High-Resolution Imaging Flow Cytometry Reveals Impact of Incubation Temperature on Labeling of Extracellular Vesicles with Antibodies

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Abstract
Extracellular vesicles (EVs) are released from basically all cells. Over the last decade, small EVs (sEVs; 50–150 nm) have gained enormous attention in diagnostics and therapy. However, methodological limitations coupled to the lack of EV standards leave many questions in this quickly evolving field unresolved. Recently, by using enhanced green fluorescent protein (eGFP)-labeled sEVs as biological reference material, we systematically optimized imaging flow cytometry for single sEV analysis. Furthermore, we showed that sEVs stained with different fluorescent antibodies can be analyzed in a multiparametric manner. However, many parameters potentially affecting the sEV staining procedure still require further evaluation and optimization. Here, we present a concise, systematic evaluation of the impact of the incubation temperature (4°C, room temperature and 37°C) during sEV antibody staining on the outcome of experiments involving the staining of EVs with fluorescence-conjugated antibodies. We provide evidence that both the staining intensity and the sample recovery can vary depending on the incubation temperature applied, and that observed differences are less pronounced following prolonged incubation times. In addition, this study can serve as an application-specific example of parameter evaluation in EV flow cytometry. © 2020 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms
extracellular vesicles; EVs; exosomes; microvesicles; microparticles; vesicles; imaging flow cytometry; IFCM

Extracellular vesicles (EVs) are membranous biological nanovesicles released by basically all cells and can be isolated from cell culture supernatants and body fluids (1). Due to their capability of transmitting biological signals to recipient cells across tissues, EVs are currently explored for both therapeutic and diagnostic use (2, 3). EVs can be enriched by different techniques based on their density and/or size, which provides rather heterogeneous mixtures of different EV types of comparable sizes. Analysis of this complexity has been hampered by their small size, which for small EVs such as exosomes and small microvesicles (sEVs, ~50–150 nm) is below the diffraction limit of light. For now, there are only few methods facilitating the analysis of sub-micron particles below 300 nm, all of them including methodological limitations (4–6). In contrast to other single sEV analysis methods such as nanoparticle tracking analysis (NTA), dynamic light scattering, or resistance pulse sensing, flow cytometry (FCM) provides several advantages. However, most conventional flow cytometers are not sensitive enough to detect EVs below sizes of 300 nm (7, 8). Within the last few years, advanced flow cytometers with increased resolution and sensitivity have become available for analyzing single EVs by using exosomal marker...
proteins fused to fluorescent proteins such as CD63-GFP, fluorescent dyes, or fluorescently labeled antibodies (8–13). However, the EV flow cytometry field is still in need of suitable standards and validated protocols. In this context, the ISEV-ISAC-ISTH EV flow cytometry working group recently provided MiFlowCyt-EV, which is a framework for standardized reporting of extracellular vesicle flow cytometry experiments in order to improve comparability between studies and standardization (14).

Recently, we have optimized an imaging flow cytometry (IFCM)-based single EV analysis method by using CD63-enhanced green fluorescent protein (eGFP)-tagged EVs as biological reference material. We demonstrated that IFCM is sensitive enough to robustly detect and quantify fluorescently labeled EVs from cell culture supernatants or human biological fluids, even if these EV-containing liquids remain unprocessed in terms of EV preparation (8, 10). Aiming to further optimize IFCM-based EV analysis by staining with fluorescently labeled antibodies, we here investigated the impact of the incubation temperature during the EV staining over time on experimental results and provide evidence that both the staining intensity and the sample recovery can vary depending on the incubation temperature applied.

