

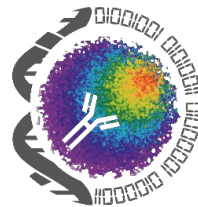
# Interlaboratory and benchmarking studies: experience from ISEV-ISAC-ISTH

Marca Wauben  
Edwin van der Pol

December 2<sup>nd</sup>, 2019



**INTERNATIONAL SOCIETY** *for*  
**EXTRACELLULAR VESICLES**



**ISAC**  
INTERNATIONAL SOCIETY FOR  
ADVANCEMENT OF CYTOMETRY

**ISTH**<sup>TM</sup>  
International Society on  
Thrombosis and Haemostasis

# Outline

- ISEV-ISAC-ISTH EV flow cytometry working group
  - History
  - MIFlowCyt-EV reporting framework
- Experience from previous interlaboratory study
  - Problems
  - Calibration
  - Results
  - What have we learned
- Outlook

# Need for high throughput single EV-based analysis

- EV subset analysis → Flow cytometry-based EV analysis
- Rare event analysis (EV-biomarkers)

## What do we need?

- Sensitive instruments
- Reference materials (calibrators), EV-control materials
- Tools (fl-labels, fl-labeled Abs, single chain Abs, aptamers,...)
- Good practise
- Minimal requirements for publication, Data repository

# Standardisation of EV concentration based on light scatter

*Journal of Thrombosis and Haemostasis*, 7: 190–197

DOI: 10.1111/j.1538-7836.2008.03200.x

ORIGINAL ARTICLE

Standard  
calibrate  
a first st

*Journal of Thrombosis and Haemostasis*, 8: 2571–2574

DOI: 10.1111/j.1538-7836.2010.04047.x

OFFICIAL COMMUNICATION OF THE SSC

S. ROBERT,\*  
J. SAMPOL\*‡  
\*Unité Mixte de R,  
Méditerranée, Unité  
d'hématologie, Ce

Standardization of platelet-derived microparticle enumeration  
by flow cytometry  
International Standardization  
Collaborative Workshop

*Journal of Thrombosis and Haemostasis*, 15: 187–193

DOI: 10.1111/jth.13514

ORIGINAL ARTICLE

To cite this article:  
derived microparticle  
J Thromb Haemost

R. LACROIX,\* S. ROBERT\*  
ON BEHALF OF THE INTERNATIONAL  
\*UMR-S 608 INSERM-Aix-Marseille  
Marseille, France; and ‡Departmen

Standardization of microparticle enumeration across different  
flow cytometry platforms: results of a multicenter  
collaborative workshop

To cite this article: Lacroix R, Robert  
platelet-derived microparticle enumeration  
Haemostasis SSC Collaborative Workshop

S. COINTE,\*† C. JUDICONE,†‡ S. ROBERT,\* M. J. MOOBERRY,§ P. PONCELET,‡ M. WAUBEN,¶  
R. NIEUWLAND,\*\* N. S. KEY,§ F. DIGNAT-GEORGE\*† and R. LACROIX\*†  
\*VRCM, UMR-S1076, Aix-Marseille Université, INSERM, UFR de Pharmacie; †Hematology and Vascular Biology Department, CHU La  
Conception, Assistance Publique-Hôpitaux de Marseille; ‡R and T Department, BioCytex, Marseille, France; §Department of Medicine,  
University of North Carolina, Chapel Hill, NC, USA; ¶Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht  
University, Utrecht; and \*\*Laboratory of Experimental Clinical Chemistry, Academic Medical Center, Amsterdam, the Netherlands

Polystyrene reference beads (0.5 - 0.9 µm) used for gating  
→ Concentration of EVs within gates

# Added value to join efforts

## ISEV2015: Kick-off EV-flow cytometry work group (ISEV-ISAC-ISTH)



- Knowledge on biological samples/EV isolation
- Need for high throughput single EV analysis
- Development of EV analysis technology
- Many end-users



International Society for Advancement of Cytometry

- Technology development (cytometry)
- Education
- Strong connections with Industry
- Interface of users

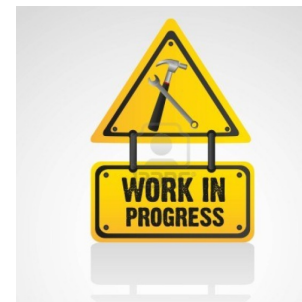


- Development guidelines for plasma EV isolation
- Standardization of plasma EV analysis
- Many end-users

# ISEV-ISAC-ISTH EV flow cytometry working group

## Defined needs for single EV analysis

- Comprehensive reporting of well-controlled experiments
- Instrument calibration and definition of resolution limits
- EV-like references to calibrate, compare, spike-in
- Enhanced (fluorescent) signals / (fluorescence) detection



## How to achieve our aims?

- Satellites/workgroups at the annuals meetings of the Societies
- Interlaboratory collaboration to define standards & references for EV-research
- Publication of position papers, good practises/recommendations in the Society journals
- Involve industry to show the specific needs for the EV-field (instruments, tools)

# Frameworks for comprehensive reporting of well-controlled experiments

MISEV2018 (JEV): a community-based effort

JOURNAL OF EXTRACELLULAR VESICLES  
2018, VOL. 00, 1535750  
<https://doi.org/10.1080/20013078.2018.1535750>

382 authors



OPEN ACCESS Check for updates

Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines

Clotilde Théry<sup>10394</sup>, Kenneth W. Witwer<sup>217,21844</sup>, Elena Aikawa<sup>19,79</sup>, Maria Jose Alcaraz<sup>112</sup>, Johnathon D Anderson<sup>288</sup>, Ramarosan Andriantsitohaina<sup>29</sup>, Anna Antoniou<sup>70,265</sup>, Tanina Arab<sup>257</sup>, Fabienne Archer<sup>18</sup>, Georgia K Atkin-Smith<sup>131</sup>, D Craig Ayre<sup>15,158</sup>, Jean-Marie Bach<sup>254</sup>, Daniel Bachurski<sup>301</sup>, Hossein Baharvand<sup>195,353</sup>, Leonora Balaj<sup>143</sup>, Shawn Baldacchino<sup>321</sup>, Natalie N Bauer<sup>354</sup>, Amy A Baxter<sup>131</sup>, Mary Bebaw<sup>357</sup>, Carla Beckham<sup>350</sup>, Apolonija Bedina Zavec<sup>165</sup>, Abderrahim Benmoussa<sup>260</sup>, Anna C Berardi<sup>179</sup>, Paolo Bergese<sup>39,111,283</sup>, Ewa Bielska<sup>282</sup>, Cherie Blenkiron<sup>2779</sup>, Sylwia Bobis-Wozowicz<sup>119</sup>, Eric Boilard<sup>260</sup>, Wilfrid Boireau<sup>58</sup>, Antonella Bongiovanni<sup>106</sup>, Francesc E Borràs<sup>72,73,250</sup>, Steffi Bosch<sup>254</sup>, Chantal M Boulanger<sup>100,261</sup>, Xandra Breakfield<sup>140</sup>, Andrew M Breglio<sup>92,169</sup>, Meadhbh A Brennan<sup>82,144,258</sup>, David R Brigstock<sup>174,221</sup>, Alain Brisson<sup>298</sup>, Marika LD Broekman<sup>78,134,142</sup>, Jacqueline F Bromberg<sup>155,379</sup>, Paulina Bryl-Górecka<sup>136</sup>, Shilpa Buch<sup>334</sup>, Amy H Buck<sup>305</sup>, Dylan Burger<sup>128,180,337</sup>, Sara Busatto<sup>148,283</sup>, Dominik Buschmann<sup>212</sup>, Benedetta Bussolati<sup>360</sup>, Edit I Buzás<sup>160,2019</sup>, James Brian Byrd<sup>330</sup>, Giovanni Camussi<sup>359</sup>, David RF Carter<sup>181</sup>, Sarah Caruso<sup>131</sup>, Lawrence W Chamley<sup>279</sup>, Yu-Ting Chang<sup>170</sup>, Chihchen Chen<sup>17,112</sup>, Shuai Chen<sup>133</sup>, Lesley Cheng<sup>131</sup>, Andrew R Chin<sup>25</sup>, Aled Clayton<sup>23</sup>, Stefano P Clerici<sup>239</sup>, Alex Cocks<sup>23</sup>, Emanuele Cocucci<sup>220,222</sup>, Robert J Coffey<sup>373</sup>, Yvonne Couch<sup>340</sup>, Frank AW Coumans<sup>7</sup>, Beth Coyle<sup>229</sup>, Rossella Crescitelli<sup>308</sup>, Miria Ferreira Criado<sup>352</sup>, Crislyn D'Souza-Schorey<sup>335</sup>, Anabela Cordeiro-da-Silva<sup>346</sup>, Saumya Das<sup>141</sup>, Amrita Datta Chaudhuri<sup>218</sup>, Paola de Candia<sup>116</sup>, Eliezer F De Santana Junio<sup>225</sup>, Olivier De Wever<sup>22,75</sup>, Hernando A del Portillo<sup>101,104,117</sup>, Tanguy Demaret<sup>256</sup>, Sarah Deville<sup>62,377</sup>, Andrew Devitt<sup>12</sup>, Bert Dhondt<sup>22,74,75</sup>, Dolores Di Vizio<sup>24</sup>, Lothar C Dieterich<sup>49</sup>, Vincenza Dolo<sup>315</sup>, Ana Paula Dominguez Rubio<sup>243</sup>, Marina Domini<sup>1234,333</sup>, Maurício P. Dornelas<sup>298,338</sup>, Tom AD Dziadosz<sup>369</sup>, Filipa M Duarte<sup>53</sup>

