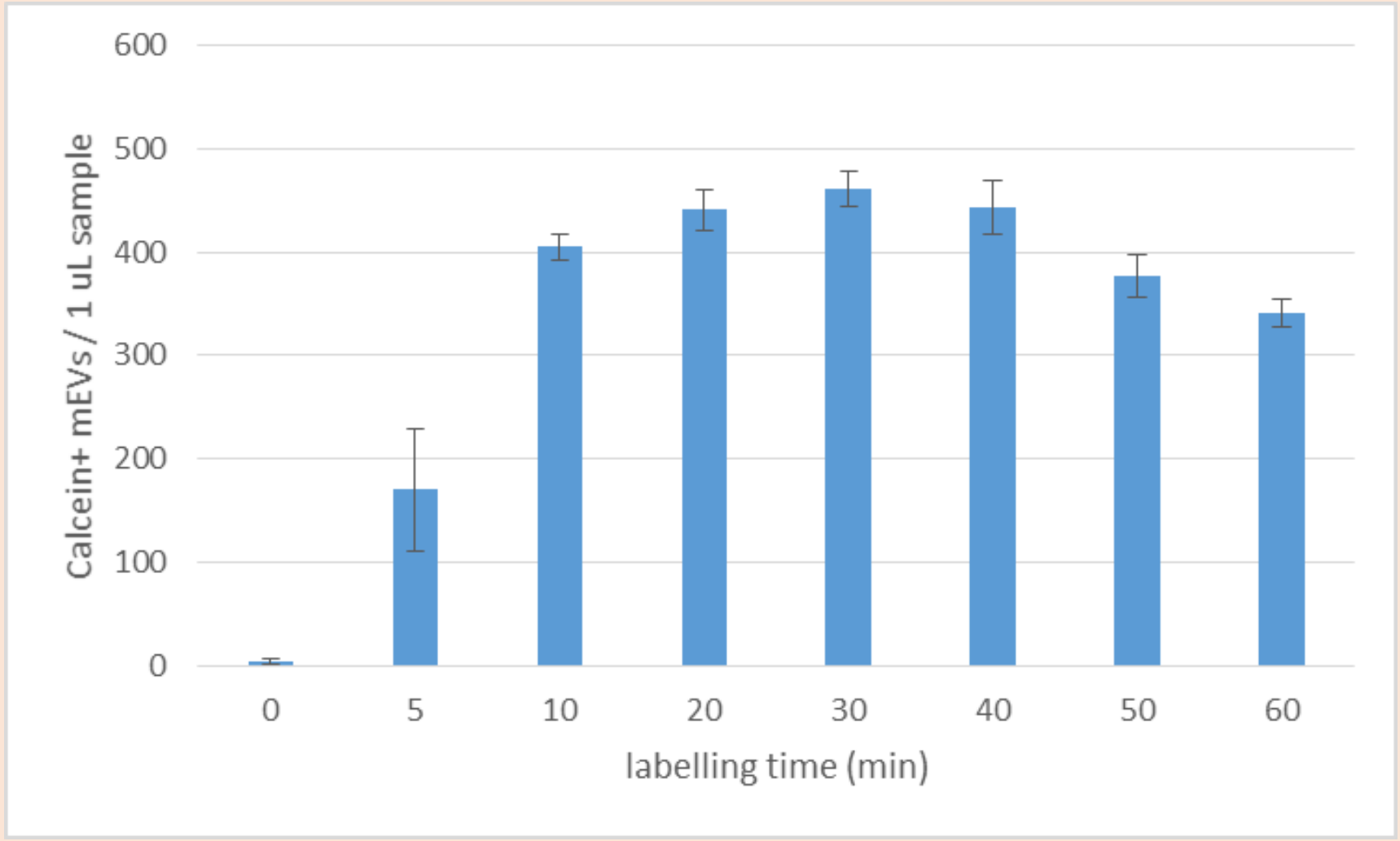
Experimental conditions:
Same cell number,
same volume of cell culture medium,
same culture time,
same mEV isolation time,
same volume for mEVisolation

Control tests must be performed on all cell lines!

Both samples could be labelled by Annexin V.

3.6. Optimization of staining (e.g. time, temperature and concentration dependence)

Calcein+ mEVs: time dependence



Multicolor staining

My suggestions:

1. Given that the surface area of EV is small compared to cells, I propose labeling with up to 3 different antibodies.

2. For the combination of membrane labeling and immunophenotyping, titration of the membrane dye is very important. This is necessary because fluorochrome, which stains the membrane uniformly, will give a much larger fluorescent signal than the antibody. Therefore, fluorescence compensation will be very difficult.

Fluorescence compensation

My suggestions:

Use the VersaComp Antibody Capture Kit, because vesicles surface protein expressions are different, so it is possible, that the detected fluorescent signals may not be enough for compensation.

Never accept compensation results automatically.

Think!