Materials and Methods

Staining of eGFP-Tagged EVs and IFCM Data Acquisition

The staining of THP-1:CD63:eGFP EVs was performed as described previously (8) unless mentioned otherwise. In brief, conditioned media (CM) were preclarified by centrifugation (900g for 5 min and 2000g for 15 min) and filtered through 0.22 μm filters (Sartorius, Minisart, cat 16,534). For staining, CM was incubated with PE-conjugated mouse anti-human CD63 (EXBIO, cat 1P-343-T100, clone MEM-259) or mouse IgG1 isotype control antibodies (BD Biosciences, cat 555,749, clone MOPC-21). More specifically, CM samples were mixed at a ratio of 1:1 with PBS-containing antibodies at 20/60 μM (resulting in 10/30 nM final antibody concentration in a total volume of 40 μl), incubated for indicated time points (2 or 20 h) and at 4°C, RT or 37°C in the dark and diluted eightfold in PBS (Gibco, pH 7.4, cat 10,010,023) to a total volume of 320 μl post staining. About 100 μl were then transferred to wells in U-bottom 96-well plates (Gorning Falcon, cat 353,077) and measured with an ImageStreamX Mark II instrument (Amnis/Luminex, Seattle, WA) as described recently without further washing (8). Controls comprised unstained samples, buffer controls without EVs but incubated with antibodies and detergent lysis controls as described before and as shown in Figure 2C and Supporting Information Figure S3A (8). All samples were acquired for 5–10 min acquisition time per well (details provided in figure legends) and a wash well containing PBS only for 1 min after each sample to avoid sample carry-over (example shown in the Supporting Information Fig. S2A). All data were acquired at 60X magnification at low flow rate (volumetric assessment for samples in this study: 0.3795 ± 0.0003 μl/min; mean ± SD; n = 12) and with removed beads option deactivated as described previously (8). More details are given in the Supporting Information Tables S1 and S2, respective figure legends of Figures 1 and 2 and Supporting Information - Figures S1–S3.

Staining of Human Plasma-Derived EVs and IFCM Data Acquisition

Human blood samples were obtained as approved by the medical ethics committee of the Chamber of Physicians in Hamburg. Informed consent was obtained from all patients. EDTA was used as anti-coagulant for all plasma samples. EVs were isolated by differential centrifugation as described previously (10). Briefly, plasma was centrifuged at 1,000g for 7 min. Supernatants were then centrifuged at 10,000g for 30 min. EVs were pelleted from supernatants by ultracentrifugation (Beckman, TW601) at 100,000g for 70 min. EVs were stained in 0.22 μm filtered PBS containing 2% exosome-depleted FBS (Invitrogen, cat A2720801) supplemented with protease-inhibitor (Roche, cat 11,836,170,001) and phosphatase-inhibitor (Roche, cat 0,406,845,001). Antibodies used to stain EVs were PE-conjugated anti-human CD9 (Biolegend, clone H9A [4 μg/ml]), PacificBlue-conjugated anti-human CD63 (Biolegend, clone H5C6 [40 μg/ml]), and FITC-conjugated anti-human CD81 (Biolegend, clone 5A6 [40 μg/ml]). EVs and antibodies were incubated in a total volume of 16 μl (with 4 μl of each antibody added to 4 μl EVs) for 45 min at 4°C, RT and 37°C in the dark. EVs were then washed using a 300 kDa filter (NanoSep, 4,000 μl) and resuspended in washing buffer (0.22 μm-filtered PBS + 2% exosome-depleted-FBS) for IFCM analysis. For control purposes, EVs were lysed by NP40 (0.5%) for 30 min at RT as described previously (15). Data were acquired on an ImageStreamX Mark II Imaging Flow Cytometer (Amnis/Luminex, Seattle). Laser powers were adjusted so that the fluorescence intensity was inside the detection range or run at maximum power (Supporting Information Table S1). Fluorescent signals were collected as follows: PacificBlue was measured in Channel 7 (435–505 nm filter), FITC was measured in channel 2 (480–560 nm filter), phycoerythrin (PE) was detected in Channel 3 (560–595 nm filter). All readings were acquired for 5 min at 60X magnification collected at low flow rate and removed beads option activated. More details are given in the Supporting Information Tables S1, S2 and S6, the figure legend of Figure 3 and Figure S5.
unstained EVs) as described previously with details given in respective figure legends (8, 10). Absolute fluorescence calibration in molecules of equivalent soluble fluorophores (MESF) for data shown in Figures 1 and 2 was described previously (8) and is provided in the Supporting Information Figure S1. In brief, FITC MESF beads (Quantibrite-FITC custom beads, lot MM2307-153, kindly provided by Majid Mehrpouyan, BD Biosciences, FL) and PE MESF beads (BD Quantibrite Beads, PE Fluorescence Quantitation Kit, cat 340,495, lot 60,057) with known absolute FITC/PE fluorescence values for each peak from respective bead populations were acquired for experiments shown in Figures 1 and 2, and FITC MESF beads (Quantum FITC-5 MESF, Bangs Laboratories, cat 555A, lot 14,318), PE MESF beads (BD Quantibrite Beads, PE Fluorescence Quantitation Kit, cat 340,495, lot 96,354), and PacificBlue MESF beads (Quantum PacificBlue, Bangs Laboratories, cat 821A, lot 13,935) were acquired for experiments shown in Figure 3. MESF beads were acquired with the same respective settings used for EV measurements with the exception that the SSC laser was turned off. Linear regressions were performed (Supporting Information Figs. S1 and S5; Table S6), and the resulting equations were used to convert fluorescence intensity values into MESF values. Flow cytometric plots using MESF unit axes were created with FlowJo v 10.5.3 (FlowJo, LLC, Ashland, OR).