## Cytometry



## MIFlowCyt: The Minimum Information About a Flow Cytometry Experiment

Jamie A. Lee,<sup>11</sup> Josef Spidlen,<sup>21</sup> Keith Boyce,<sup>3</sup> Jennifer Cai,<sup>1</sup> Nicholas Crosbie,<sup>4</sup> Mark Dalphin,<sup>5</sup>

228 | VOL.14 NO.3 | MARCH 2017 | NATURE METHODS

## EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research

EV-TRACK Consortium\*

We argue that the field of extracellular vesicle (EV) biology needs more transparent reporting to facilitate interpretation and replication of experiments. To achieve this, we describe EV-TRACK, a crowdsourcing knowledgebase (<http://evtrack.org>) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice.



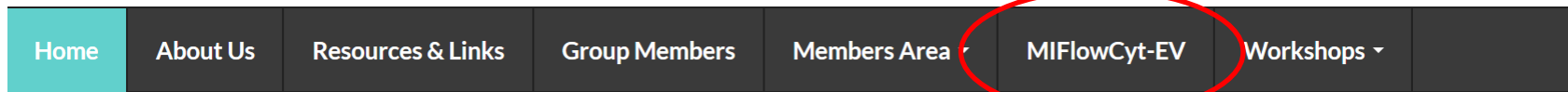
Journal of  
Extracellular Vesicles

(Submitted)

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

Joshua A. Welsh<sup>1\*</sup>, Edwin van der Pol<sup>2,3,4</sup>, Ger J.A. Arkesteijn<sup>5</sup>, Michel Bremer<sup>6</sup>, Alain Brisson<sup>7</sup>, Frank Coumans<sup>2,3,4</sup>, Françoise Dignat-George<sup>8</sup>, 9, Erika Duggan<sup>10</sup>, Ionita Ghiran<sup>11</sup>, Bernd Giebel<sup>6</sup>, André Görgens<sup>6</sup>, 12, 13, An Hendrix<sup>14</sup>, Romaric Lacroix<sup>8</sup>, 9, Joanne Lannigan<sup>15</sup>, Sten F.W.M. Libregts<sup>5</sup>, 16, Estefania Lozano-Andrés, Aizea Morales-Kastresana<sup>1</sup>, Stephane Robert<sup>13</sup>, Leonie de Rond<sup>2,3,4</sup>, Tobias Tertel<sup>6</sup>, John Tigges<sup>11,17</sup>, Olivier de Wever<sup>14</sup>, Xiaomei Yan<sup>18</sup>, Rienk Nieuwland<sup>3,4\*</sup>, Marca H.M. Wauben<sup>5\*</sup>, John P. Nolan<sup>10\*</sup>, Jennifer C. Jones<sup>1\*</sup>

# ISEV-ISAC-ISTH EV flow cytometry working group



## Home

Welcome to the EV Flow Cytometry Working Group Website!

The purpose of this website is to serve as a central hub for information and links to sites that will advance scientific progress and education relating to these topics along with the progress and materials of the working group.

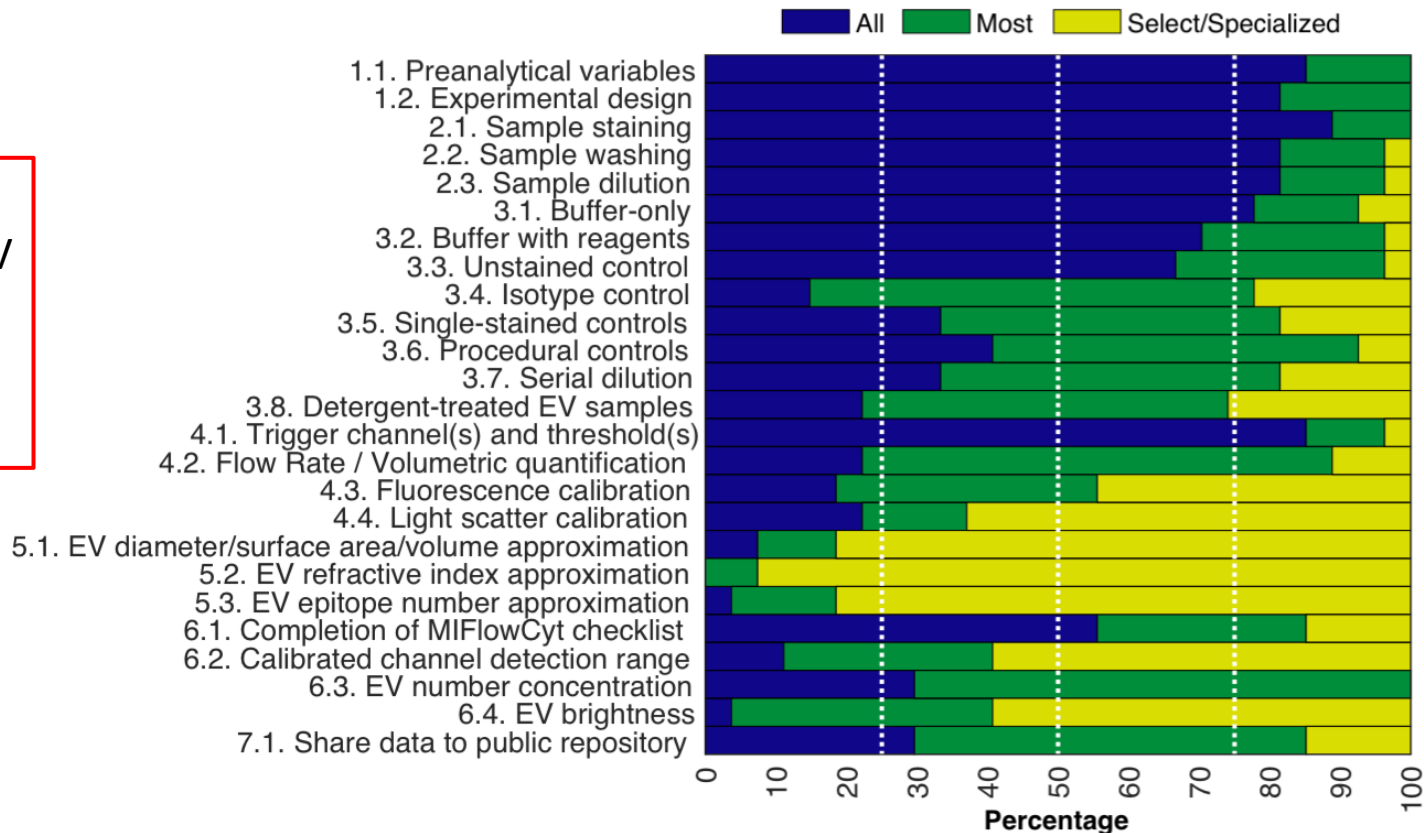
Because many extracellular vesicles (especially exosomes) are 100 nm or smaller, conventional flow cytometric instruments and methods are not suitable for analysis of individual EVs. There have been several efforts worldwide to develop and refine methods for EV flow cytometry, but until 2015, there was no rigorous, consistent framework established for validating these methods or for conducting EV flow cytometry experiments with appropriate controls. In collaboration with flow cytometry experts from the International Society for Extracellular Vesicles (ISEV), International Society for the Advancement of Science (ISAC), and International Society on Thrombosis and Hemostasis (ISTH), we have established an EV Flow Cytometry Working Group that is working to establish guidelines for best practices for this area of research. The working group webpage contains links to the societies, tools, reports, and working group materials.



