Small Particle Fluorescence and Light Scatter Calibration Using FCM$_{PASS}$ Software

Joshua A. Welsh[1,2] and Jennifer C. Jones[1]

[1]Laboratory of Pathology, Translational Nanobiology Section, Center for Cancer Research, National Institute of Health, National Cancer Institute, Bethesda, Maryland
[2]Corresponding author: *Joshua.welsh@nih.gov*

Use of flow cytometry to analyze small particles has been implemented for several decades. More recently, small particle analysis has become increasingly utilized owing to the increased sensitivity of conventional and commercially available flow cytometers along with growing interest in small particles such as extracellular vesicles (EVs). Despite an increase in small particle flow cytometry utilization, a lack of standardization in data reporting has resulted in a growing body of literature regarding EVs that cannot be easily interpreted, validated, or reproduced. Methods for fluorescence and light scatter standardization are well established, and the reagents to perform these analyses are commercially available. Here, we describe FCM$_{PASS}$, a software package for performing fluorescence and light scatter calibration of small particles while generating standard reports conforming to the MIFlowCyt-EV standard reporting framework. This article covers the workflow of implementing calibration using FCM$_{PASS}$ as follows: acquisition of fluorescence and light scatter calibration materials, cataloguing the reference materials for use in the software, creating cytometer databases and datasets to associate calibration data and fcs files, importing fcs files for calibration, inputting fluorescence calibration parameters, inputting light scatter calibration parameters, and applying the calibration to fcs files. Published 2020. U.S. Government.

Basic Protocol 1: Acquisition and gating of light scatter calibration materials
Basic Protocol 2: Acquisition and gating of fluorescence calibration materials
Alternate Protocol: Cross-calibration of fluorescence reference materials
Basic Protocol 3: Cataloguing light scatter calibration materials
Basic Protocol 4: Cataloguing fluorescence calibration materials
Basic Protocol 5: Creating cytometer databases and datasets
Basic Protocol 6: Importing fcs files
Basic Protocol 7: Fluorescence calibration
Basic Protocol 8: Light scatter calibration
Basic Protocol 9: Performing and reporting fcs file calibration

Keywords: calibration • extracellular vesicles • fluorescence • light scatter • standardization

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INTRODUCTION

The FCM$_{PASS}$ software was developed to perform calibration and keep records on multiple instrument platforms. The workflow is intended to be ergonomic for flow cytometry core facilities and researchers with multiple instruments interested in tracking their

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instrument performance longitudinally and keeping records of their experimental calibration. To enable this, the FCM_{PASS} software is centered around keeping databases for each cytometer, with a dataset added for each calibration (Basic Protocols 5 and 6). For ergonomic use and to maintain high-quality reporting outputs for publication, FCM_{PASS} utilizes a cataloguing system for reference materials (Basic Protocols 3 and 4). The reference-material catalogue enables users to only be required to input complete records for their calibration reference materials once. With this information stored, it can be referenced when performing future calibrations, and it enables completion of documentation in the automated calibration reports.

FCM_{PASS} has been developed to perform fluorescence calibration for any number of fluorescence parameters, with the ability to allow the user to alter the method of unit scaling for regression and to define fluorophore-to-protein ratios for converting the outputs from molecules of equivalent soluble fluorophore (MESF) to epitope number (Basic Protocol 7). This article will cover acquisition and gating of fluorescence calibration materials for calibration of sample data (Basic Protocol 2) and cross-calibration of other fluorescence reference materials (Alternate Protocol).

Light scatter calibration using the FCM_{PASS} software is a semi-automated process, whereby the software approximates the collection half-angle to produce a scatter-diameter curve (Basic Protocol 8). This article will cover acquisition and gating of light scatter calibration materials for calibration of sample data (Basic Protocol 1). By default, the software outputs core-shell models based on EV light scattering characteristics. The software does, however, allow users to customize core-shell model outputs along with homogenous-sphere outputs.

Finally, once calibration is performed, all calibrated parameters are written to fcs files, which preserve the metadata from the source file (Basic Protocol 9). This allows users to share their data in calibrated units. Upon calibration, quality-control plots for both light scatter and fluorescence are outputted along with a spreadsheet. The spreadsheet contains a report conforming to the MIFlowCyt-EV standard reporting framework, with the fields for calibration automatically completed by the software (Welsh, van der Pol, et al., 2020). This spreadsheet also contains the limits of detection for files that used a trigger threshold with a calibrated parameter. All metadata associated with the fluorescence and light scatter calibration to allow reproducibility are also shared.

BASIC PROTOCOL 1

ACQUISITION AND GATING OF LIGHT SCATTER CALIBRATION MATERIALS

This protocol will outline how to prepare, analyze, and gate light scatter calibration materials for use downstream in the FCM_{PASS} software in order to perform light scatter calibration (Basic Protocol 8). Correct use of light scatter reference materials and instrument settings will allow a good approximation of the instrument collection half-angle using Mie theory. By approximating the collection half-angle, light scatter calibration can be performed, allowing for the determination of instrument sensitivity in standard units if a light scatter trigger threshold is used.

Materials

NIST-traceable size calibration reference beads:
- NIST-traceable polystyrene size standards (Thermo Fisher Scientific, 3000 series: 3100A, 3150A, 3200A, 3269A, 3300A, 3350A, 3400A, 3450A, 3500A, 3600A)
- NIST-traceable silica size standards (Thermo Fisher Scientific, 8000 series: 8050A, 8070A)

DPBS, no calcium, no magnesium (Thermo Fisher Scientific, cat. no. 14190250)

Vortex
500-µl low-protein-binding Eppendorf tubes
FACS tubes (round-bottom polystyrene test tubes; Falcon, cat. no. 14-959-2A)
Flow cytometer

1. Calculate stock NIST-traceable size calibration reference bead particle concentration using the percent solids value and particle density provided by the manufacturer and the equation below, where N_P is the concentration (particles ml^{-1}), $W_V\%$ is the percent solids, ρ_ρ is the particle density (g ml^{-1}), and D is the average diameter (µm):

$$N_P = \frac{W_V\% \ \cdot 6 \times 10^{12}}{\pi \rho_\rho D^3}$$

For example, for 100-nm polystyrene beads at 1% and 1.05 g ml^{-1}, the calculation would be as follows:

$$1.82 \ \times 10^{13} = \frac{0.01 \cdot 6 \times 10^{12}}{\pi \ \times 1.05 \times 0.1^3}$$

2. Thoroughly vortex NIST-traceable size calibration reference bead stock bottles (NIST-traceable polystyrene or silica size standards) to homogenize the mixtures before dispensing one drop (~50 µl) of each into separate 500-µl low-protein-binding Eppendorf tubes.

3. Using the working stocks from step 2, make up 500-µl solutions at 1×10^7 particles ml^{-1} in DPBS in FACS tubes.

It is recommended that serial dilutions are used and that volumes of ≥10 µl are used to avoid pipetting errors. The optimal particle concentration at which to run the reference materials will vary depending on several factors, including the flow rate, beam height, and electronic sampling rate. If running for the first time, it is recommended that serial dilutions are performed to determine the optimal concentration for preparation of the beads.

4. On a flow cytometer, set trigger threshold to the most sensitive light scatter detector and ensure that parameter is using log scaling (not linear or biexponential).

5. Running DPBS, lower trigger threshold until the noise floor of the instrument becomes visible.

This is most clear when using a histogram (see Fig. 1).

Plotting the trigger-channel height parameter against time and monitoring while running DPBS is a good way to determine whether an instrument is clean. If the spread of noise (and event rate) decreases over time, it is indicative that the instrument was dirty and is becoming cleaner.

The extent to which the opto-electronic noise of an instrument can be sampled will vary between instruments. Legacy flow cytometers will tolerate 1000 to 2000 events/s while allowing room to sample desired events, whereas high-speed jet-in-air sorters are capable of sampling 10,000+ events/s.

Triggering using a light scatter parameter on the opto-electronic noise of the instrument has benefits in determining and tracking the lower limit of detection as well as being informative for buffer + reagent controls, where background fluorescence will show clear shifts due to many events being triggered from sampling the noise. Use of this method comes at the cost of having high event rates and therefore larger files. Before utilizing this method, the instrument should be validated to determine 1) its ability to detect and accurately process particles, 2) the event rate at which single dim particles are detected, and 3) the degree to which the opto-electronic noise can be sampled without creating artifacts or reducing the ability to detect genuine events.