**Figure 1.** IFCM facilitates robust analysis of fluorescent sEVs. (A) Gating strategy applied to identify SSClow eGFP+ sEV events [eGFP+] with X-axis showing FITC MESF calibrated data. Uncalibrated data and MESF regression analysis are shown in the Supporting Information Figure S1. (B) Evaluation of reproducibility of IFCM analyses in 96-well plates. Precleared and filtrated identical, unstained CM samples derived from THP-1:CD63:eGFP cells were pipetted into a 96-well plate (A1-H12; with PBS-containing wash wells between all samples, see Supporting Information Fig. S2 for details) and measured with the built-in autosampler in three independent experiments to compare, respectively, obtained concentration (left graph) and fluorescence intensity. Cutoffs for gating on SSC(low) eGFP(+) events were chosen based on background events in PBS only controls for eGFP (>40 FITC MESF) and based on previous characterization of eGFP-tagged sEVs (8) and to include the main eGFP(+) population for SSC (<1,000 a.u.). Coefficients of variation (cVs) were calculated dependent on which rows of wells were measured. Arbitrary units of median fluorescence intensity as shown in the Supporting Information Figure S1A were converted to FITC MESF units and plotted against measured wells (right graph). Considering only data from the first 48 wells (A1-D12, indicated by the dotted line) resulted in reduced variation. Detailed plate layout, data from individual experiments and analysis by row and column of the plates are shown in the Supporting Information Figure S2.
RESULTS
In two recent studies, we optimized and qualified IFCM for the analysis of single fluorescent sEVs and demonstrated that fluorescence-conjugated antibodies can be used to identify sEV subpopulations in both cell culture supernatants and human plasma samples (8, 10). Since we used CD63eGFP-tagged sEVs as biological reference material to define initial

Figure 2 legend on next page
parameters of such an antibody staining protocol before (8), we here aimed to use the same setup to investigate the impact of respective incubation temperatures on the staining intensity and overall assay robustness.

First, to evaluate how consistent the results from EV samples acquired from 96-well plates through the built-in autosampler overall would be, we compared data obtained from the same sample quantified in respective wells of the plate over time. To that end, identical THP1:CD63:eGFP tagged sEV containing samples were pipetted into every second well of a U-bottom 96-well plate and recorded for 10 min each. SSClow eGFP+ sEVs were identified as described previously (Fig. 1A; Information Fig. S1A) (8). We obtained relatively consistent values for the estimated concentration of SSClow eGFP+ events (cV = 8.01%) and their mean fluorescence intensity (cV = 1.00%) for all samples over time. However, since variation was increasing considerably after running rows A–D of the 96-well plate, we decided to only use those four rows of a 96-well plate for all subsequent experiments (cV = 5.96% and 0.96%, respectively) (Fig. 1B; Supporting Information Fig. S2).