# Needs for reporting in EV-FC manuscripts

## Objectives:

- Proof of single EV detection
- Reproducibility
- Standardization



EV-FC-WG survey. Bar graph summarizes the personal expectations of all coauthors (n=27) regarding criteria to be reported in EV-FC manuscript published in ISEV, ISAC, and ISTH journals. The expectations fall into categories of all (blue), most (green), or select/specialized (yellow) manuscripts.

## MIFlowCyt-EV Reporting Framework

1	Preamerical variables & experimental design	<ul style="list-style-type: none"><li>1.1. Report preanalytical variables conforming to MISEV guidelines</li><li>1.2. Report experimental design according to MIFlowCyt guidelines</li></ul>
2	Sample preparation	<ul style="list-style-type: none"><li>2.1. Sample staining</li><li>2.2. Sample washing steps</li><li>2.3. Sample dilution</li></ul>
3	Assay controls	<ul style="list-style-type: none"><li>3.1. Buffer only</li><li>3.2. Buffer with reagents</li><li>3.3. Unstained controls</li><li>3.4. Isotype controls</li><li>3.5. Single-stained controls</li><li>3.6. Procedural controls</li><li>3.7. Serial dilution</li><li>3.8. Detergent-treated EV samples</li></ul>
4	Instrument calibration & data acquisition	<ul style="list-style-type: none"><li>4.1. Trigger channel(s) and threshold(s)</li><li>4.2. Flow rate &amp; volumetric quantification (<math>\mu\text{L min}^{-1} / \mu\text{L}</math>)</li><li>4.3. Fluorescence Calibration (MESF/ERF units)</li><li>4.4. Light Scatter Calibration (<math>\text{nm}^2</math>)</li></ul>
5	EV characterization	<ul style="list-style-type: none"><li>5.1. EV diameter/surface area/volume approximation</li><li>5.2. EV refractive index approximation</li><li>5.3. Epitope number approximation</li></ul>
6	FC data reporting	<ul style="list-style-type: none"><li>6.1. Complete MIFlowCyt checklist</li><li>6.2. Calibrated channel detection range</li><li>6.3. EV number concentration</li><li>6.4. EV brightness</li></ul>
7	FC data sharing	<ul style="list-style-type: none"><li>7.1. Share data to public repository</li></ul>

Why? When? & How?

# Implementation of MIFlowCyt-EV reporting framework

- Proper reporting of sample preparation & EV analysis
- Developed guidelines will apply to other EV analysis methods
  - EV counting, sizing, fluorescence
- Guidelines will support the development and validation of new EV analysis methods & materials

# Acknowledgements

## ISEV ISAC ISTH EV-Flow cytometry working group



Marca Wauben, Ger Arkesteijn, Sten Libregts,  
Estefania Lozano Andres (Utrecht)

Rienk Nieuwland, Edwin van der Pol, Frank  
Coumans, Leonie de Rond (Amsterdam)

John Nolan, Erika Duggan (San Diego)

Jennifer Jones, Aizea Morales-Kastresana,  
**Joshua Welsh** (Bethesda)

Joanne Lannigan, Uta Erdbrugger  
(Charlottesville)

Alain Brisson (Bordeaux)

Romarc Lacroix, Stephane Robert, Fracoise  
Dignat-George (Marseilles)

John Tigges, Ionita Ghiron, Vasilis Toxivaidis  
(Boston)

Bernd Giebel, Andre Goergens, Tobias Tertel  
(Essen)

James Higgenbotham, Bob Coffey (Vanderbilt)

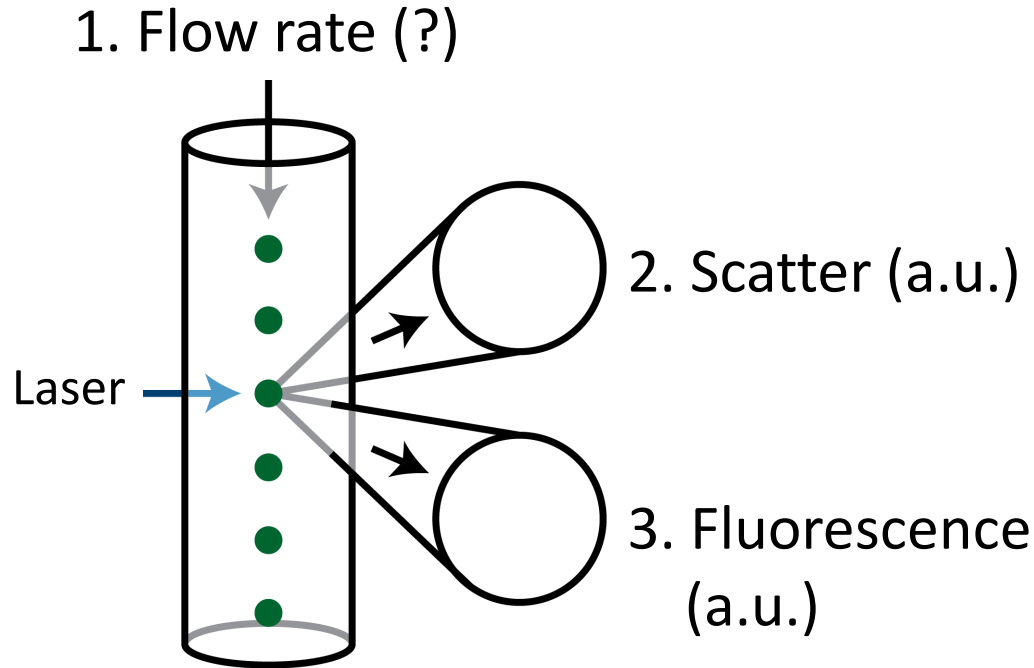
An Hendrix, Oliver de Wever (Ghent)

Xiaomei Yan (Xiamen)