On some instruments that utilize peristaltic pumps, there can appear to be an increase and decrease in the baseline, corresponding to the turnover of the pump. This is a result of the

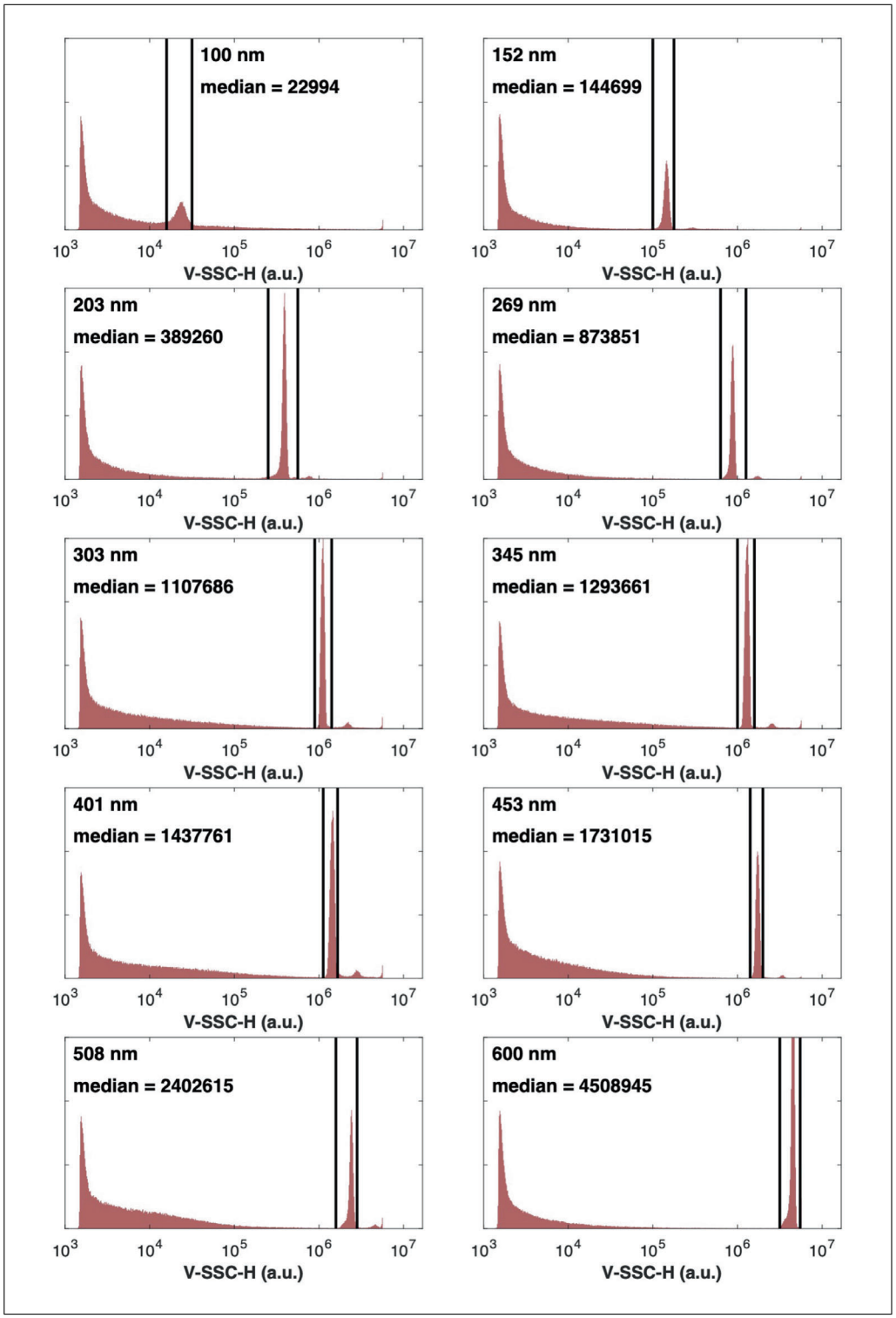

Figure 1 Gating light scatter reference beads. Each panel shows the gating of polystyrene NIST-traceable reference beads ranging in mean diameter from 100 to 600 nm. The median light scatter statistic of the gated population is given in each panel.

threshold being set close to (but above) the electronic noise, resulting in an increase and decrease in trigger events in light scatter. This can be overcome by lowering the threshold so that the noise is being sampled regardless of the peristaltic pump turnover or by increasing the threshold and therefore decreasing the instrument's limit of sensitivity.

6. Analyze each bead sample (see step 3) at the same acquisition settings until >5000 bead events are recorded.

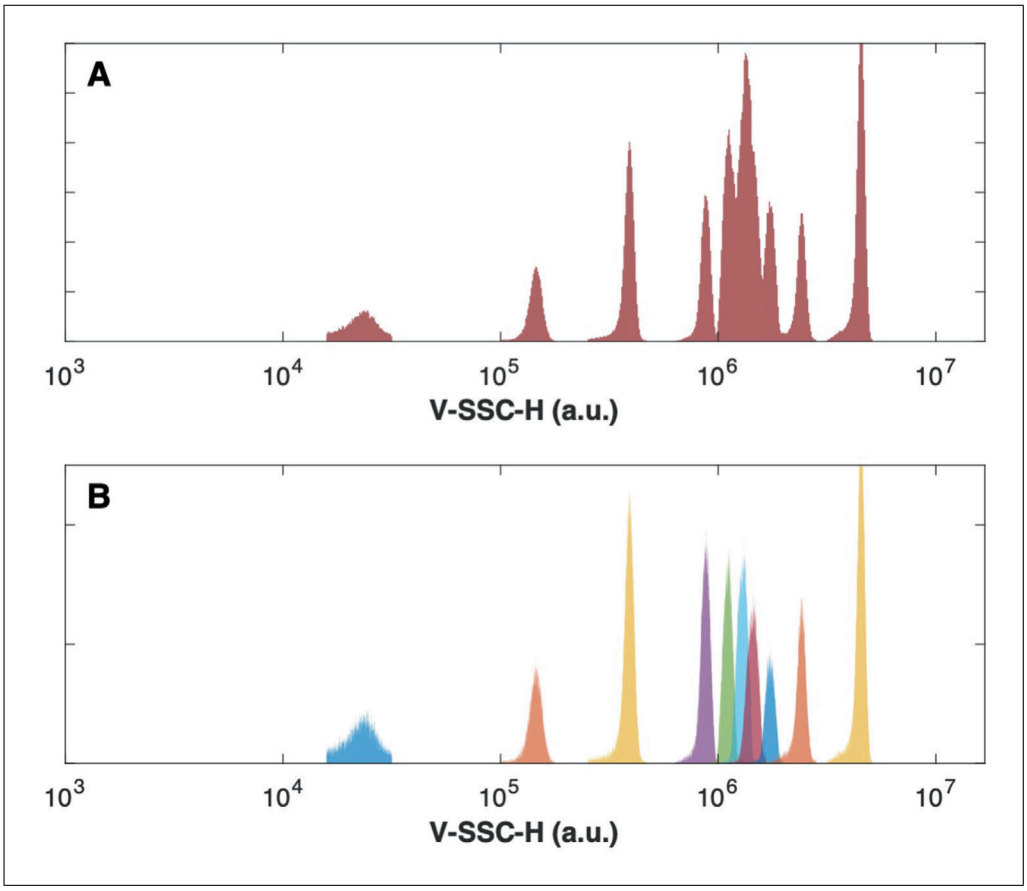

Figure 2 Analyzing light scatter reference beads. (**A**) The cumulative distribution of the gated populations from Figure 1 when mixed together. Whereas some populations are clearly distinguished, some are not. The areas where bunching of populations occurs are dependent upon the cytometer and are useful in determining the collection angle. (**B**) The overlaid and colored gated bead population from Figure 1.

It is preferable to analyze and store bead populations individually. This will minimize population overlap, aggregates, background noise, and artifacts (see Fig. 2).

7. Gate each bead population using parameter Height vs. Area in a dot plot to remove doublets/aggregates and then use a histogram on light scatter parameter (Height) to obtain statistics for each population.

   The light scatter parameter should use log scaling (see Figs. 1 and 2).

8. Obtain median statistic for each of the bead populations (see Fig. 1).

   By default, flow cytometers trigger the acquisition of an event using the pulse-height parameter. In cases where a trigger threshold is being defined (e.g., SSC), it is recommended that the pulse height is used so that the limit of detection can be defined in calibrated units. There is no consensus within the small particle research community over the use of pulse height vs. area. We recommend that, in general, if the parameter being calibrated was not used as a trigger channel, the pulse-area statistic should be used due to the tendency for low signal intensities to be linear and is therefore a more reliable method for extrapolation.