Next, to address how the incubation temperature would affect the staining of sEVs with antibodies, we incubated eGFP-tagged sEV with anti-CD63 antibodies at 4°C, RT, and 37°C, at two different concentrations (10 nM and 30 nM; Fig. 2A). Also, while we applied a 2 h incubation time in our previous study, we here included an additional 20 h timepoint to additionally study potential effects over time. Samples were analyzed with previously optimized settings and data was presented in following fluorescence calibration with FITC MESF beads as described before (Supporting Information Fig. S1B) (8). As shown before, the usage of PE-labeled anti-CD63 antibodies resulted in a distinct double positive EV population being separable from nonfluorescent background events. Non-EV-containing controls and isotype controls were devoid of notable PE staining, and NP40-treated lysis controls were devoid of both PE staining and eGFP(+) events (Fig. 2B,C; Supporting Information Fig. S3). Of note, the low frequent eGFP(−) PE(+) events visible in all samples including non-EV-containing samples and buffer controls (Fig. 2C; Supporting Information Fig. S3A) were described previously in the exact same context and assumingly relate to mostly antibody-mediated background events and possible (for EV-containing samples) to a minor proportion of non-engineered eGFP(−) CD63(+/-) EVs (8).

Interestingly, it became apparent that the eGFP intensity dropped clearly after staining with PE-conjugated anti-CD63 antibodies compared to unstained samples and samples incubated with isotype control antibodies. This further was in line with an increase in concentration of eGFP+ sEVs after staining with CD63-PE antibodies compared to unstained measurements (Fig. 2C,D; Supporting Information Fig. S3). This suggests that a proportion of rather dim eGFP+ sEVs not being detected based on eGFP fluorescence became detectable only after antibody labeling. While being ultimately out of the scope of this manuscript, this observation is of general interest and would imply that antibody staining procedures against abundant EV surface markers generally can be used to more accurately quantify otherwise fluorescently labeled sEVs, especially when a proportion of those sEVs would be rather dim. In context of this study, this further indicates that obtained values of unstained and stained samples may not be comparable, and thus we subsequently focused on the comparison of stained samples only to evaluate how respective incubation temperatures influence the staining outcome.

When quantifying the PE fluorescence intensity following staining with PE-labeled anti-CD63 antibodies, we observed higher values at higher antibody concentrations in all cases, as expected. The staining intensity was comparable for all samples incubated at 4°C or RT, respectively, but generally higher at 37°C after 2 h, while this difference was similar but less drastic after 20 h (Fig. 2E). After short-term incubation (2 h), the detected concentration of CD63-PE+ events was up to 3.61-fold higher when sEVs were stained at 37°C for both concentrations compared to 4°C. However, incubation at RT also resulted in up to 1.86-fold higher detected concentrations of CD63-PE-positive events. After incubation for 20 h, we generally measured higher concentrations of CD63-PE positive events compared to results after 2 h incubation. Also, we detected higher concentrations at RT compared to staining at 4°C or 37°C at higher antibody concentrations. In any combination of constant time and constant temperature, a higher antibody concentration resulted