# Experience from previous interlaboratory study

- Problems involved in EV standardization
- Avoid arbitrary units: calibrate
  - Calibration
  - Examples
- Unexpected results of previous standardization study
- What we have learned
- Outlook

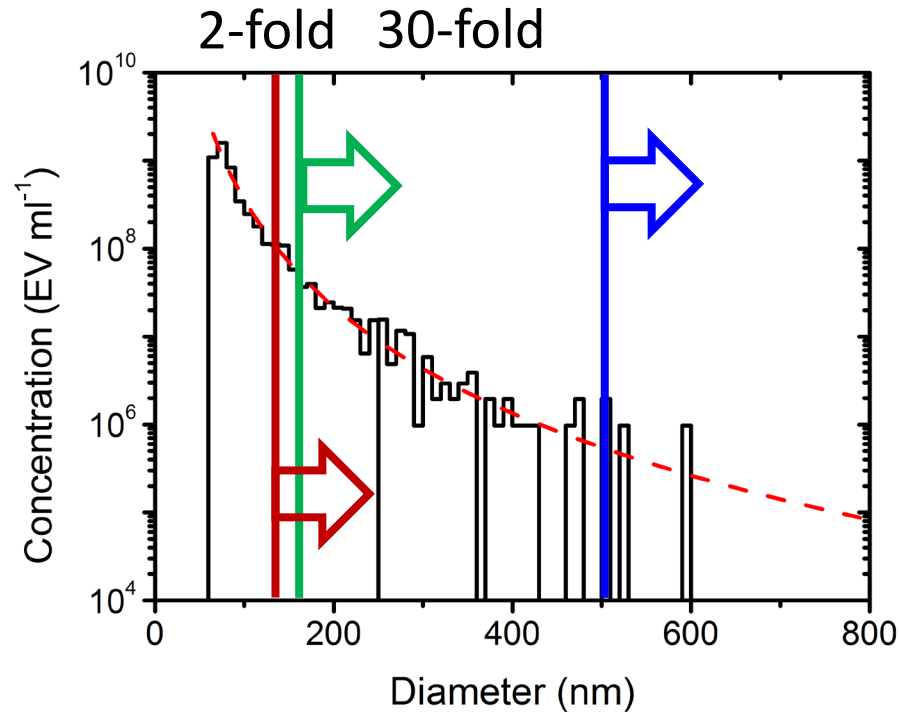
# Flow cytometry



Other EV properties to standardize:

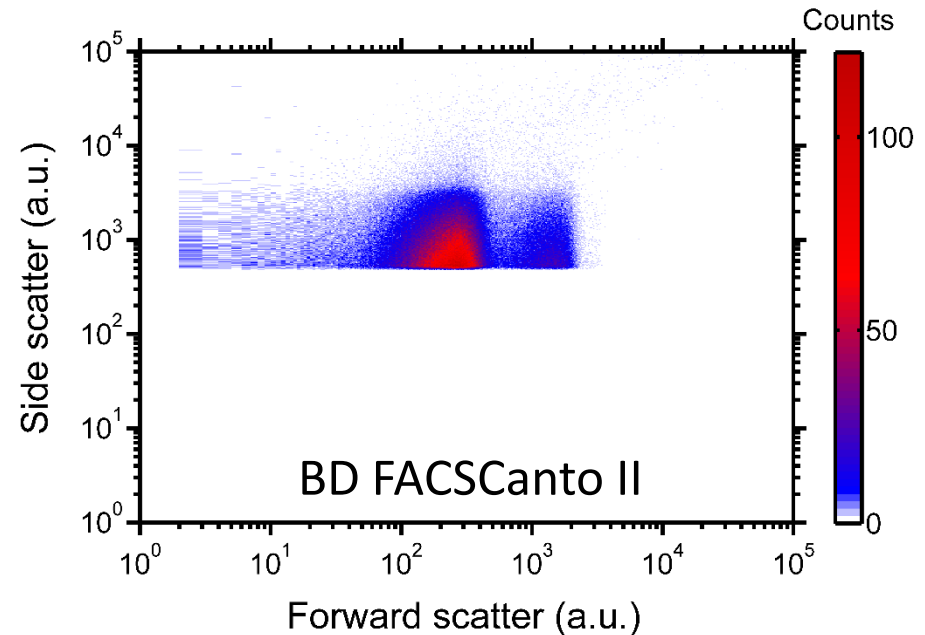
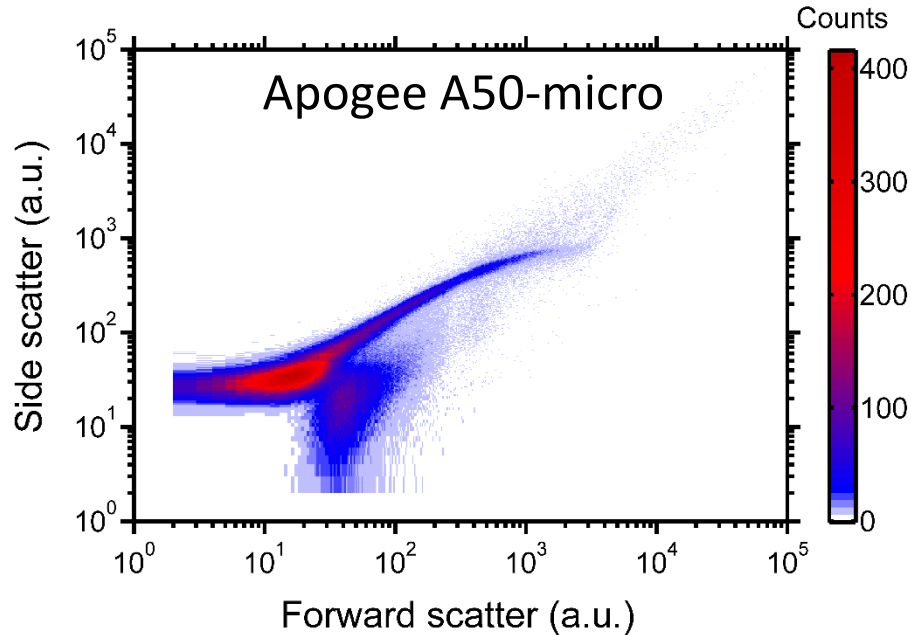
- Bioactivity, function
- Conductivity
- Density
- Diffusion constant
- Lipid content
- Protein content
- Nucleic acids content (DNA, RNA)
- Refractive index
- Morphology (size, shape)
- Stiffness

# Problem 1: instruments differ in sensitivity



# Problem 2: arbitrary units

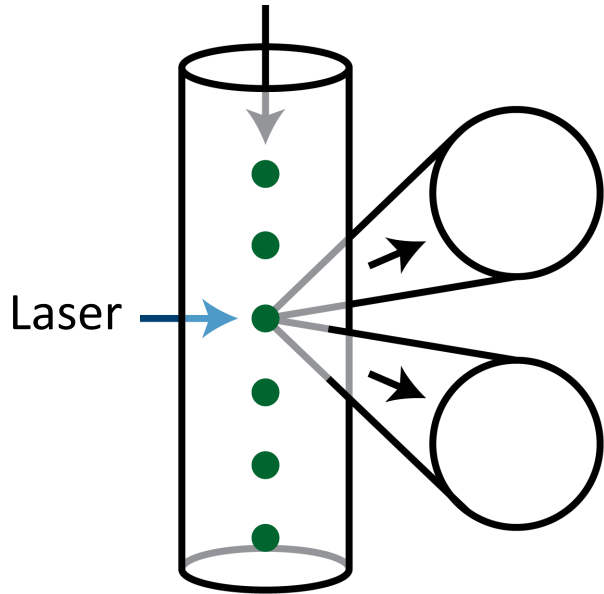
same population of erythrocyte EVs





# Avoid arbitrary units: calibrate “the unknown”

1. Calibrate flow rate ( $\mu\text{L}/\text{min}$ )

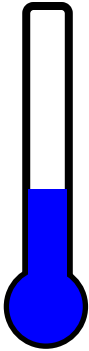


2. Calibrate scatter ( $\text{nm}^2$ )

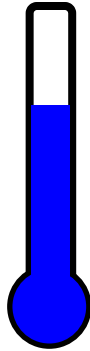
3. Calibrate fluorescence (MESF)