4.1. – 5.3. Instrument settings

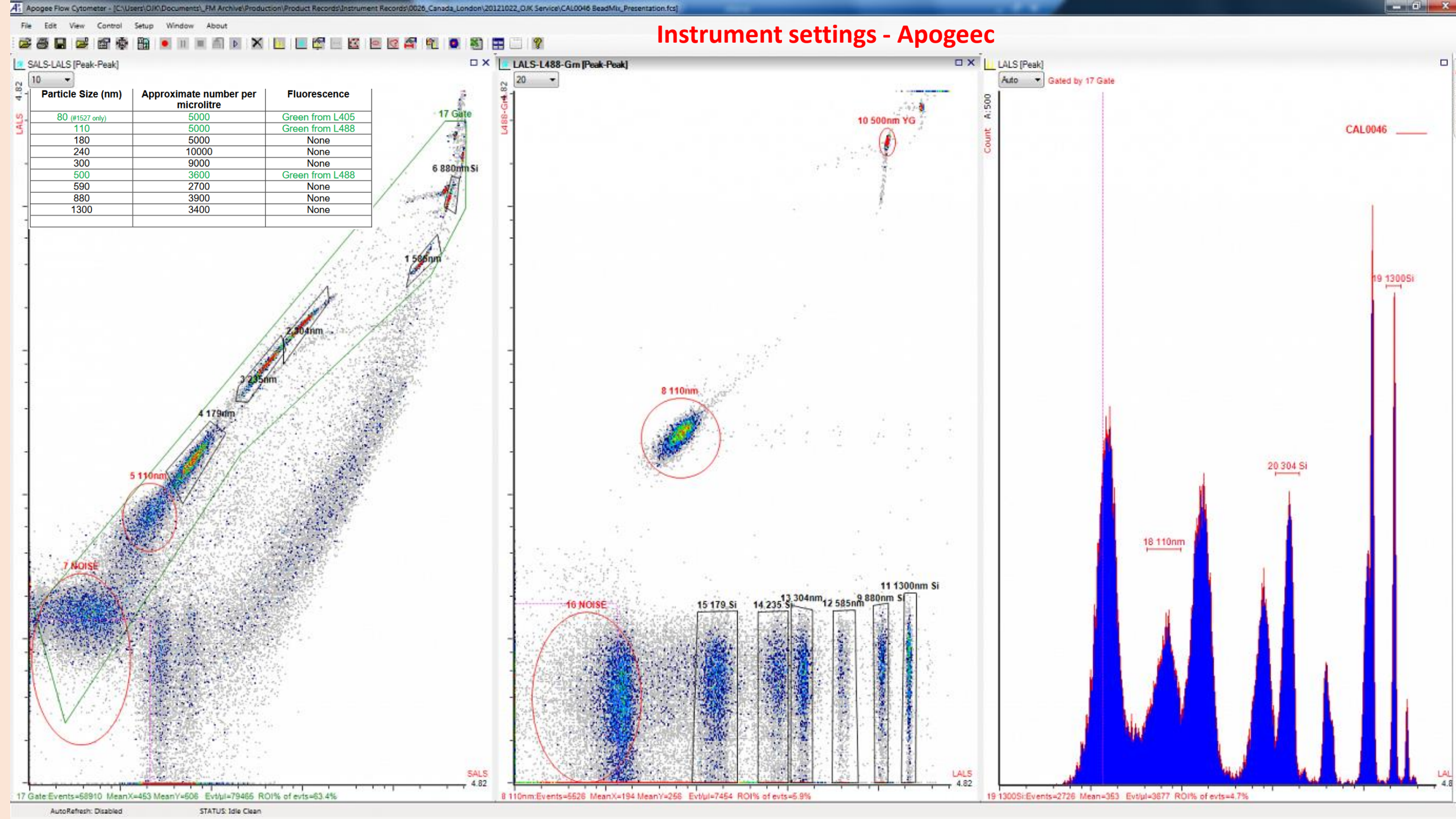
4.1 Trigger channel(s) and threshold(s)
4.2 Flow rate/volumetric quantification
4.3 Fluorescence calibration
4.4 Scatter calibration
5.1 EV diameter/surface area/volume approximation
5.2 EV refractive index approximation
5.3 EV epitope number approximation

My suggestions:

1. Instrument dependent:
   - If you have question, you can choose the best instrument for your experiment
   - Use calibration beads (refractive index!). I think it is not necessary every time, but important in the first attempt.
2. Experiment dependent
   - You have to choose your special controls for the detection of background noise
   - You have to create your own fluorescence compensation
   - It is possible that you would like to use the fluorescence intensity as a marker (e.g. protein expression level, enzyme activity etc). In these cases you have to use special calibrations beads, e.g. Fluorescence Quantitation Kits.

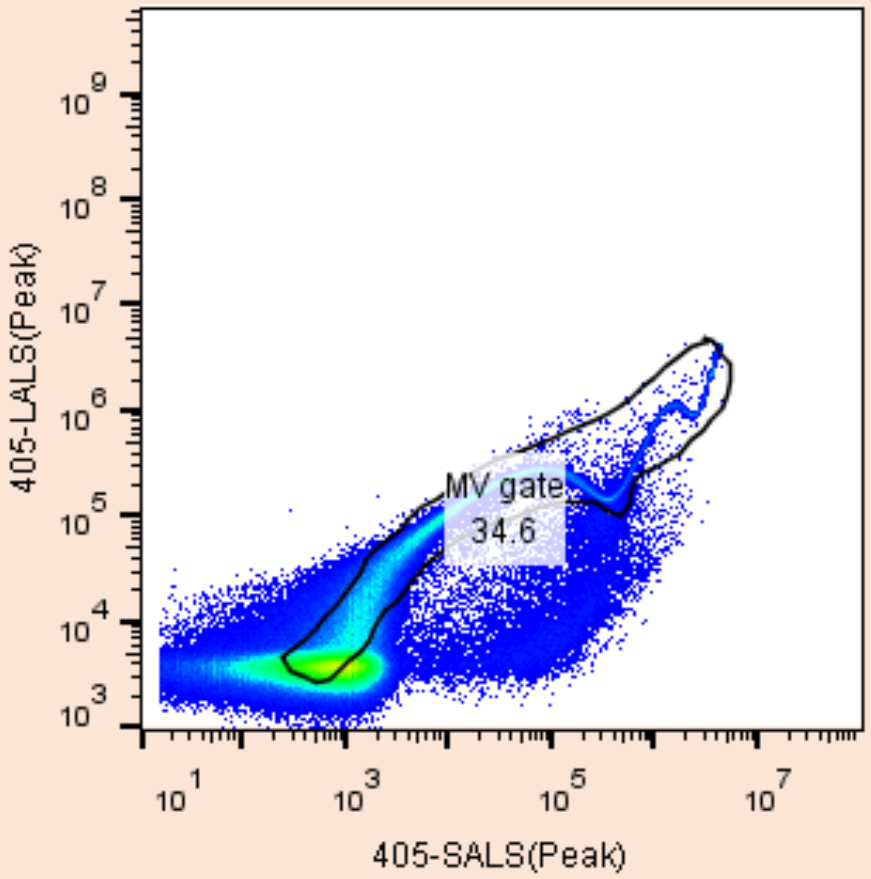
Instrument settings - Apogeec

Particle Size (nm)	Approximate number per microlitre	Fluorescence
80 (#1527 only)	5000	Green from L405
110	5000	Green from L488
180	5000	None
240	10000	None
300	9000	None
500	3600	Green from L488
590	2700	None
880	3900	None
1300	3400	None