Figure 2. Comparison of different incubation temperatures for staining EVs with antibodies. (A) Experimental outline: Preclarved, 0.22 μm filtered THP-1:CD63:eGFP CM was incubated at different concentrations (10 nM and 30 nM final concentration) with anti-CD63-PE or IgG1-PE antibodies, or left unstained as control. sEVs were incubated with antibodies for 2 or 20 h at three different temperatures (4°C, RT, or 37°C) protected from light. The processed samples were diluted eightfold in PBS before IFCM measurement without further washing. Sample wells were acquired for 5 min with PBS wash wells (1 min acquisition) between sample wells. (B) Pregating on SSC(low) for downstream analysis of CD63-eGFP EVs, including non-fluorescent events to visualize low-frequent eGFP(−)PE(+) events. (C) The fluorescence intensity of eGFP (Channel 2) in calibrated units of FITC MESF was plotted against PE (Channel 3; PE MESF) as described before (8). Contour plots are shown exemplarily for data after 2 h of incubation at room temperature. Uncalibrated example dotplots are provided in the Supporting Information Figure S3A. Examples plots for all conditions are provided in the Supporting Information Figure S4. Cutoffs for gated populations background events in PBS, only controls for eGFP (>50 FITC MESF), based on unstained eGFP+ sEV samples for PE (>40 PE MESF) and based on previous characterization of eGFP-tagged sEVs (8) and to include the mean eGFP(+) population for SSC (<1,000 a.u.). (D) FITC MESF converted eGFP mean fluorescence intensity (MFI) values for unstained versus stained samples (left) and volumetric quantification of [eGFP+] gated events (right). The example shows data after incubation at RT. See Supporting Information Figure S3B/C for complete data set. (E) MESF converted (PE) mean intensity values of [eGFP+] gated events. (F) Volumetric quantification of double positive gated (DP) event concentrations. All bar graphs show mean ± SEM for triplicates. [Color figure can be viewed at wileyonlinelibrary.com]
in more detectable CD63-PE-positive events, while no time-
or temperature-related consistent difference of unspecific
binding of PE-labeled mIgG1 isotype controls was detected
(Fig. 2F). In conclusion, these results indicate that staining of
sEVs with antibodies for shorter timepoints (here: 2 h) generally seems to be more temperature dependent than prolonged

Figure 3. Analysis of anti-tetraspanin-stained single human plasma EVs by IFCM. (A) Experimental scheme for staining of human plasma-
derived EVs with anti-tetraspanin antibodies at different incubation temperatures. EVs were isolated by differential ultracentrifugation
from 2 ml of human plasma and subsequently stained with anti-tetraspanin antibodies at 4 °C, RT or 37 °C. Samples were washed by using
a 300 kDa filter before IFCM analysis. (B) Representative histograms for anti-tetraspanin (anti-CD9-PE, anti-CD63-PacificBlue and anti-
CD81-FITC) stained EVs measured by IFCM as described previously (10). Histograms are pregated on SSC(low). Gating, controls and data
before fluorescence calibration are provided in the Supporting Information Figure S5A. See Supporting Information Figure S5B-D and
Table S6 for MESF calibration details and 2D contour plots. (C) Quantiﬁcation of the EV concentration from three individual EV
preparations from plasma of healthy donors stained and incubated at respective temperatures. Events are measured as positive events
positive for either CD9, CD63, or CD81 (SSC(low) total events), or as EVs being speciﬁcally positive for CD9, CD63 or CD81. (D) EV
concentrations of each individual donor are shown, respectively. (E) MESF converted mean ﬂuorescence intensity values of positively
gated events for CD9, CD63, or CD81 after staining at indicated staining temperatures. All bar graphs show mean ± SD for triplicates.
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staining (here: 20 h). Observed differences regarding the overall concentration of PE-positive events also suggests that the recovery of EV samples from multwell plates can differ depending on the incubation temperature applied.

In another recent study (10), we described the usage of combinations of fluorescently labeled anti-CD9, anti-CD63, and anti-CD81 antibodies to detect otherwise unlabeled sEVs in blood plasma samples from patients. To address how different incubation temperatures would influence the staining results for such settings, we performed a similar comparison as above with three independently processed samples of healthy donor human plasma-derived sEVs. As described before (10), sEVs were stained for 45 min at either RT, 4°C, or 37°C and analyzed by IFCM (Fig. 3A). Non-EV-containing buffer controls, unstained controls, and detergent-treated controls displayed background at negligible levels, while similar as described in the original study (10), CD9 positive sEVs were the most abundant sEVs in human plasma in comparison to CD63 and CD81 (Fig. 3B; Supporting Information Fig. S5A). Quantification of detectable EV concentrations showed a trend to slightly higher mean recovery of detectable events when staining at RT or 37°C in all cases when compared to samples stained at 4°C (Fig. 3C). While detectable EV concentrations of respective phenotypes varied between donors, the overall trend was similar in most cases (Fig. 3D). Obtained values for fluorescence intensity were not consistently different between the different incubation temperatures used for this dataset (Fig. 3E; Supporting Information Fig. S5D).