ACQUISITION AND GATING OF FLUORESCENCE CALIBRATION MATERIALS

BASIC PROTOCOL 2

This protocol will outline how to prepare, analyze, and gate fluorescence calibration materials for use downstream in the FCM_{PASS} software in order to perform fluorescence calibration (Basic Protocol 7). Correct use of fluorescence reference materials and

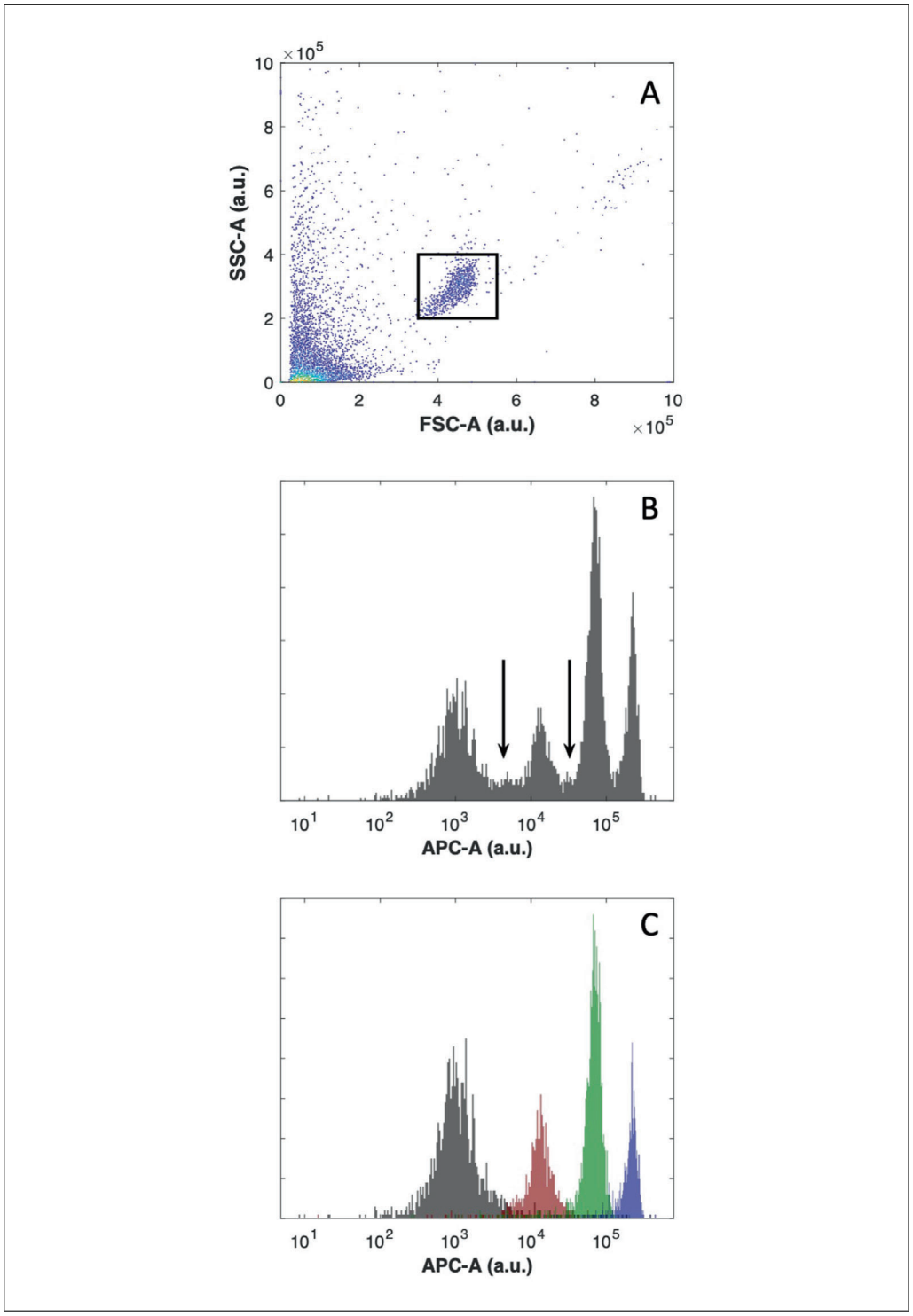

Figure 3 Gating fluorescence reference beads. (**A**) Gating of the bead population using FSC-A and SSC-A. (**B**) Histogram of all four APC MESF bead populations in a cumulative distribution. Arrows highlight areas of overlap between beads that may lead to subjectivity regarding where to manually draw gates. (**C**) Histogram of individual APC MESF bead populations.

instrument settings will allow a good approximation of instrument sensitivity in standard units.

Additional Materials *(also see Basic Protocol 1)*

MESF calibration beads (Bangs Laboratories, cat. no. 823)

1. Vortex each MESF calibration bead bottle before use.
2. Add one drop (~50 µl) of each bead population to separate FACS tubes containing 250 µl DPBS.

   Due to the high autofluorescence of the "Blank" beads, it is not recommended to use them as 0 MESF.

   Many commercially available fluorescence calibration beads are bright and will require extrapolation instead of interpolation to obtain the dim fluorescence values. The accuracy of the extrapolation will therefore be influenced by several factors, including the gating of the populations. Although less ergonomic, it is preferable to analyze one bead population at a time. This allows for gating on scatter parameters, rather than fluorescence parameters, making the statistics less biased by the gating strategy. Analyzing one bead population at a time will also minimize the subjectivity when gating fluorescence populations that overlap, sometimes causing small peaks (see Fig. 3).
3. Ensure that cytometer fluorescence settings are those used for the assay being calibrated.
4. If the beads are >1 µm in diameter, use a forward scatter trigger threshold.
5. Analyze each bead sample (see step 2) at the same acquisition settings until >5000 bead events are recorded.
6. Gate each bead population on FSC-A vs. SSC-A and obtain median area statistic for the fluorescence parameter being calibrated.

   By default, flow cytometers trigger the acquisition of an event using the pulse-height parameter. In cases where a trigger threshold is being defined, e.g., SSC, it is recommended that the pulse height is used so that the limit of detection can be defined in calibrated units. There is no consensus within the small particle research community over the use of pulse height vs. area. We recommend that, in general, if the parameter being calibrated was not used as a trigger channel, the pulse-area statistic should be used due to it tending to be linear at low signal intensities and therefore a more reliable method for extrapolation.

   This step must be completed to be able to move on to the fluorescence calibration protocol (see Basic Protocol 7).

CROSS-CALIBRATION OF FLUORESCENCE REFERENCE MATERIALS

ALTERNATE PROTOCOL

A more economical method (compared to Basic Protocol 2) for calibrating fluorescence parameters over time and using high detector settings that may cause some MESF standards to be off scale is to cross-calibrate eight-peak rainbow beads. These beads fluoresce over a wide region of the visible spectrum and range in fluorescence intensity from very bright to very dim. Typically, the dimmest population of an eight-peak sample has a lower MESF value than the dimmest population among MESF calibration beads on the same channel.

Additional Materials *(also see Basic Protocol 1)*

APC MESF calibration beads (Bangs Laboratories, cat. no. 823)
Eight-peak rainbow beads (Spherotech, cat. no. RCP-30-5A)

1. Vortex each fluorescence reference bead bottle before use.
2. Add one drop (~50 µl) of each APC MESF calibration bead population to separate FACS tubes containing 250 µl DPBS.
3. Add one drop (~50 µl) of the eight-peak rainbow bead population to a FACS tube containing 250 µl DPBS.
4. Ensure that cytometer fluorescence settings are those used for assays being calibrated.