Instrument settings - Apogee

Embryo-culture medium – unstained sample



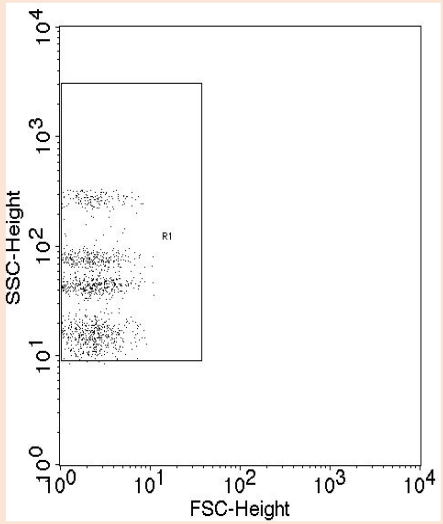
„MV gate" was defined on the basis of SALS/LALS Signals of Apogee calibration beads.

My opinion:

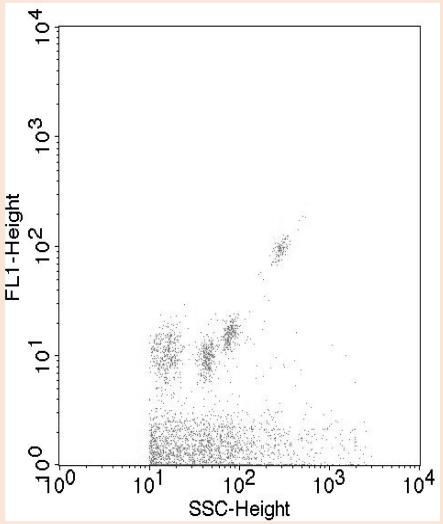
1. You will always detect noise.
2. You have to create the correct gate.
3. The correct gate changes from time to time and from sample to sample. It must be set for each measurement. Changes must be made for each analysis.

Instrument settings - Calibur

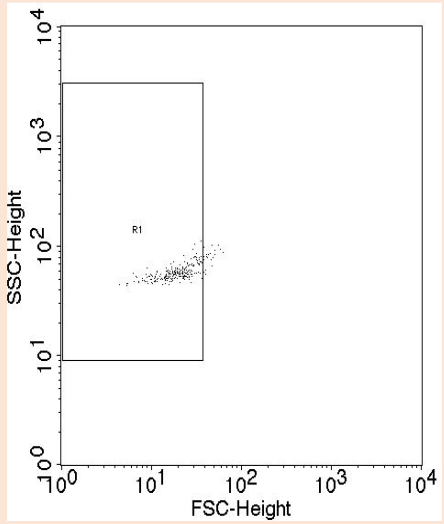
A)



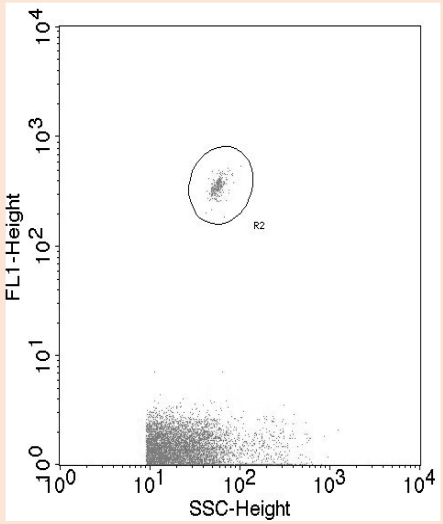
B)



C)



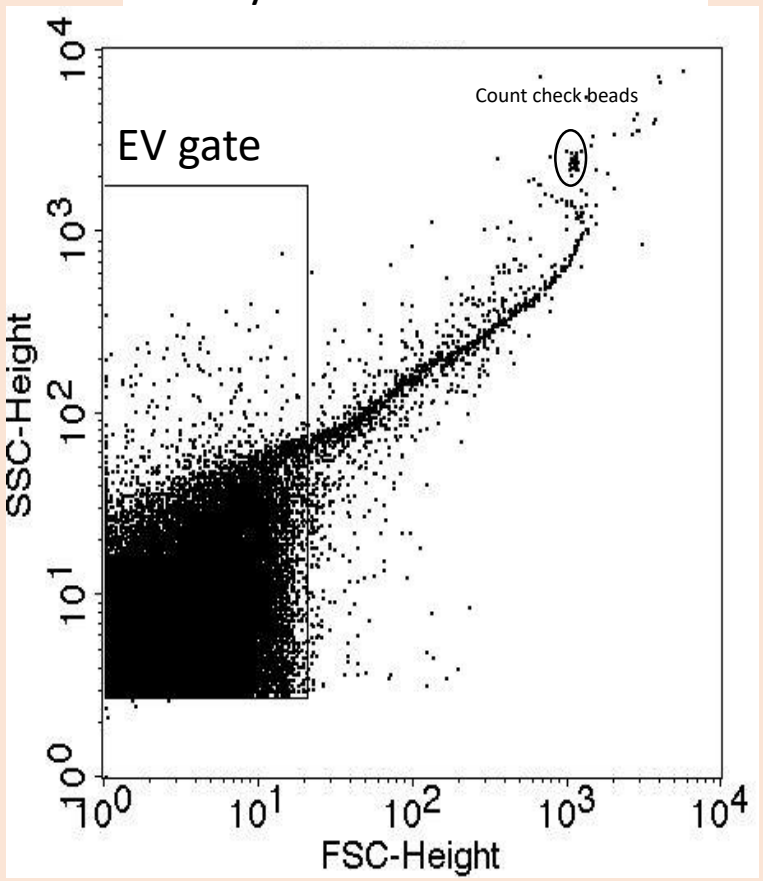
D)



Megamix-Plus SSC is a mix of beads with following diameters: 0.16 µm, 0.20 µm, 0.24 µm and 0.5 µm.

