Aug 05, 2020

FCMPASS Protocol Collection

Joshua A Welsh[1], Jennifer Jones[1]

[1]Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

1 *Works for me* dx.doi.org/10.17504/protocols.io.bjcqkivw

Translational Nanobiology Section

Joshua Welsh

ABSTRACT

This is collection contains the protocols required for each step in the fcmpass software pipeline for performing small particle calibration using the fcmpass software package.

DOI

dx.doi.org/10.17504/protocols.io.bjcqkivw

PROTOCOL CITATION

Joshua A Welsh, Jennifer Jones 2020. FCMPASS Protocol Collection. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bjcqkivw

KEYWORDS

fcmpass, flow cytometry, calibration, EVs

LICENSE

This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

Aug 05, 2020

LAST MODIFIED

Aug 05, 2020

PROTOCOL INTEGER ID

40048

MATERIALS TEXT

FCMPASS software can be accessed at https://nanopass.ccr.cancer.gov.

DISCLAIMER:

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization. By using the FCMPASS software you agree to the following terms and conditions.

Terms & Conditions of use for FCMPASS software.

Definitions: The term “SOFTWARE” throughout this agreement means the machine readable, binary, object code form, and the related documentation for FCMPASS, a software package that is designed to allow flow cytometer calibration for small particles. The term “RECIPIENT” means the party that downloads the software. The term “PROVIDER”

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

means the National Cancer Institute (NCI), a participating institute of the National Institutes of Health (NIH), and an agency of the United States Government.By downloading or otherwise receiving the SOFTWARE, RECIPIENT may use the SOFTWARE subject to RECIPIENT's agreement to the following terms:

1. THE SOFTWARE SHALL NOT BE USED IN THE TREATMENT OR DIAGNOSIS OF HUMAN SUBJECTS. RECIPIENT is responsible for compliance with all laws and regulations applicable to the use of the SOFTWARE.
2. RECIPIENT shall not distribute the SOFTWARE, in whole or in part without express advance written approval of PROVIDER.
3. The SOFTWARE may be used for research, academic, and educational purposes only. The SOFTWARE may not be used for commercial purposes. RECIPIENT will not license or sell or use the SOFTWARE for commercial purposes or applications.
4. The SOFTWARE that is distributed pursuant to this Agreement has been created by United States Government employees. In accordance with Title 17 of the United States Code, section 105, the SOFTWARE isnot subject to copyright protection in the United States. Other than copyright, all rights, title and interest in the SOFTWARE shall remain with the PROVIDER.
5. RECIPIENT shall not modify, extend, decompile, make derivatives of, merge, publish, reverse engineer or distribute the SOFTWAREwithout written permission from PROVIDER.
6. RECIPIENT may publish or otherwise publicly disclose the results of using the SOFTWARE. RECIPIENT agrees to acknowledge PROVIDER's contribution of the SOFTWARE in all written publications containing any data or information regarding or resulting from use of the SOFTWARE.
7. THE SOFTWARE IS PROVIDED "AS IS" AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE AND NONINFRINGEMENT ARE DISCLAIMED. IN NO EVENT SHALL THE PROVIDER OR THE INDIVIDUAL DEVELOPERS BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL, EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES; LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION) HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE. PROVIDER makes no representations that the use of SOFTWARE will not infringe any patent or proprietary rights of third parties.
8. RECIPIENT may, on an as-needed basis, send to PROVIDER reports regarding the application of the SOFTWARE and the effectiveness and problems encountered in using the SOFTWARE, without disclosing RECIPIENT's confidential information. Information from general reports may be used by the PROVIDER to enhance the capabilities of the SOFTWARE. Reports can be forwarded to the PROVIDER at one of the following addresses: joshua.welsh@nih.gov or jennifer.jones2@nih.gov
9. No indemnification for any loss, claim, damage, or liability is intended or provided by either Party under this Agreement. Each Party shall be liable for any loss, claim, damage, or liability that said Party incurs as a result of said Party's activities under this Agreement, except that Provider, as an agency of the United States, assumes liability only to the extent as provided under the Federal Tort Claims Act (28 U.S.C. Chapter 171 Sections 2671-2680).
10. RECIPIENT agrees not to claim, infer, or imply endorsement by the United States Government, or any of its organizational units, contractors or employees. RECIPIENT agrees not to use any trademarks, service marks, trade names, logos or product names of NCI or NIH to endorse or promote products derived from the SOFTWARE without specific, prior and written permission.
11. Title in the SOFTWARE shall remain with the PROVIDER. It is understood that nothing herein will be deemed to constitute, by implication or otherwise, the grant to either party by the other of any license or other rights under any patent, patent application or other intellectual property right or interest. PROVIDER reserves the right to distribute the SOFTWARE to others and to use it for PROVIDER's own purposes. The United States Government explicitly retains all rights to use the SOFTWARE for any purpose, to have it used on the Government's behalf or to allow others to use it.