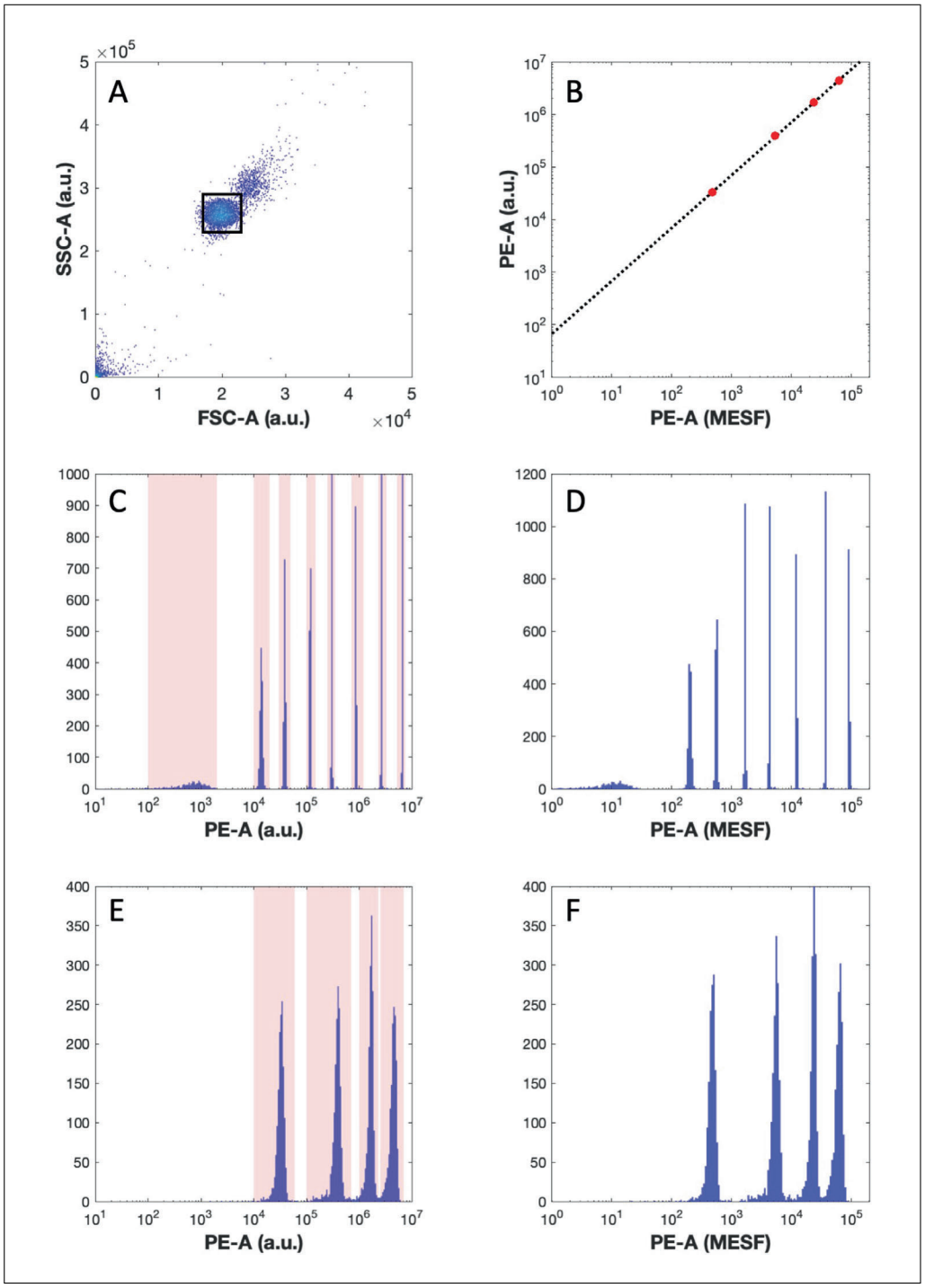

Figure 4 Gating and cross-calibration of fluorescence reference beads. (**A**) Gating of the eight-peak bead population using FSC-A and SSC-A. (**B**) Regression of PE MESF bead reference values vs. acquired arbitrary statistics for each population. (**C**) Histogram of gated (A) eight-peak reference beads and gating of each population (pink). (**D**) Histogram of gated (A) eight-peak reference beads converted to PE MESF units using regression (B). (**E**) Histogram of gated PE MESF beads and gating of each population (pink). (**F**) Histogram of gated PE MESF beads converted to PE MESF units using regression (B).

5. If the beads are >1 μm in diameter, use a forward scatter trigger threshold.

6. Analyze each bead sample (see steps 2 and 3) at the same acquisition settings until >2000 bead events are recorded.

   For the eight-peak beads, this will be >16,000 events.

7. Gate each APC MESF calibration bead population on FSC-A vs. SSC-A and obtain median statistic for the parameter before performing calibration of the MESF and eight-peak bead files (see Basic Protocols 5, 6, 7, and 9).

8. Once the eight-peak rainbow beads are calibrated in PE MESF units, gate population on FSC-A vs. SSC-A to obtain singlets (see Fig. 4). Using the singlet population gate each of the eight-peak populations.

   The gating the individual fluorescent bead populations can be done in the parameter that best separates each population. This may be a different fluorescence detector than the calibrated parameter. Although the gating of each population does not have to be on the MESF parameter itself, the MESF parameter should be checked to ensure that all populations are on scale. In some third-party software, the scale limits (minimum and maximum values) will influence the outputted statistic due to how the data are binned.

9. Once each of the eight-peak populations has been gated (see Fig. 4), obtain median MESF value for each of the populations.

   These values are now the cross-calibrated values for these beads and can be used on the same instrument at different gains.

   If an instrument is re-aligned or filters are changed, these values will no longer be valid and will require cross-calibration to be determined again. In general, it is good practice to regularly cross-calibrate eight-peak bead reference values, e.g., once a month.

CATALOGUING LIGHT SCATTER CALIBRATION MATERIALS

BASIC PROTOCOL 3

This protocol outlines how to catalogue light scatter calibration reference materials for use in the FCM_{PASS} software. By cataloguing each reagent, including relevant metadata such as manufacturer, catalogue number, and lot number, the automated output report can include all relevant information for reporting. By calling upon a catalogue during the calibration input steps within the software, time is saved because the user does not have to repeat inputting the same information.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Additional Materials *(also see Basic Protocol 1)*

FCM_{PASS} software (v3+, Windows, MacOS compatible, available at *https://nano.ccr.cancer.gov/fcmpass*)
Appropriate computer (quad core, 8 GB+ RAM recommended)

1. Open FCM_{PASS} software on an appropriate computer.

2. Click "Catalogue" in top menu bar.

3. Under the "Light Scatter" tab, fill out entry fields for each of the pertinent metadata for reporting with light scatter calibration (see Fig. 5):

   a. "Diameter" is the mean diameter of the solid bead population provided on the certificate of analysis.
   b. "Diameter CV" is the percent coefficient of variation of the mean diameter provided on the certificate of analysis.
   c. "Refractive Index" is the provided refractive index of the bead population on the certificate of analysis.

   If a refractive index is not available, an approximate guide for the polystyrene refractive index is 1.59 at 589 nm. Silica can vary more in refractive index than polystyrene but tends to be ∼1.45 at 589 nm.

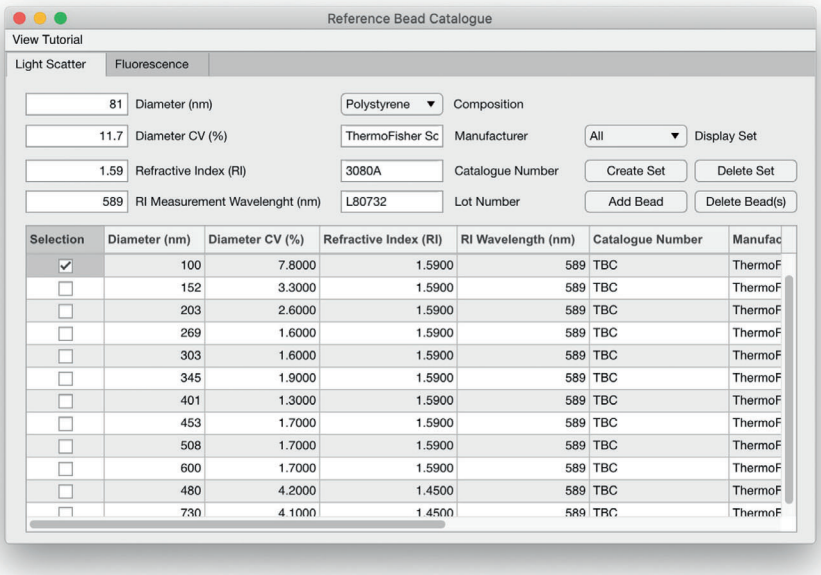

Figure 5 Light scatter reference bead catalogue input window.

d. "RI Measurement Wavelength" is the wavelength at which the refractive index was measured and should be provided on the certificate of analysis.

This is often 589 nm.

e. "Composition" can be selected as polystyrene, silica, or other.

If polystyrene or silica is selected, changes in detection wavelength, e.g., 488 nm to 405 nm, are accounted for using the appropriate Sellmeier equations. If "Other" is selected, then the refractive index change is made proportionally to the sheath refractive index.

f. "Manufacturer," "Catalogue Number," and "Lot Number" should all be completed appropriately.

4. Once the fields have been completed for a bead population, click "Add Bead."

The population should then appear in the table below.

5. Once the relevant beads have been added, create "Bead Sets":

a. In the "Selection" column of the table, check all bead populations to be included within a bead set.

Bead sets are the bead populations that are used for calibration. Any number of bead sets and combinations can be made.

b. Click "Create Set," enter a unique name, and click "OK."

Once a bead set has been defined, you will be able to perform light scatter calibration (see Basic Protocol 8).

CATALOGUING FLUORESCENCE CALIBRATION MATERIALS

BASIC PROTOCOL 4

This protocol outlines how to catalogue fluorescence calibration reference materials for use in the FCM_{PASS} software. By cataloguing each reagent, including relevant metadata such as manufacturer, catalogue number, and lot number, the automated output report

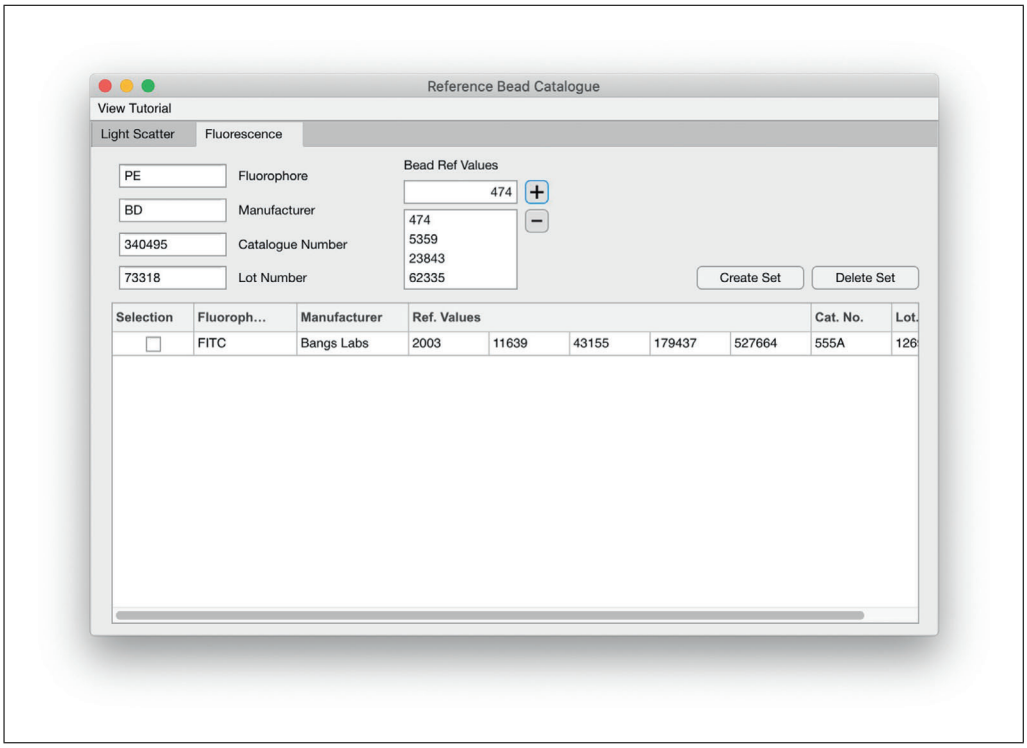

Figure 6 Fluorescence reference bead catalogue input window.