# Thermometer: no calibration

Lab 1



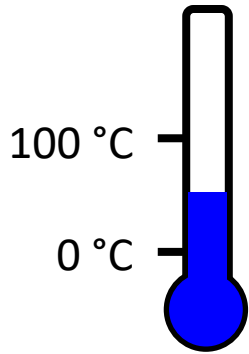
Lab 2



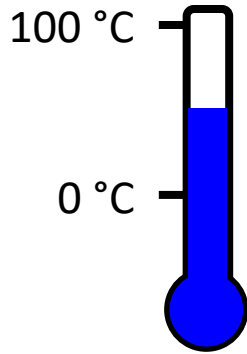
- Data interpretation
  - What is the temperature?
- Data comparison
  - Is the temperature equal?

# Thermometer: measuring reference values

Lab 1



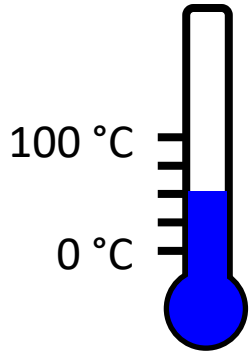
Lab 2



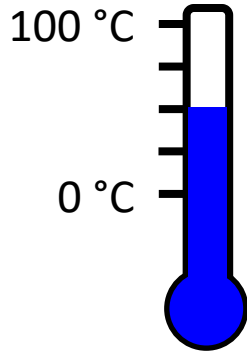
- Data interpretation
  - What is the temperature?
- Data comparison
  - Is the temperature equal?

# Thermometer: calibration

Lab 1



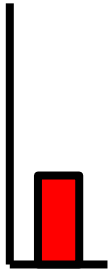
Lab 2



- Data interpretation
  - What is the temperature?  
50 °C
- Data comparison
  - Is the temperature equal?  
Yes!

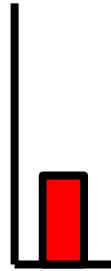
# Flow cytometer: no calibration

BD LSR



Side scatter

BD Influx

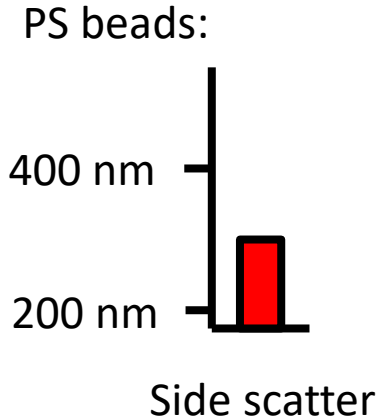


Forward scatter

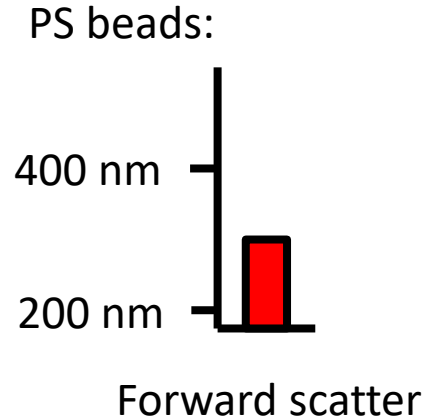
- Data interpretation
  - What is the EV size?
- Data comparison
  - Do we study equal EV sizes?

# Flow cytometer: measuring reference materials

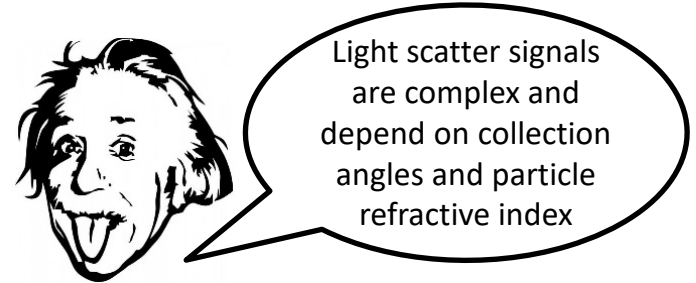
BD LSR



BD Influx

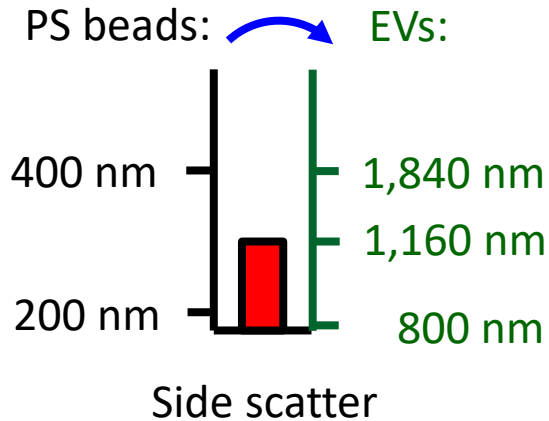


- Data interpretation
  - What is the EV size?  
~~300 nm?~~
- Data comparison
  - Do we study equal EV sizes?  
~~Yes?~~