RESULTS

While there currently is no better way to calibrate fluorescence-coupled antibodies to the antigen on cells but also to a lower binding kinetic compared to 37°C (16, 17).

In addition to varying staining intensities, we also observed that the measured concentration of recovered cell culture derived sEVs consistently differed when stained at different temperatures. For cell culture-derived EV samples, measured EV concentrations were lower both after 2 h compared to overnight and after incubation at 4°C compared to higher temperatures, suggesting time- and temperature-dependent adsorption to plasticware surfaces under these conditions.

While some tendencies of temperature-related effects observed for cell culture derived sEVs were also observed for human plasma sEVs, obtained results for plasma sEVs were less affected by the different applied incubation temperatures. This indicates that such temperature-related effects can further depend on other factors such as sample type, EV purity or plastic ware used and implies that temperature related effects should ideally be evaluated separately for all studies aiming at highly accurate EV subset quantification. While we cannot give a final recommendation based on these results, overnight staining at RT or 37°C seems preferable to achieve more accurate results. Of note, for interpretation of obtained results, it should be considered that plate runs with the ImageStream took several hours and that samples thus were incubated also for different amounts of times with residual antibody following an eightfold dilution for data in Figures 1 and 2, which might contribute to further variation in that context. It should be further noted that fluorescence calibration and presentation of fluorescence intensity values in units of MESF were included to ensure standardized reporting of EV flow cytometry results and enable comparison of results between instruments, for example. The importance of this has been described at more detail in the recently published MiFlowCyt-EV framework for standardized reporting of EV flow cytometry experiments (14). In this context, the authors would like to emphasize that MESF values assigned for relatively dim sEVs in this approach are extrapolated by assuming linearity from measurements based on much brighter MESF calibration beads, and those assigned values are likely inaccurate in terms of actual fluorophores/antibodies being bound to each EV. For example, this is illustrated by the drastic variation of MESF scales for stained EVs in Figure 3. While there currently is no better way to calibrate fluorescence data due to the lack of more suitable calibration materials, results should not be overinterpreted based on the current approach, however data presented in MESF units should still be preferable to ensure reproducibility and comparability of results.

We further observed clearly decreased eGFP intensities and detected higher concentrations of SSC(low) eGFP(+) sEVs after CD63-PE labeling (Fig. 2C/D). This side finding is not directly
relevant for the main focus of this study but indicates that antibody labeling against abundant epitopes such as tetraspanins might be an efficient way to label (and subsequently detect) events that otherwise are too dim for another fluorescent marker. In this case, it appears that CD63-PE labeling of sEVs resulted in more complete detection of dim SSC(low) eGFP(+) sEVs. While this is relevant for the EV flow cytometry field in general, it also might be specifically useful in future studies to more accurately assess the number of engineered versus non-engineered EVs or enhance sEV subset detection by combining brighter with dimmer fluorescent markers.

In summary, by using previously optimized settings for single sEV quantification by IFCM, we here provide evidence for temperature dependent effects on the outcome of EV antibody staining procedures. We propose that such parameters should be further investigated, and further that sEV analysis by IFCM due to its high sensitivity is highly suitable to reveal such kind of effects on the experimental outcome.

After all, the aim of this study was to specifically use experimental settings published in our recent studies and vary respective antibody incubation temperatures to study related effects on the experimental outcome. As secondary parameters we here also varied the antibody concentration during incubation and the incubation time, however, we like to emphasize that neither antibody concentrations applied nor timepoints chosen may be ideal and ultimately may vary in different experimental contexts, and probably are additionally dependent on several other parameters such as antigen density, EV purity and instrument used. Thus, for quantitative experiments involving staining of sEVs with fluorescence-conjugated antibodies, it is highly recommended to evaluate methodical parameters, choose antibodies with low background and standardize all materials involved in respective protocols to ensure reproducible results.

Acknowledgments
A. G. is an International Society for Advancement of Cytometry (ISAC) Marylou Ingram Scholar (2019–2023).

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