can include all relevant information for reporting. By calling upon a catalogue during the calibration input steps within the software, time is saved because the user does not have to repeat inputting the same information.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Materials

FITC MESF calibration beads (Bangs Laboratories, cat. no. 555A)
PE MESF calibration beads (Becton Dickinson, cat. no. 340495)

FCM_{PASS} software (v3+, Windows, MacOS compatible, available at *https://nano.ccr.cancer.gov/fcmpass*)
Appropriate computer (quad core, 8 GB+ RAM recommended)

1. Open FCM_{PASS} software on an appropriate computer.
2. Click "Catalogue" in top menu bar.
3. Under the "Fluorescence" tab, fill out entry fields exist for each of the pertinent metadata for reporting with fluorescence calibration (see Fig. 6):
   a. Enter name of the fluorophore on the fluorescence reference beads (FITC or PE MESF calibration beads).
   b. Enter manufacturer, catalogue number, and lot number fields appropriately.
   c. In the "Bead Ref Values" field, enter each fluorescence bead sample's reference values. After each reference value, click "+" button.

      This may be in MESF, equivalent reference fluorophore, or antibody-binding capacity.

   d. Once all fields and reference values have been added, click "Create Set."

      The beads will then appear in the table below and will be available for selection when performing fluorescence calibration.

CREATING CYTOMETER DATABASES AND DATASETS

This protocol outlines how to create cytometer databases and datasets. Storing the calibration information by dataset for each cytometer is an ergonomic approach for users who need to perform calibration and track multiple instruments simultaneously.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Materials

FCM_{PASS} software (v3+, Windows, MacOS compatible, available at *https://nano.ccr.cancer.gov/fcmpass*)
Appropriate computer (quad core, 8 GB+ RAM recommended)

1. Open FCM_{PASS} software on an appropriate computer (see Fig. 7).
2. Click "+" icon next to the "Cytometer IDs" list and enter a unique name to identify instrument.
3. Select relevant cytometer ID to which to add the dataset.
4. Click "+" icon next to the "Datasets" list. In the window, enter acquisition date of the calibration data and enter dataset/experiment name.

   If there are any notes related to the experiment that are beneficial, they can be entered in the "Dataset Notes" field.

5. Proceed to Basic Protocol 6.

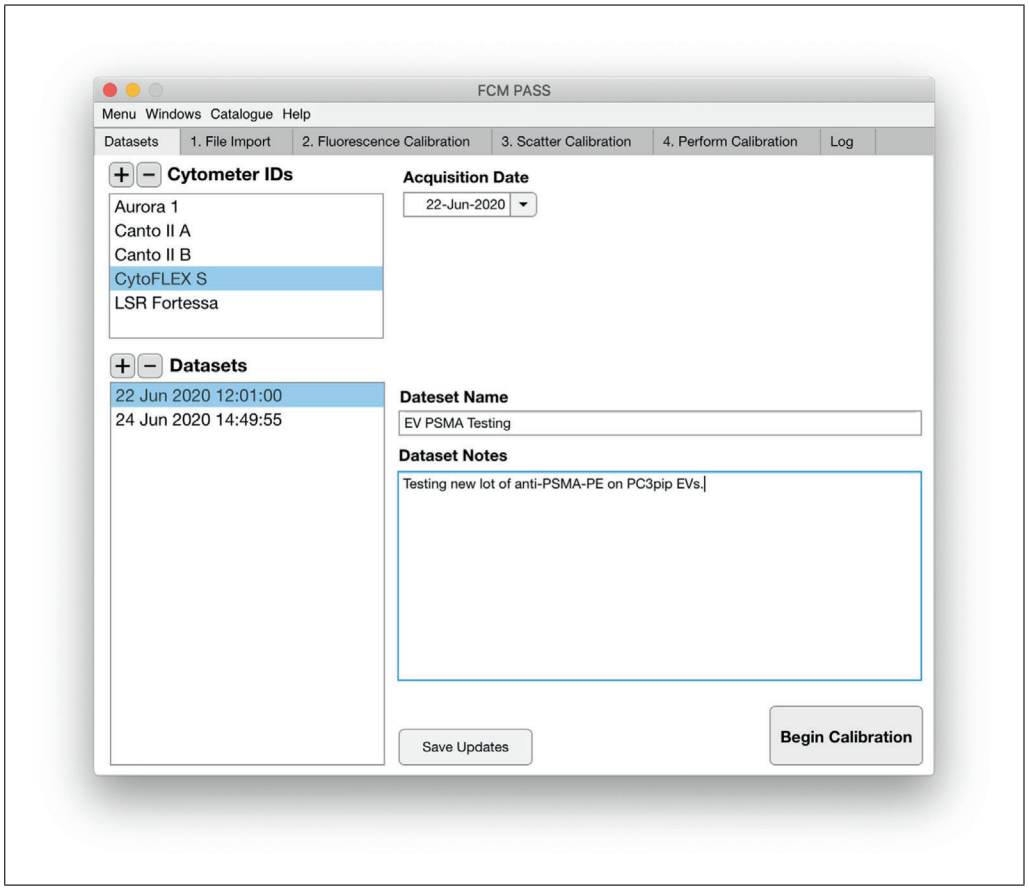

Figure 7 Main window of FCM_{PASS} v3. Components include cytometer database selection (top left) and cytometer dataset selection (bottom left), and loaded dataset metadata (right).

IMPORTING FCS FILES

This protocol outlines how to import fcs files into the FCM_{PASS} software for calibration. Importing fcs files into the software is the first step in beginning calibration for a dataset. At this step, the software can extract the parameters associated with the fcs file along with the instrument settings used to acquire the data associated with the fcs file.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Materials

See Basic Protocol 5.

1. Once a dataset has been created (see Basic Protocol 5), click "Begin Calibration" button.
2. Import fcs files by selecting "+" icon next to the "Files to calibrate" table (see Fig. 8). In the new window, navigate to folder containing the fcs files to calibrate and select "OK."

   The fcs files and related metadata will now be imported. If the folder contains fcs files that you do not wish to use for calibration, select them and click the "–" icon. The metadata related to the remaining files will then be reprocessed.

   The parameters, e.g., SSC-H or SSC-A, that are available in further steps in the software are those that are common to all the loaded fcs files. If files that are loaded do not have any common parameter names, a selection will not be available in these steps.

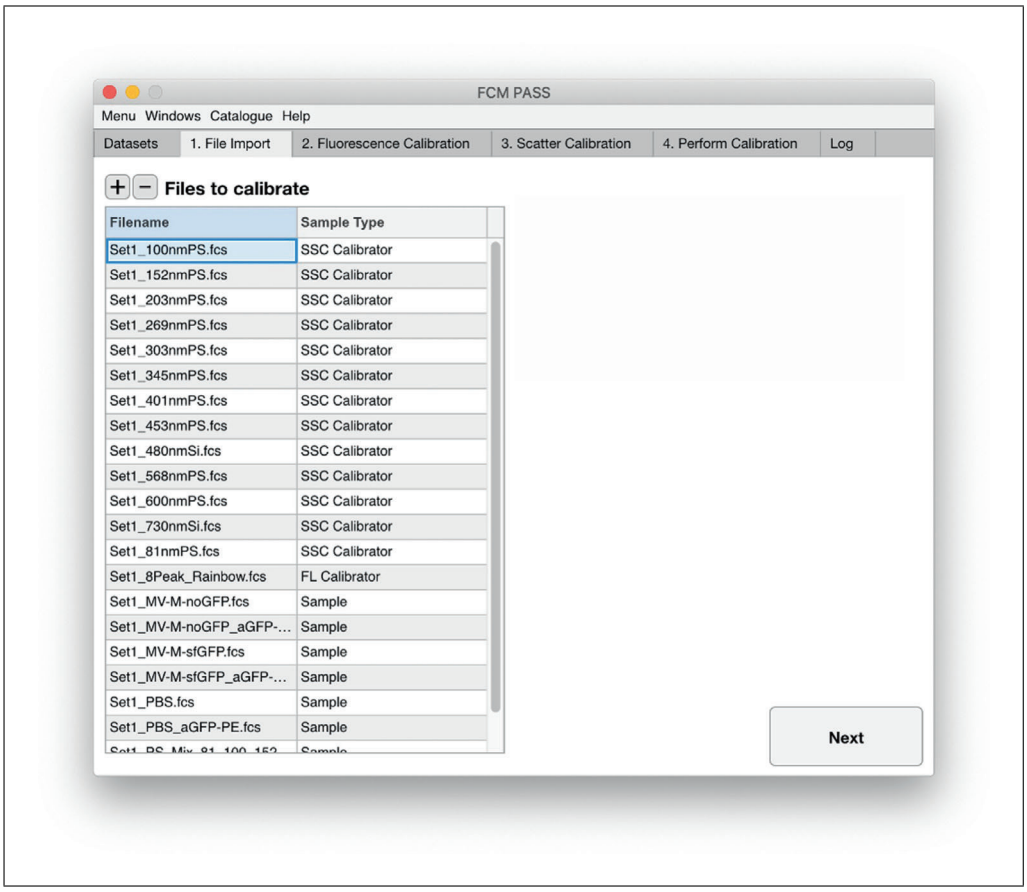

Figure 8 Import window for fcs files.