E)

Embryo-culture medium



Bead-based measurements

Facts:

1. Exosomes are under the detection sensitivity of most cytometers
2. If we would like to detect exosomes we have to bind them to latex beads

Difficulties and facts:

1. We have no information about the biding sites of latex beads
2. We are able to standardize the binding conditions BUT we are NOT able to guarantee the binding efficiency

Which type of controls have to be used?

1. **Autofluorescence detection**: Latex beads conjugated with unstained vesicles
2. **Staining control**: Latex beads incubated with dyes or fluorochrome conjugated antibodies without vesicles
3. **Multicolor staining**: Latex beads incubated with all the fluorochrome conjugated antibodies without vesicles and with separated fluorochrome conjugated antibodies (You can use it to fluorescence compensation as well.)

What does it mean? – My opinion

1. We can use these type of measurements as qualitative measurements.
2. We can not calculate the number of exosomes in the sample.
3. Because we can not guarantee the binding of sEVs and / or antibodies to latex particles, therfore the negative result (absence of detected fluorescence) is not equal with the absence of antigen / protein on the surface of sEVs. $\rightarrow$ it is necessary to validate the results by other methods including Western blot

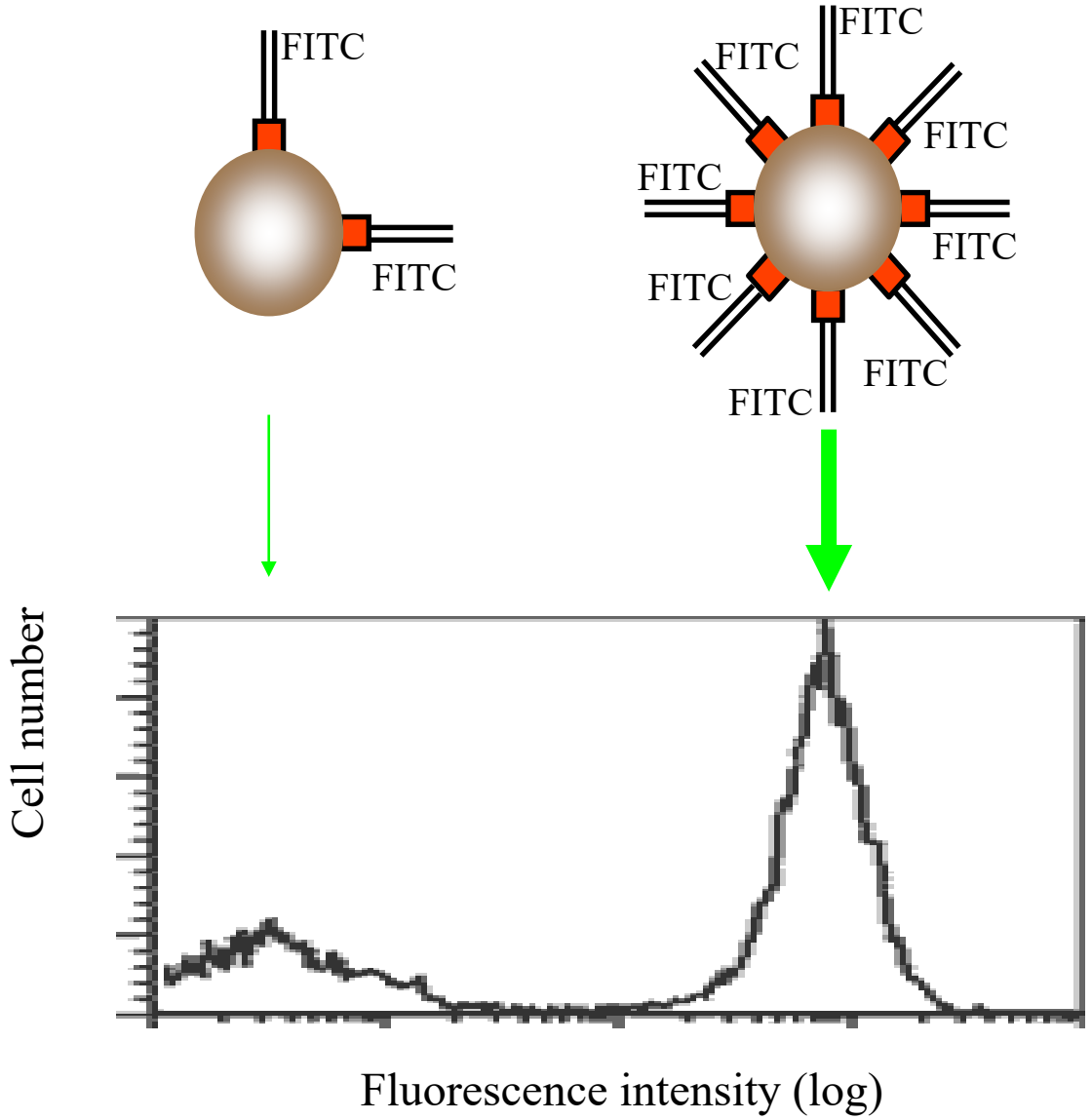
MFI FACS analysis

(mean fluorescence intensity)