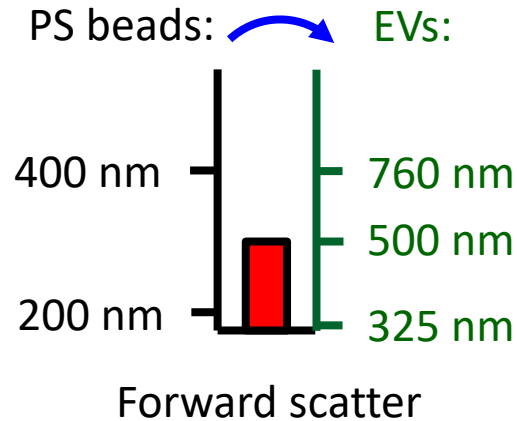


# Flow cytometer: calibration

BD LSR



BD Influx



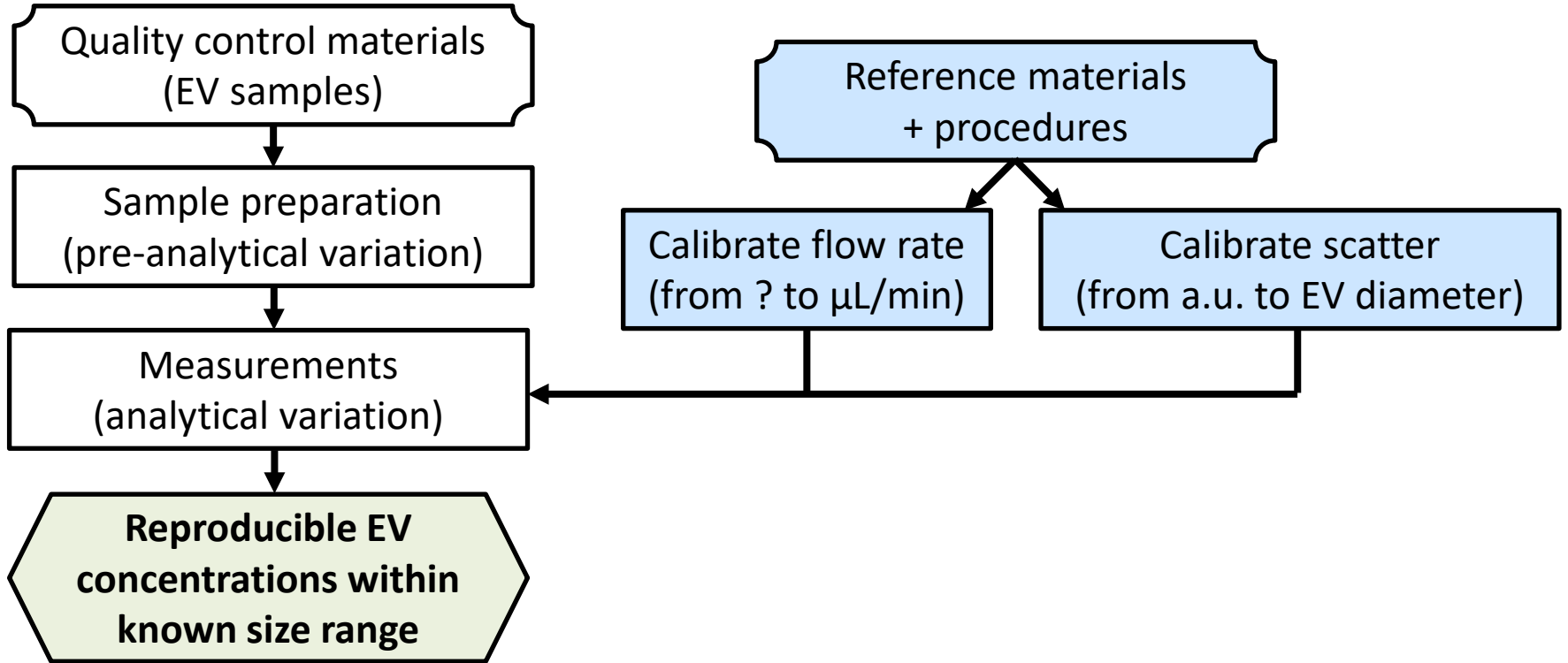
- Data interpretation
  - What is the EV size?  
1,160 nm & 500 nm
- Data comparison
  - Do we study equal EV sizes?  
No!
- de Rond et al. *Curr Protoc Cytom* 2018
  - FCMPASS
  - Rosetta Calibration:  
Exometry.com