3. Under the "Sample Type" column, all loaded files by default are listed as "Sample." For the relevant files these can be adjusted to "SSC Calibration" or "FL Calibration" depending on what the sample was used for.
4. Once completed, select "Next."
5. Proceed to Basic Protocol 7.

BASIC PROTOCOL 7

FLUORESCENCE CALIBRATION

This protocol outlines how to input the parameters required for the FCM_{PASS} software to perform fluorescence calibration using imported fcs files. Fluorescence calibration allows users to report their data in standard units as well as determine their instrument's limit of sensitivity and dynamic range. Fluorescence calibration can also allow users to determine the best instruments and instrument settings for their assay.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Additional Materials *(also see Basic Protocol 6)*

FlowJo or equivalent gating software for fcs files

1. Using FlowJo or equivalent gating software for fcs files, if fluorescence calibration is being performed, click "+" button to add a calibration parameter to the table (see Fig. 9). If fluorescence calibration is not required, click "Next" and proceed to Basic Protocol 8.

   If you have not yet added the MESF reference bead information that will be used for calibration into the catalogue, click "Catalogue" in the top menu bar and complete Basic Protocol 4.

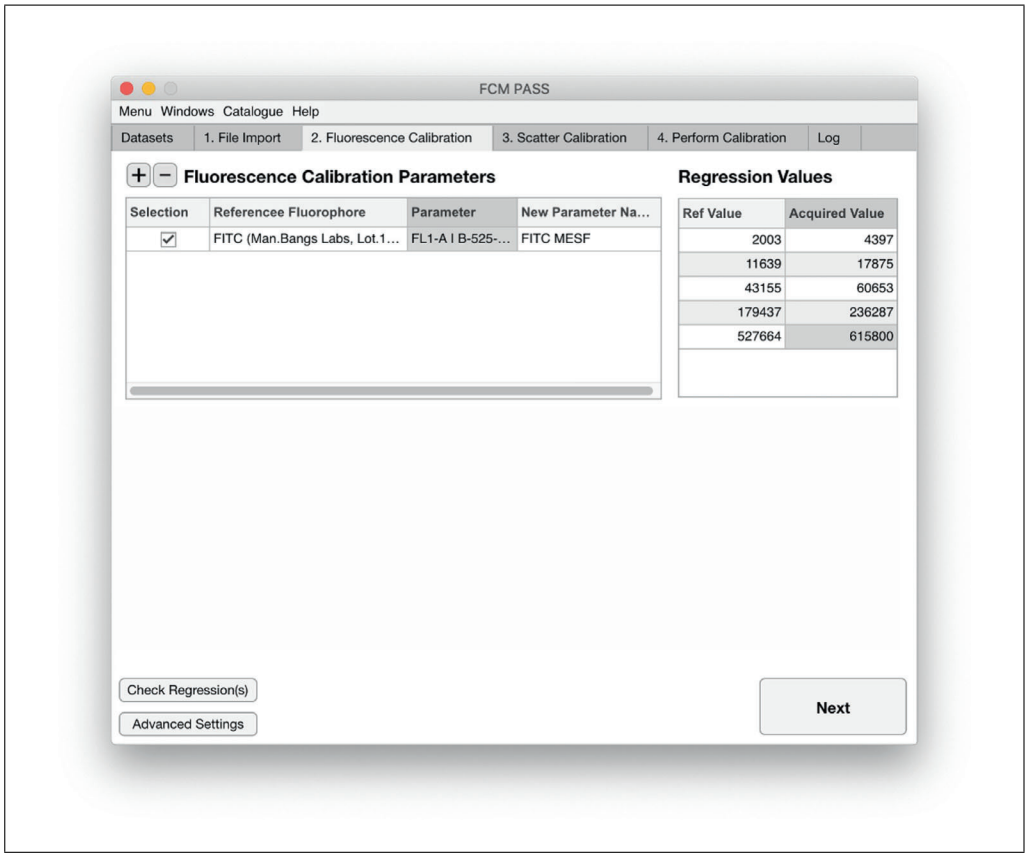

Figure 9 Fluorescence calibration window.

2. Once a parameter is added, double click "Reference Fluorophore" item and select bead set used for calibration.

   The displayed sets are those that have previously been added to the catalogue in Basic Protocol 4.

3. Double click parameter to select the associated parameter with the correct fluorophore.

4. Double click relevant cell in the "New Parameter Name" column to adjust how the calibrated parameter's name will appear once written to the fcs file.

5. Once the reference bead values for the selected parameter appear in the "Regression Values" table, click in "Acquired Value" box next to each bead reference value and input acquired statistic.

6. Repeat steps 1 to 5 for any further parameters that need to be calibrated. To change the "Ref Value" table for other fluorophores, select them in reference "Fluorescence Calibration Parameters" table.

7. Once completed, click "Next."

   The regression plots for the inputted fluorescence calibration parameters can be checked at any time using the "Check Regression(s)" button. The "Advanced Settings" button can be used to specify a fluorophore-to-protein (F/P) ratio or alter the regression method among linear, log, weighted linear, and weighted log.

8. Proceed to Basic Protocol 8.

LIGHT SCATTER CALIBRATION

BASIC PROTOCOL 8

This protocol outlines how to input the parameters required for the FCM_{PASS} software to perform light scatter calibration using imported fcs files. Light scatter calibration allows users to report their data in standard units as well as determine their instrument's limit of sensitivity and dynamic range. Light scatter calibration can also allow users to determine the best instruments and instrument settings for their assay.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Materials

See Basic Protocol 7.

1. Using FlowJo or equivalent gating software for fcs files, if light scatter calibration is being performed, click "+" button to add a calibration parameter to the table (see Fig. 10). If light scatter calibration is not required, click "Next" and proceed to Basic Protocol 9.

   If you have not yet defined the light scatter bead sets in the catalogue, click "Catalogue" and complete Basic Protocol 3.

2. Double click "Scatter Parameter" field to change which parameter is being used for light scatter calibration.

3. Alter "Scatter Wavelength (nm)" to the relevant wavelength for the parameter being used to calibrate light scatter.

   You will see that the "Sheath RI" field will automatically update when this is altered. In the background, refractive indices for reference beads and core-shell and homogenous-sphere models will all also be updated.

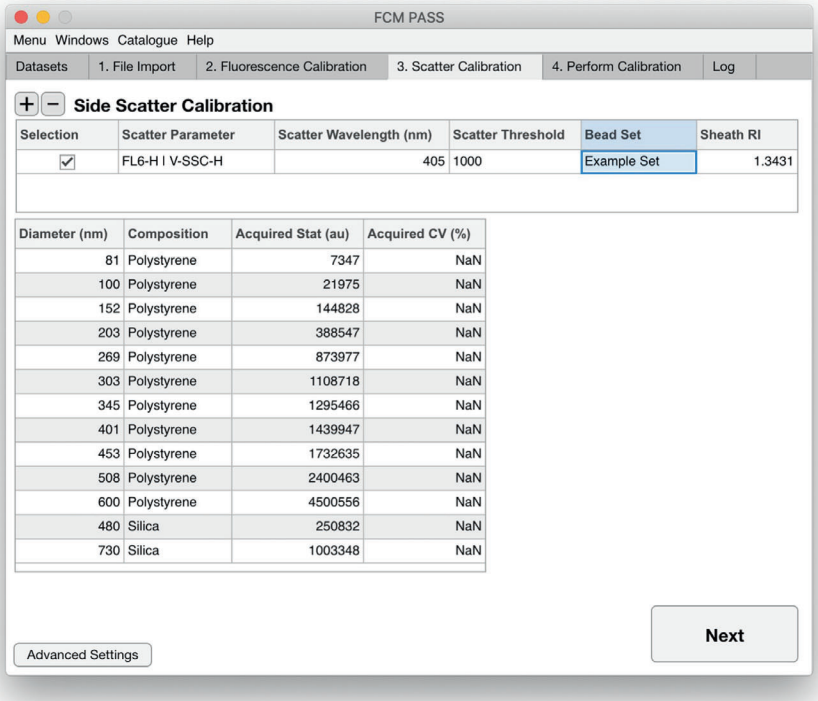

Figure 10 Light scatter calibration window.

If the selected "Scatter Parameter" was used as a trigger threshold, then the "Scatter Threshold" field will automatically update to show the values used as thresholds in the fcs files loaded.