Aim: comparison of expression levels of proteins

Fluorescence intensity

Fluorescence intensity is proportional with the number of fluorochromes

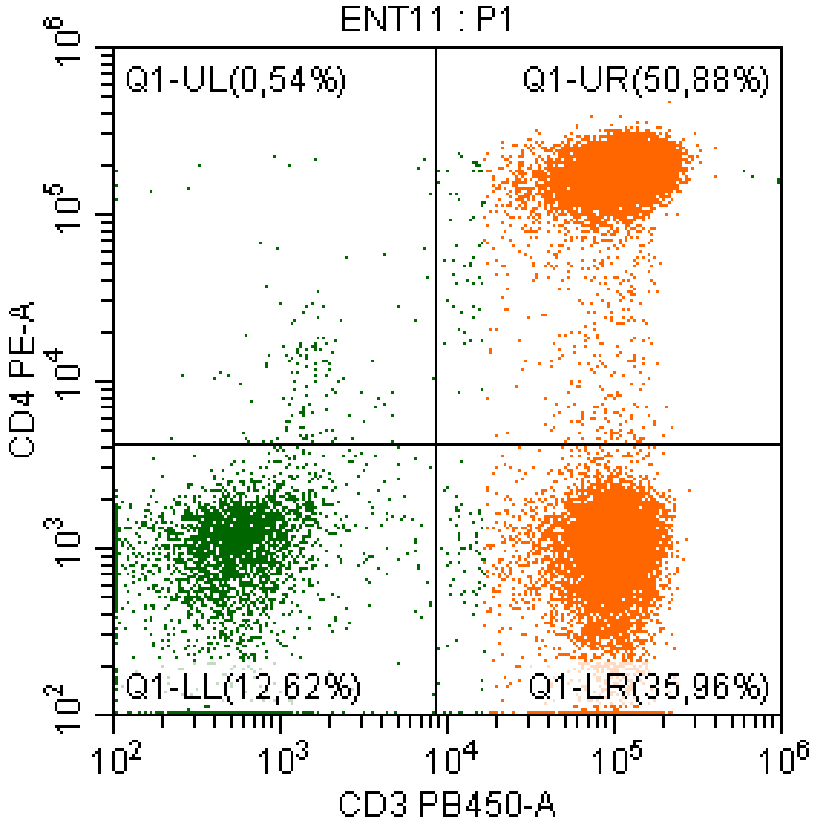


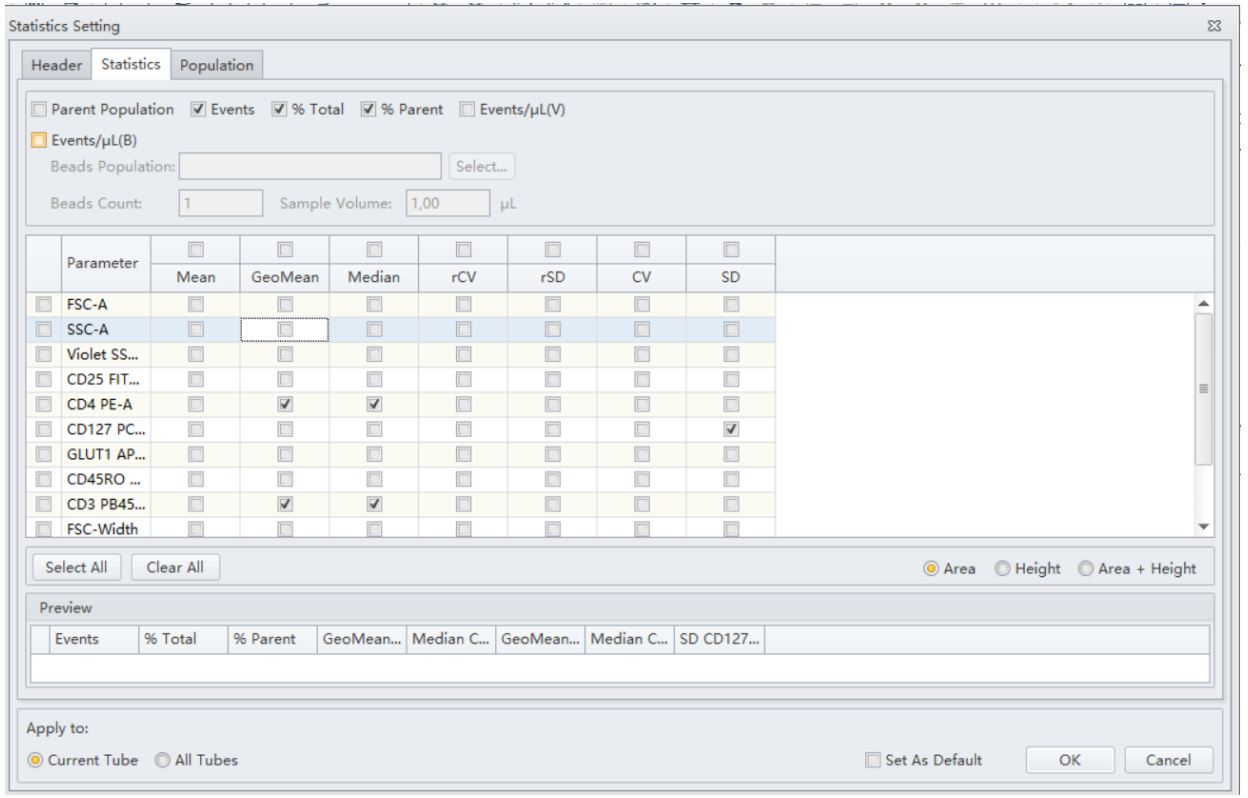
„Biological questions”:

1. EXPRESSION LEVELS OF MOLECULES (RELATIVE OR ABSOLUTE): How many molecules are on the surface or in the cytoplasm?

2. COMPARISON: Can we compare the expression levels of antigens?

Important: we have to saturate all the binding sites. (previous titration of antibodies)





Tube Name: ENT11

Sample ID:

Population	Events	% Total	% Parent	GeoMean CD4 PE-A	Median CD4 PE-A
All Events	50000	100,00%	100,00%	9761,8	4010,0
P1	25879	51,76%	51,76%	13375,8	39874,4
P2	33236	66,47%	66,47%	17897,9	95626,1
Q1-UR	13167	26,33%	50,88%	165424,6	176532,1
Q1-UL	140	0,28%	0,54%	13752,3	11646,5
Q1-LL	3267	6,53%	12,62%	862,0	1029,1
Q1-LR	9305	18,61%	35,96%	860,2	996,2

MFI is typically understood as mean fluorescence intensity. However, it is important to know which kind of mean we are talking about.

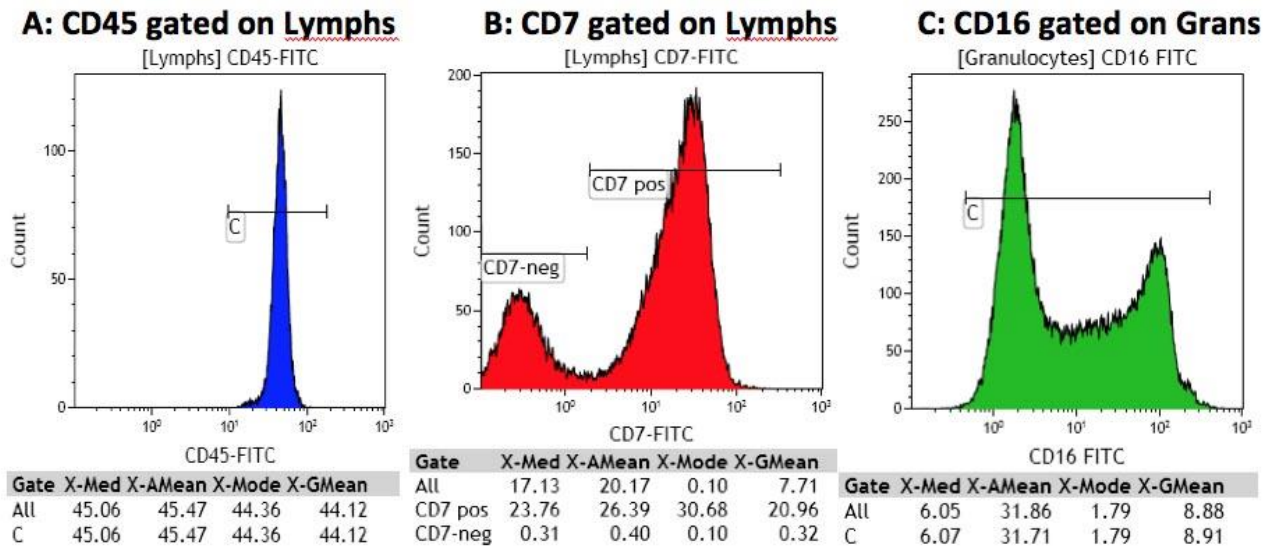
1. Median: midpoint of population (middle channel). Preferred method to measure MFI of a logarithmic histogram.

2. Arithmetic mean: number of events in each fluorescent channel divided by the number of channels. Because fluorescent intensity increases logarithmically (and most flow data are logarithmic), arithmetic mean quickly becomes useless to generalize a population of events, as a right-hand skew causes even more exaggeration of the mean and accurate MFI measurements cannot be made of events off scale either at the negative/dim or bright ends of the histogram.

3. Geometric mean: Without going into too much mathematical detail, the geometric mean compensates for that and is considered the second best choice of describing the MFI of a logarithmic histogram.

4. Mode: refers to the channels which are most frequented.

In an even distribution (example A) the median, arithmetic mean, mode and the geometric mean is almost identical. However, if we look at example B, a skewed antigen expression causes the mean to drift in the direction of the skewed area (in this case to the left). In order to compensate for this, the geometric mean (gMFI) is often used to account for the log-normal behavior of flow data. The MFI should NOT be used in a bimodal distribution (example C) as any average only holds true for normal distributions, and a bi-modal population is by definition not normal. Gating each population and presenting percentages will provide much more useful information.