# Study comprised 33 sites (64 instruments) worldwide



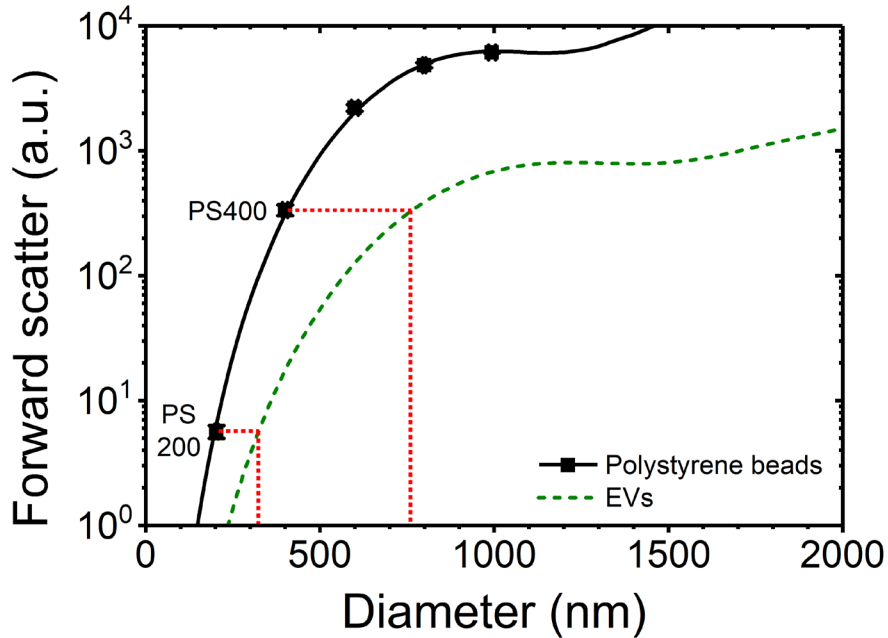


# Goal and approach

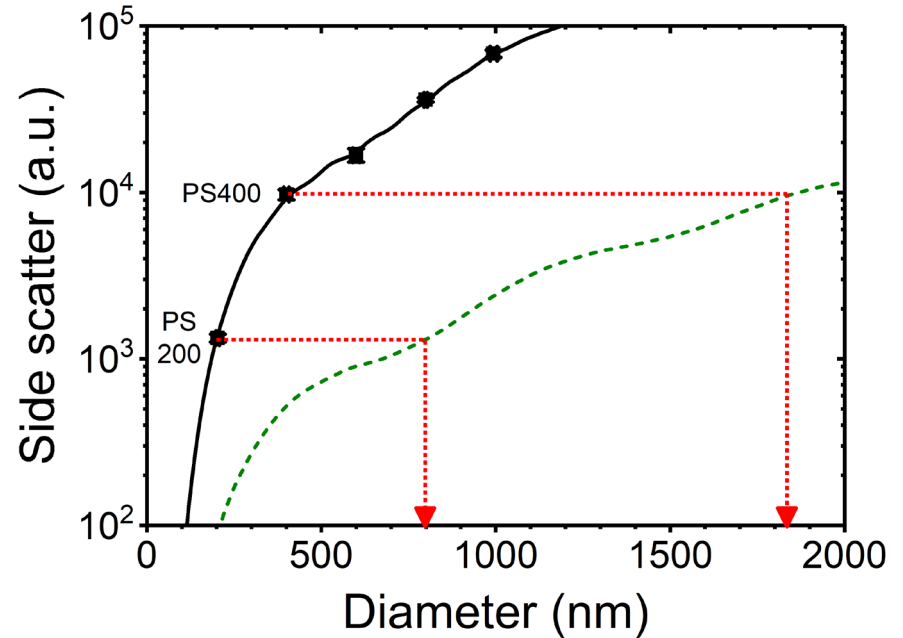


# Example of the need for scatter calibration

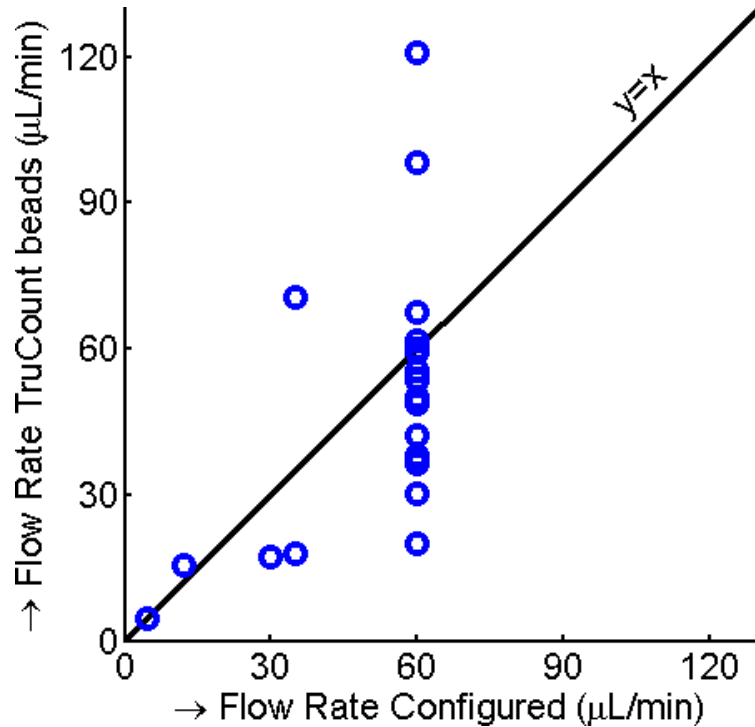
## BD Influx



## BD LSR



# Example of the need for flow rate calibration

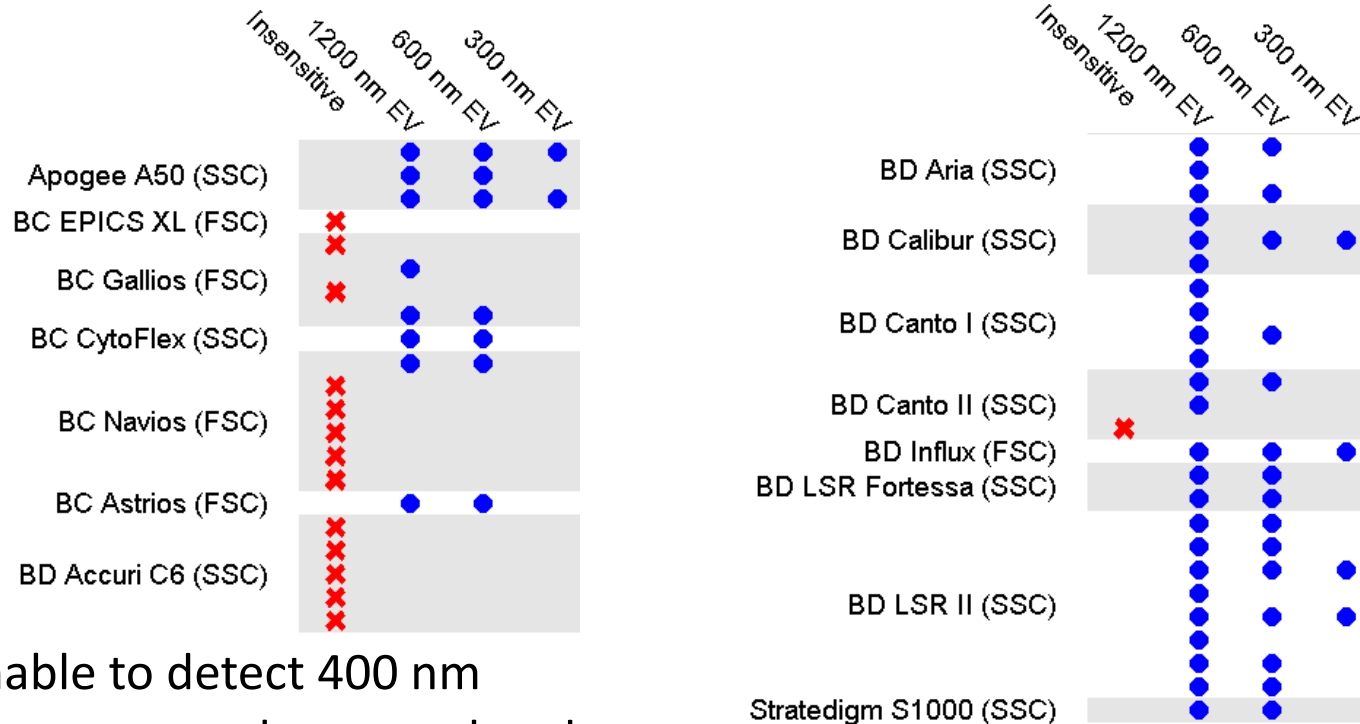


$$\text{concentration} = \frac{\text{\# of EV}}{\text{flow rate} \times \text{measurement time}}$$

# Example of the need for fluorescence calibration

- Next presentation (Joshua Welsh)
  - FCMPASS v3 utilizes MIFlowCyt-EV criteria and extracts flow cytometry setting information to allow transparent reporting irrespective of file sharing repository

# Sensitivity of 46 flow cytometers in the field



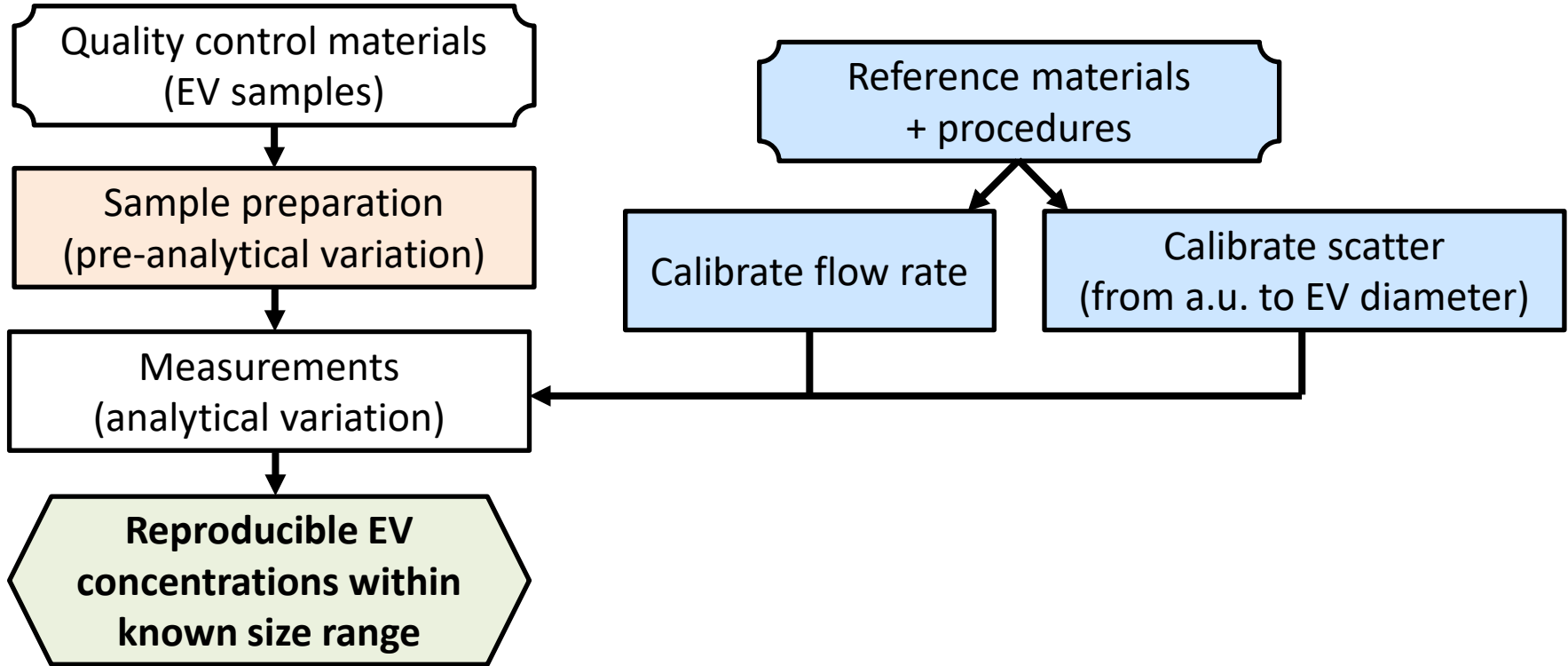
× = unable to detect 400 nm fluorescent polystyrene beads

# Reproducibility of 1200-3000 nm EVs, 31 FCMs

	CV(%)
Gate on beads	139%
Gate on EV size with light scatter theory	81%

Requires improvement!