4. Select "Scatter Threshold" by double clicking the field and selecting an option from the dropdown menu or by inputting a custom entry.
5. Load light scatter reference beads used by double clicking the "Bead Set" field. Once loaded the beads within the set will populate the bottom table.
6. The "Sheath RI" field automatically accounts for "Scatter Wavelength" but can be updated manually by double clicking the field.
7. In the bottom table, enter median scatter parameter statistic for each population.

   The acquired CV can optionally also be completed. It will, however, only be used for plotting purposes and will not alter the model calculations.

8. Once completed, click "Next."

   Custom core-shell models, solid-sphere models, plot data points, modeling parameters, and output settings can be entered or altered by clicking the "Advanced Settings" button. By default, three EV core-shell models relating to high, medium, and low EV refractive indices are calculated. All core-shell models assume a 5-nm shell thickness.

9. Proceed to Basic Protocol 9.

PERFORMING AND REPORTING FCS FILE CALIBRATION

This protocol outlines how parameters from the fluorescence and light scatter calibration input steps (Basic Protocols 7 and 8) can be written to fcs files using FCM_{PASS}. This will generate a folder that contains the information required for sharing and reporting

single-EV flow cytometry data. The folder will contain calibrated fcs files, a spreadsheet with all metadata required to replicate calibration, a MIFlowCyt-EV report with light scatter and fluorescence calibration fields completed, and quality-control plots for light scatter and fluorescence calibrations.

NOTE: Updates and videos related to this protocol can be found at doi: *10.17504/protocols.io.bjcqkivw*.

Materials

See Basic Protocol 8.

1. Upon completing Basic Protocol 7 and/or Basic Protocol 8, click "Calibrate" button.

   If this is the first time using a particle composition, whether it be for a light scatter calibration reference material or a new homogenous-sphere or core-shell model, the software will need to generate a database for that composition. The processing time for this can vary depending upon computing processor speed and the number of processor cores. Generally, this will take 5 to 10 min (2 GHz, four-core processor). Any subsequent calibration using the same light scatter reference materials or custom models will be loaded instantly.

   The FCM$_{PASS}$ software will perform fluorescence and light scatter calibration. An FCM$_{PASS}$ export folder will be created in the directory from which the fcs files were imported. This folder will contain calibrated fcs files, a MIFlowCyt-EV report with fields relevant to fluorescence and light scatter calibration completed, and supplementary sheets for reproducing each of the calibrations. A calibration output report file will also be generated, which contains the relevant figures to support the fluorescence calibration and light scatter calibration that were performed. For complete and transparent records, all of these files should be kept together when fcs files are shared.

2. Complete remaining fields within the MIFlowCyt-EV report as recommended in the associated position paper (Welsh, van der Pol, et al., 2020).

COMMENTARY

Background Information

Small particle analysis using flow cytometry has been demonstrated since the late 1970s (Hercher, Mueller, & Shapiro, 1979), initially with viruses. Today, a variety of small particles are being researched for their potential in biomedical sciences, many of which fall under the umbrella term "extracellular vesicles" (EVs) (Thery et al., 2018). The definition of EVs continues to evolve but currently encompasses a variety of particles with overlapping nomenclature, e.g., exomeres, exosomes, microparticles, microvesicles, ectosomes, retroviral-like particles, and retroviruses (Lotvall et al., 2014; Thery et al., 2018). The lack of clarity within the nomenclature is in part due to the lack of an ability to separate and characterize all EVs. Today, as flow cytometers become ever more sensitive due to technological advancements, the ability to characterize particles of 100 nm in diameter or smaller is feasible using some commercially available instruments (de Rond et al., 2019; Gasecka et al., 2020; Morales-Kastresana et al., 2019; Stoner et al., 2016; Tian et al., 2020; Tian et al., 2018; Welsh, Jones, & Tang, 2020; Zhu et al., 2014). As the use of small particle flow cytometry increases, it is critical for researchers to understand the particular controls and considerations that are distinctively necessary in small particle flow cytometry, as compared to what is necessary in cellular flow cytometry (see Current Protocols article; Nolan, 2015).

Some major hurdles for the field to overcome are in utilization of calibration and controls along with clarity in reporting. Researchers with a demonstrated history of small particle analysis from the International Society for Extracellular Vesicles (ISEV), International Society for Advancement of Cytometry (ISAC), and International Society on Thrombosis and Haemostasis (ISTH) formed an inter-societal EV flow cytometry working group in 2015. At the time, few controls were agreed upon, and only a handful of publications utilized any form of calibration. A key product of this working group was the development of the MIFlowCyt-EV framework in 2020 (Welsh, van der Pol, et al., 2020). The MIFlowCyt-EV framework was published as a position paper outlining a

reporting framework of key metadata, controls, calibration, and data reporting fields that should be completed when performing single-EV measurements using flow cytometry, all of which have been demonstrated to be useful in the reproducibility or characterization of small particle data. Implementation of the metrics outlined in this framework will improve transparency and reproducibility in the current literature, where the reported concentration of EVs can differ by several orders of magnitude depending on the equipment or assay being used (Gasecka, Boing, Filipiak, & Nieuwland, 2017; Johnsen, Gudbergsson, Andresen, & Simonsen, 2019).

As the MIFlowCyt-EV framework provides insight into the controls required and a framework for transparent and standardized reporting and given the rigor that is required for accurate and reproducible reporting of measurements obtained near the limits of an instrument's detection, the practice of reporting data in calibrated standard units is increasingly encouraged in the EV research community. Fluorescence calibration methods have been in use since the late 1990s, with fluorescence reference materials also commercially available (Gaigalas, Wang, Schwartz, Marti, & Vogt, 2005; see Current Protocols article; Hoffman, 2005; Schwartz et al., 2004; Schwartz et al., 2002; see Current Protocols article; Wang & Hoffman, 2017; Wood, 1998; Wood & Hoffman, 1998). Fluorescence calibration allows for the conversion of arbitrary units into standardized units of fluorescence, such as MESF or the equivalent number of reference fluorophore (ERF) (Gaigalas et al., 2005; see Current Protocols article; Hoffman, 2005; Schwartz et al., 2004; Schwartz et al., 2002; see Current Protocols article; Wang & Hoffman, 2017; Wood, 1998; Wood & Hoffman, 1998). Light scatter calibration methods were first demonstrated for small particles in 2009 and were specifically applied to EVs in 2012 (Fattaccioli et al., 2009; van der Pol, van Gemert, Sturk, Nieuwland, & van Leeuwen, 2012). Although reference materials are available for light scatter calibration, the methodology has been relatively inaccessible owing to the lack of free software available and the knowledge required to model small particle light scattering distributions within a flow cytometer. More recently, commercially available kits and software dedicated to small particle flow cytometry calibration have become more readily available (see Current Protocols article; de Rond, Coumans, Nieuwland, van Leeuwen, & van der Pol, 2018).

In 2019, FCM_{PASS}, a small particle flow cytometer calibration software package for light scatter and fluorescence, became available (Welsh, Horak, et al., 2020). FCM_{PASS} was developed to be compatible with any commercial calibration reference materials for fluorescence and light scattering. The recent development of FCM_{PASS} v3 has enabled instrument tracking for longitudinal performance and alignment for light scatter. The outputs of the FCM_{PASS} software have also been internationally formatted into the MIFlowCyt-EV framework, so as to semi-automate the completion of the calibration fields and improve the ergonomics of the calibration and reporting process. Here, protocols for acquisition of light scatter and fluorescence reference materials (Basic Protocols 1 and 2 and Alternate Protocol), creating reference material catalogues for light scatter and fluorescence reference materials (Basic Protocols 3 and 4), and performing fluorescence and light scatter calibration (Basic Protocols 7 and 8) are outlined.

Critical Parameters

In order to approximate the collection half-angle of a flow cytometer (Basic Protocol 1) to perform light scatter calibration (Basic Protocol 8), some assumptions have to be made. One of these critical assumptions is that the light scatter calibration beads have the diameter and refractive index inputted into the software. This is because predicted data based on light scatter models using this information will be compared to acquired data. For this reason, assurance that the beads are sized accurately and have reasonable refractive indices is important. NIST-traceable size standards are therefore solely recommended as light scatter calibration reagents. Use of popular fluorescent hard-dyed beads such as Megamix beads (Biocytex) or FluoSpheres (Thermo Fisher Scientific) is not recommended for performing light scatter calibration. This is due their size being an approximation and their fluorescence creating a complex refractive that which is difficult to model accurately. The accuracy of light scatter modeling is also dependent upon the number of bead populations used and their diameters. It is recommended that no fewer than five beads of the same composition are used (more is preferable), with their diameters spanning the smallest bead detectable (preferably 80 to 100 nm) up to 600 nm. The most accurate application of light scatter calibration using the FCM_{PASS} software is when using analyzers with conventional side scatter

collection optics, i.e., collection perpendicular to the illumination source with a symmetrical collection. Non-conventional cytometer collection optics, such as the Apogee instruments, are currently not supported due to lack of testing availability. Although light scatter calibration can be applied to sorters and has been demonstrated (Morales-Kastresana et al., 2019; Welsh, Horak, et al., 2018), care must be taken in the stream alignment, and the confidence of the model fit will likely be lower. This is due to the use of laser obscuration bars in front of the collection lens that are difficult to take into account.