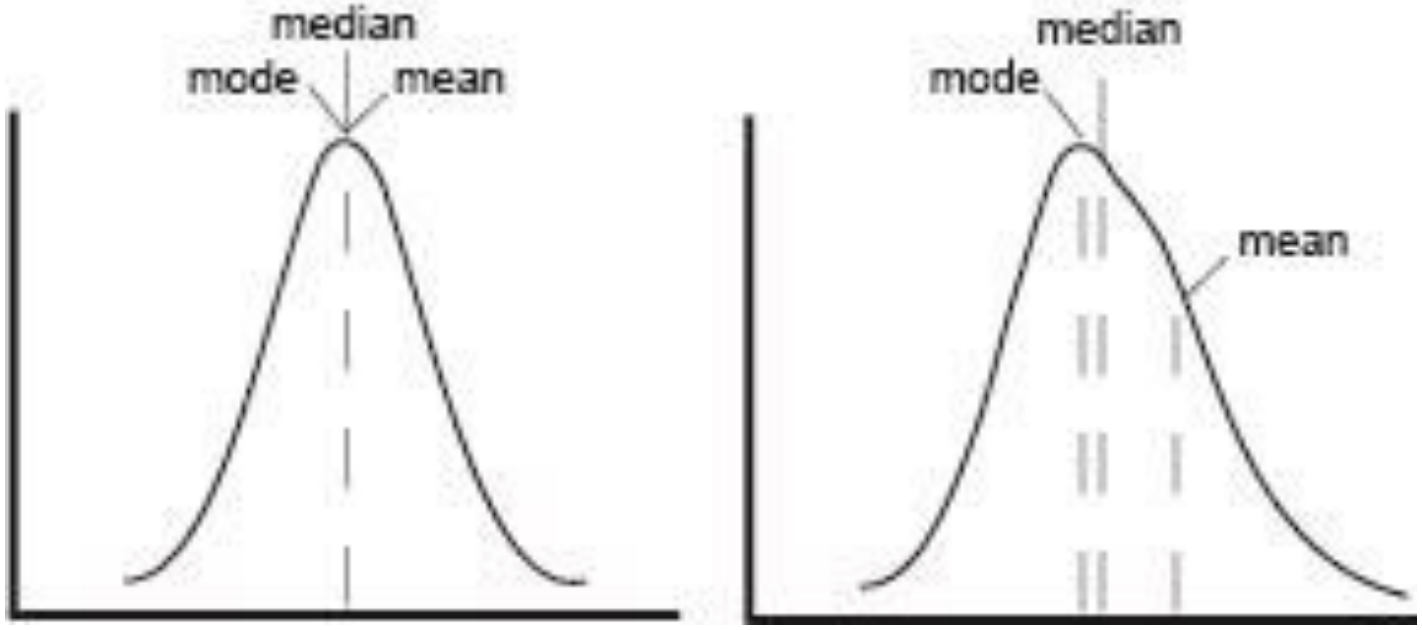


Gate	X-Med	X-AMean	X-Mode	X-GMean
All	45.06	45.47	44.36	44.12
C	45.06	45.47	44.36	44.12

Gate	X-Med	X-AMean	X-Mode	X-GMean
All	17.13	20.17	0.10	7.71
CD7 pos	23.76	26.39	30.68	20.96
CD7-neg	0.31	0.40	0.10	0.32

Gate	X-Med	X-AMean	X-Mode	X-GMean
All	6.05	31.86	1.79	8.88
C	6.07	31.71	1.79	8.91

https://www.cytometry.org/web/q_view.php?id=152&filter=Analysis%20Techniques



https://technical.sanguinebio.com/understanding-mfi-in-the-context-of-facs-data/

What is MFI?

mean mode median MFI The first point of confusion is born from the name itself. MFI is often used without explanation, to abbreviate either arithmetic mean, geometric mean, or median fluorescence intensity. In a perfect world, our data would be normally distributed and in that case means, median and mode are all equal. In reality, flow data is rarely normal and never perfect. The more that the data skews, the further the mean drifts in the direction of skew and becomes less representative of the data being analyze as seen on the graphical representation.

Because fluorescent intensity increases logarithmically, arithmetic mean quickly becomes useless to generalize a population of events, as a right-hand skew causes even more exaggeration of the mean. To combat this, geometric mean (gMFI) is often used to account for the log-normal behavior of flow data, however, even gMFI is susceptible to significant shifts. This leaves us with the median or the mid-point of the population. Median is considered a much more robust statistic in that it is less influenced by skew or outliers. Is there a “right” MFI to use to analyze flow data? No. But generally speaking, median is the safest choice and usually most representative of a “typical” cell.

Although the MFI is often used to define and describe the mean intensity and level of antibody expression, it should be noted that this assumes that the instrument is optimized including the voltage and compensation settings. Fluorescent intensity is sensitive to experimental condition (e.g. voltage, compensation, antibody dilution, tandem dye degradation, laser fluctuations, etc.), it can be misleading when comparing intensity of any kind across multiple experiments.

A more meaningful way may be to measure the signal to noise ratio (mean of an antigen-positive population / mean of antigen negative population) while no compensation setting are applied. If compensation settings are applied, the MFI's of the antigen-negative as well as the antigen-positive populations are often affected. → CONTROLS !!!

Conclusion: The experts recommend using the median (preferred) or the geometric mean (second best choice) for the evaluation of MFI on a logarithmic scale.

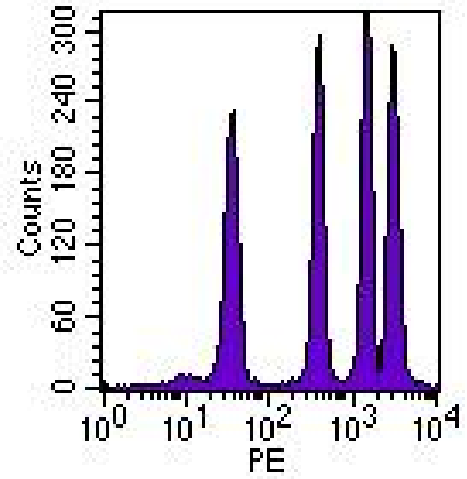
https://www.cytometry.org/web/q_view.php?id=152&filter=Analysis%20Techniques

MESF is an abbreviation for Molecules of Equivalent Soluble Fluorochrome.
The MESF concept indicates that a sample labeled with a fluorochrome has the same fluorescence intensity as an equivalent number of molecules of the fluorochrome free in a solution under the same environmental conditions.
This fluorescence unit provided researchers with tool to compare flow cytometry data in a quantitative manner over time and across instrument

Volume 107, Number 4, July–August 2002
Journal of Research of the National Institute of Standards and Technology

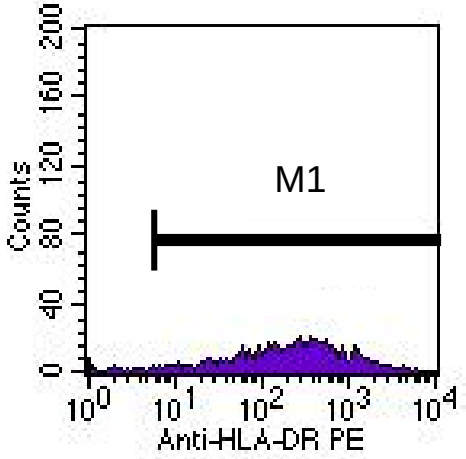
[J. Res. Natl. Inst. Stand. Technol. **107**, 339–353 (2002)]