# What we have learned



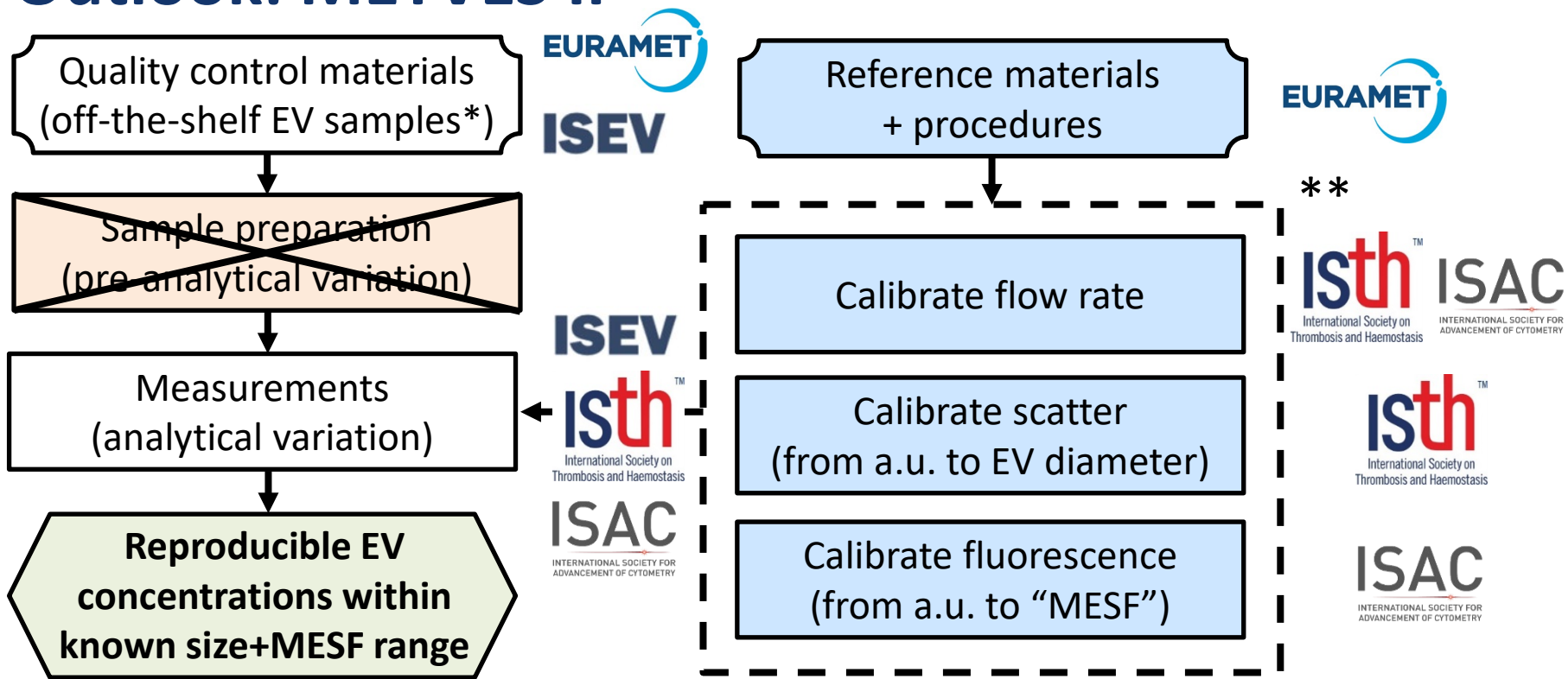
# What we have learned

	ISTH/METVES/Exometry study
Shipping	Funded by ISTH, time-consuming
Procedures	18 pages
Pre-analytical variables	Thawing, staining, diluting
Support	Frank Coumans
Backup	Data + samples
Data analysis	On-site, semi-manual





# Outlook: METVES II



\*ISEV poster 10, Britta Bettin)

\*\*ISEV posters 27 (Katariina Maaninka) & 35 (Zoltán Varga)

# Outlook: METVES II

- One traceable reference material to calibrate them all\*
  - fluorescence
    - 100 – 100,000 fluorescent molecules
  - number concentration
    - $10^9$  –  $10^{12}$  particles mL<sup>-1</sup>
  - scatter
    - discrete diameters between 50 nm – 1,000 nm
    - refractive index between 1.37 – 1.42
- Reference procedures

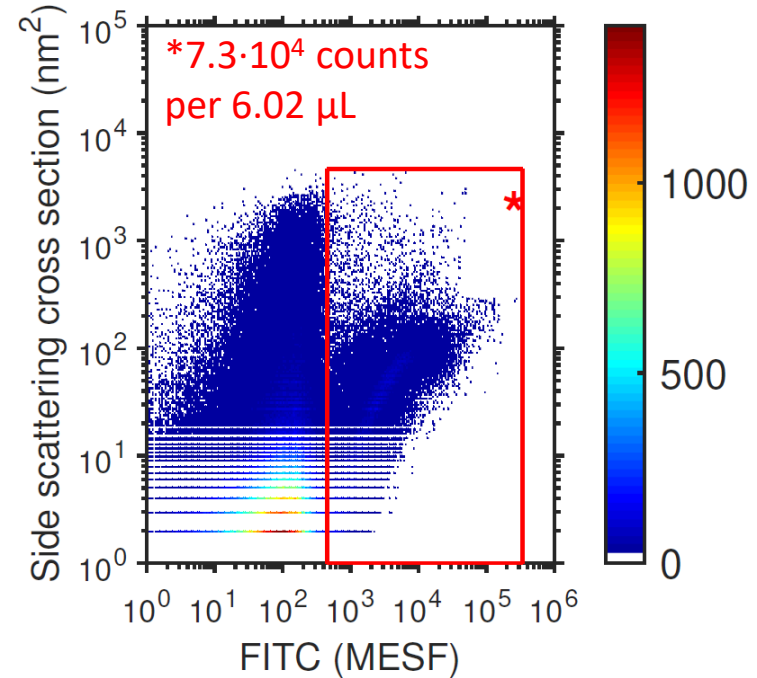
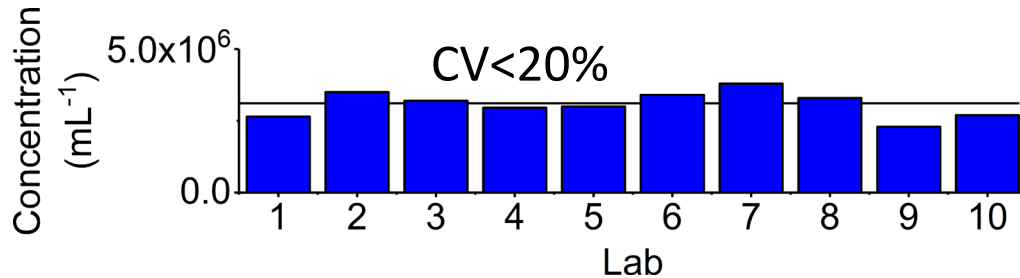


\* ISEV posters 27 (Katariina Maaninka), 35 (Zoltán Varga)

Red: properties resembling EVs or EV samples

# Anticipated outcome METVES II interlaboratory study

- Per lab:
  - flow cytometry
    - reference materials + procedures
    - control materials
    - fully automated calibration & data analysis



# Outlook ISEV-ISAC-ISTH working group

- Educate
  - MIFlowCyt-EV reporting framework
  - EV flow cytometry educational compendium
  - Webinars & workshops
- Share knowledge
  - Reference materials
- Define standards
  - Operation procedures
- Standardization
  - Participate in METVES II interlaboratory comparison study



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  - ISTH
  - NWO-TTW
  - ITN TRAIN EV
- Relevant websites
  - [evflowcytometry.org](http://evflowcytometry.org)
  - [metves.eu](http://metves.eu)
  - [train-ev.eu](http://train-ev.eu)
  - [exometry.com](http://exometry.com)