The following standards have been successfully tested by the authors for use as light scatter and fluorescence calibration reagents in Basic Protocols 1 and 2 and the Alternate Protocol:

60-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3060)

70-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3070)

81-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3080)

100-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3100)

152-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3150)

203-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3200)

240-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3240)

269-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3269)

303-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3300)

345-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3350)

401-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3400)

453-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3450)

508-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3500)

600-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3600)

707-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 3700)

1019-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 4010)

1587-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 4016)

490-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 8050)

730-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 8070)

990-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 8100)

1570-nm NIST-traceable polystyrene size standards (Thermo Fisher Scientific, cat. no. 8150)

R-PE MESF calibration beads (Becton Dickinson, cat. no. 340495)

R-PE MESF calibration beads (Bangs Laboratories, cat. no. 827)

Alexa Fluor 488 MESF calibration beads (Bangs Laboratories, cat. no. 488A)

FITC MESF calibration beads (Bangs Laboratories, cat. no. 555A)

APC MESF calibration beads (Bangs Laboratories, cat. no. 823A)

Alexa Fluor 647 MESF calibration beads (Bangs Laboratories, cat. no. 647)

Anti-human IgG capture calibration beads (Bangs Laboratories, cat. no. 816)

Troubleshooting

If the fit confidence (Fig. 11, Plot A) of the light scatter output is <80% and the majority of bead populations are not within the "Good Fit" portion of Figure 11, Plot C, the light scatter calibration (Basic Protocol 8) will likely lead to inaccuracies when extrapolating diameter for low-refractive-index materials such as EVs. If this is the case, ensure that the particles used to calibrate the light scatter calibration parameter are NIST traceable with respect to diameter, that their refractive index was inputted correctly, that the median statistic for each bead sample was inputted correctly, and that the wavelength for modeling is inputted correctly. If all of these statements are true and the instrument is a cytometer with conventional collection optics, it likely that the alignment of the optical fiber collection

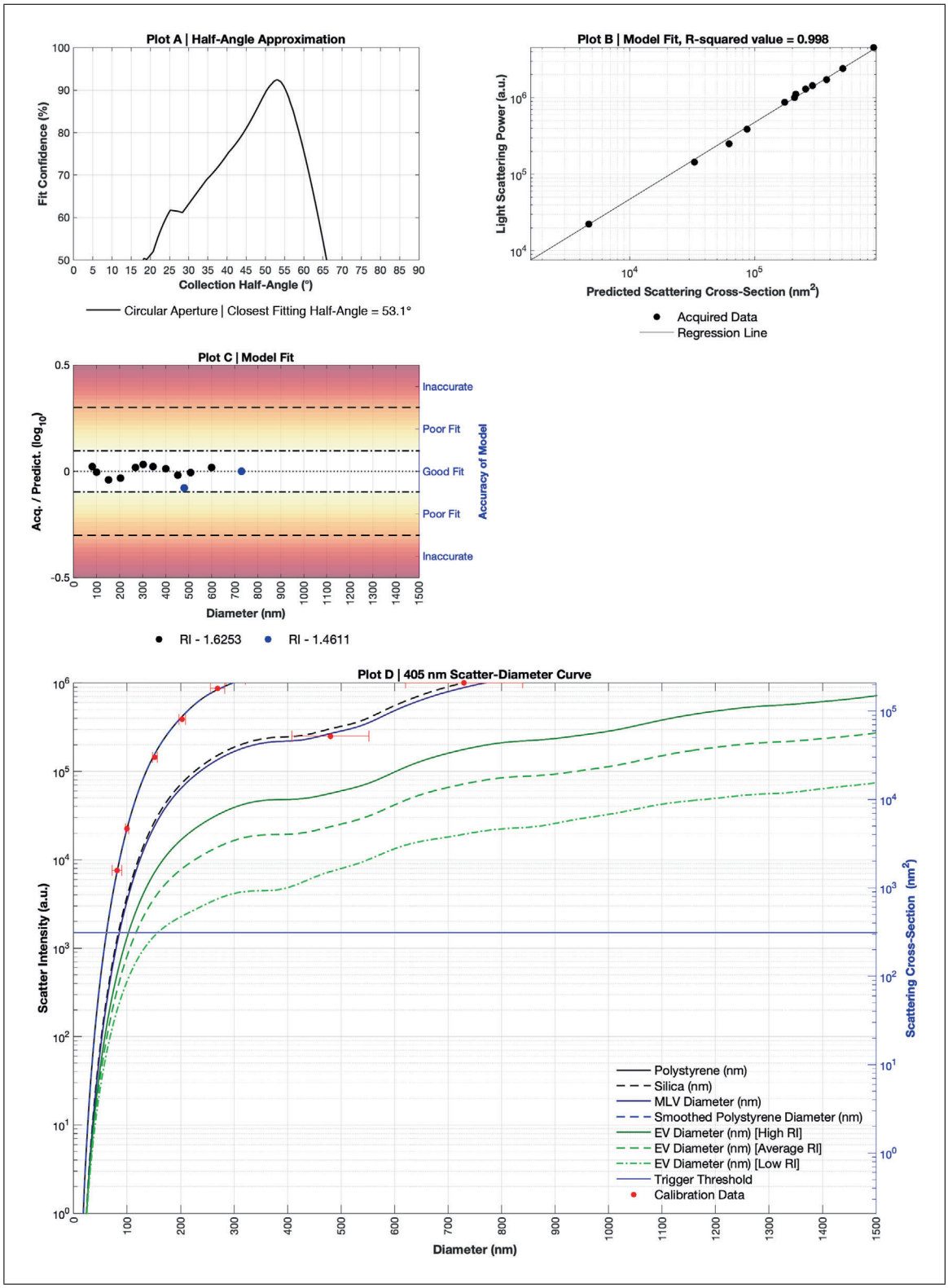

Figure 11 Example of FCM_{PASS} light scatter calibration quality-control plots.

is not quite central to the light being focused from the side scatter collection lens or that the laser beam is not quite perpendicular to the collection lens. Optimal alignment for light scatter modeling should be done using fluorescence so as not to introduce bias from the non-isotropic light scatter distribution of particles ≥100 nm (Welsh et al., 2020). Due to chromatic aberration in the collection optics, the fluorescence signal for

optimal alignment of light scatter should be the closest wavelength, e.g., a 530/30 signal for 488-nm side scatter alignment. Using this channel, the maximum separation between a dimly fluorescent particle and the instrument noise will provide the highest sensitivity for both this fluorescence channel and the similar-wavelength side scatter parameter.

Time Considerations

Basic Protocol 1: Acquisition and gating of light scatter calibration materials: 20 to 40 min.

Basic Protocol 2: Acquisition and gating of fluorescence calibration materials: 10 to 20 min.

Alternate Protocol: Cross-calibration of fluorescence reference materials: 10 to 20 min.

Basic Protocol 3: Cataloguing light scatter calibration materials: 10 min.

Basic Protocol 4: Cataloguing fluorescence calibration materials: 5 min.

Basic Protocol 5: Creating cytometer databases and datasets: 1 min.

Basic Protocol 6: Importing fcs files: 1 to 2 min.

Basic Protocol 7: Fluorescence calibration: 2 to 5 min.

Basic Protocol 8: Light scatter calibration: 2 to 5 min.

Basic Protocol 9: Performing and reporting fcs file calibration: 2 to 30 min.

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Author Contributions

Joshua A. Welsh: Conceptualization; data curation; methodology; software; writing-original draft; writing-review & editing. **Jennifer C. Jones:** Conceptualization; funding acquisition; methodology; supervision; writing-review & editing.

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Key References

Welsh, van der Pol, et al. (2020). See above.

The MIFlowCyt-EV position paper is a critical piece of literature for those considering utilizing single-EV flow cytometry measurements, outlining the critical considerations for performing single-EV flow cytometry experiments, parameters that are recommended as compulsory for reporting, and why there is a need for calibration and for general standardization of the EV literature.

Nolan (2015). See above.

Understanding the differences between cellular flow cytometry and small particle analysis is a necessity when entering the field. This comprehensive article outlines the key messages for users wishing to implement small particle flow cytometry, with a particular focus on EVs.

Welsh, Horak, et al. (2020). See above.

Small particle light scattering characteristics are dependent upon several factors, including illumination wavelength and particle composition. This paper provides a comprehensive outline of how light scatter differs between polystyrene and silica spheres when compared to core-shell models representing EVs. These differences are compared at different illumination wavelengths and cytometer collection angles, and how these differences can be accounted for with light scatter modeling is also demonstrated.

Welsh, Jones, & Tang (2020). See above.

The ability to calibrate both fluorescence and light scatter parameters to allow cross-platform comparisons of signal intensity along with

particle concentration is critical to understand. This article is the first demonstration of simultaneous fluorescence, light scatter, and concentration calibration across flow cytometry platforms and demonstrates the utility of such calibration utilizing the FCM_{PASS} software.

Internet Resources

http://doi.org/10.17504/protocols.io.bjcqkivw

A protocol collection containing up-to-date versions of each protocol due to the potential for software alterations in the future.

https://nano.ccr.cancer.gov

Software updates, background information, and learning resources.

http://evflowcytometry.org

Resources related to MIFlowCyt-EV as a reporting framework along with various educational resources and materials on the ISEV-ISAC-ISTH website.