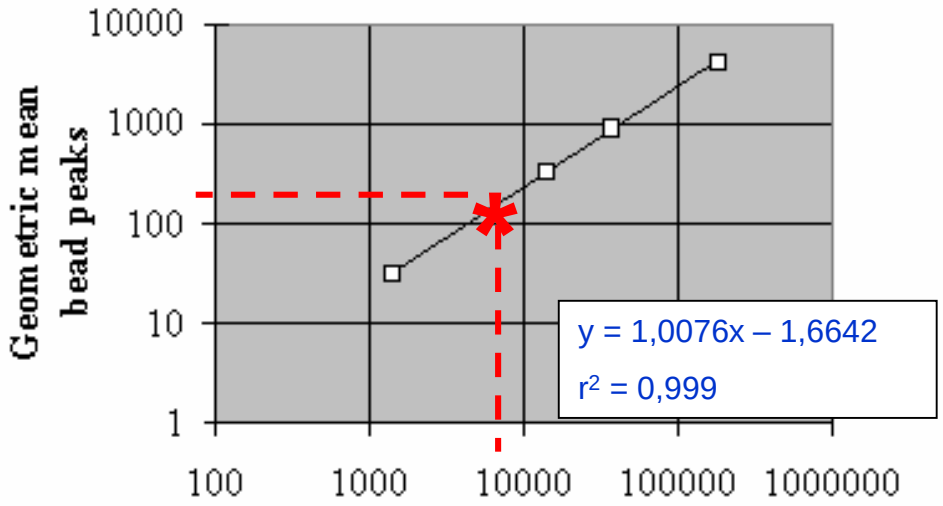
Quantitating Fluorescence Intensity From Fluorophores: Practical Use of MESF Values



# PE molecules per bead from package insert	Geometric means of bead peaks (linear fluorescence)
1400	31,2
14000	334,0
36600	900,3
182000	4144,1

Calibration curve





https://www.bangslabs.com/products/flow-cytometry/fluorescence-quantitation

Quantum™ MESF and Quantum™ Simply Cellular® beads are **external standards** that enable the standardization of fluorescence intensity units irrespective of cytometer and software. Because they are labeled with the same fluorochromes used to label cells, they provide a synchronous response to the environment. **To use, beads are run on the same day and at the same PMT & compensation settings as samples to establish a calibration curve** that relates instrument channel values and standardized fluorescence intensity units (MESF or ABC). Cell samples are then read against the curve for determination of expression (i.e. quantitation of the fluor signal from each cell population).

Catalog Number	Description	Product Data Sheet	Safety Data Sheet
488	Quantum™ Alexa Fluor® 488 MESF	PDS 821.pdf & PDS 818	SDS ST334.pdf
555	Quantum™ FITC-5 MESF	PDS 821.pdf & PDS 818	SDS ST331.pdf
555p	Quantum™ FITC-5 MESF (Premix)	PDS 821.pdf & PDS 818	SDS ST331.pdf
827	Quantum™ R-PE MESF	PDS 821.pdf & PDS 818	SDS ST334.pdf
828	Quantum™ PE-Cy™5 MESF	PDS 821.pdf & PDS 818	SDS ST334.pdf
647	Quantum™ Alexa Fluor® 647 MESF	PDS 821.pdf & PDS 818	SDS ST332.pdf
823	Quantum™ APC MESF	PDS 821.pdf & PDS 818	SDS ST334.pdf

Multicolor flow cytometry

Multicolor flow cytometry: How to design flow cytometry experiments?
https://expert.cheekyscientist.com/flow-cytometry-antibody-panel/

The flow cytometrist is **trying to optimize the ability to make a sensitive measurement to answer the biological question** the researcher has set out to answer.

Step 1: Establish a biological hypothesis.

Everything starts with understanding what the biological hypothesis for the experiments to be performed. This will dictate what populations need to be identified, and what information needs to be extracted from the data.

As you proceed, **rank your antibodies based on cellular expression level** and importance in answering the biological hypothesis.

For example, CD3 is a highly expressed antigen on T-cells and is important in making primary gating decisions, while CD86 is a dimly expressed (or emergent) marker on cells undergoing activation and may be critical to answer the biological hypothesis.

Step 2: Research your fluorochromes.

Fluorochrome brightness can be measured and the different fluorochromes ranked against each other. Use a chart like this one from BioLegend where fluorochromes are ranked from brightest (5) to most dim (https://www.biolegend.com/brightness_index).

Based on the brightness of fluorochromes and the expression density of the antigen on the cells, we want to pair highly expressed antigens (like CD3) with dimmer fluorochromes (1-2), while lower expression antigens are paired with brighter fluorochromes.

Step 3: Know the instrument you're using.

Aim to understand your flow cytometer before designing your antibody panel.
Nguyen et al. (2013) Cytometry A 83A:306-315

Step 4: Use a panel building program.

It becomes critical to find all the antigen-fluorochrome pairs that are available.

examples:
Chromocyte – a web-based system panel building system and a resource website. (https://www.chromocyte.com/)
Fluorofinder – a new resource with a web-based interface for panel building. (https://fluorofinder.com/)

Step 5: Optimize your panel.

After all is said and done, the panel must be optimized and validated. **Proper antibody titration, the correct voltages, optimized staining protocols to minimize non-specific binding** and such are all part of the long, but very critical process to ensure that the panel works appropriately.

Step 6: Bring on the OMIP.

OMIP is short for Optimized Multicolor Immunophenotyping Panel, which is a peer-reviewed, optimized flow panel. The beauty of the OMIP is the work is already done for you, including the antigens to be used, the fluorochromes, and the analysis template.