Acquisition of Calibration Bead Data

1

FCMPASS - Acquisition and gating of light scatter reference materials

PREVIEW RUN

by Joshua Welsh,
Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

1.1 Calculate the stock traceable size calibration reference bead particle concentration using percent solids value and particle density provided by the manufacturer and the following formula, whereis the concentration (particles mL^{-1}),, is the percent solids,is the particle density (g mL^{-1}), andis the average diameter (µm).

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

For example, 100 nm polystyrene beads at 1% with 1.05 g mL^{-1}would be calculated using:

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

1.2 Thoroughly vortex the traceable size calibration reference bead stock bottles to homogenize the mixtures before dispensing 1 drop (~50 µL) into separate 500 µL low-protein binding Eppendorf.

1.3 Using the working stock from step 2, make up 500 µL solution at $1x10^7$ particles mL^{-1}.

It is recommended that serial dilutions are used and volumes of no less than 10 µL 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.

1.4 On the flow cytometer, set the triggering threshold to the most sensitive light scatter detector and ensure the parameter is using log-scaling (not linear or biexponential).

1.5 Running DPBS, lower the triggering threshold until the noise floor of the instrument becomes visible. This is most clearly when using a histogram.

1.5.1 Plotting the trigger-channel height parameter against time and monitoring while running DPBS is a good indication for determining 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.

There are a number of cleaning solutions. Some shared resource lab managers have a preference. These include bleach, contrad 70, micro 90, surfanol

1.5.2 The extent to which the opto-electronic noise of an instrument can be sampled will vary between

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

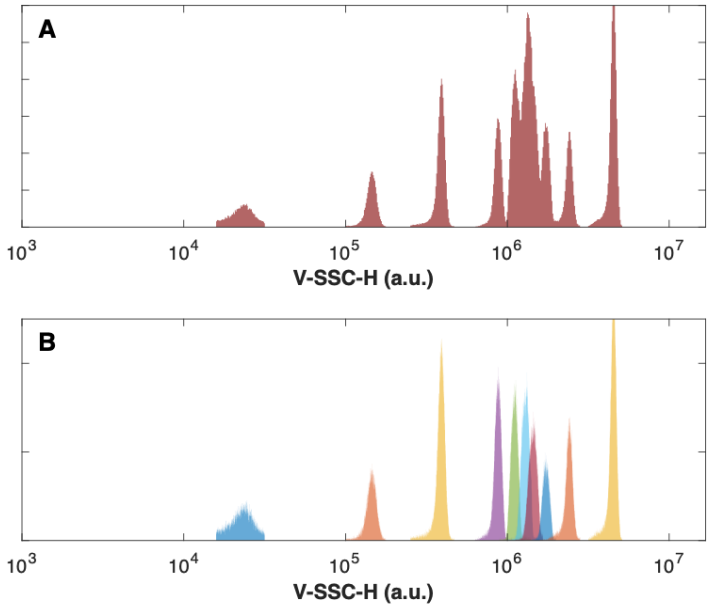
instruments. Legacy flow cytometers will tolerate a couple of 1000-2000 events/second whilst allowing room to sample desired events, while high-speed jet-in-air sorters are capable of sample 10,000+ events per second.

1.5.3 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. The 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 small particles are detected, and 3) the degree to which the opto-electronic noise can be sampled without creating artefacts or reducing the ability to detect genuine events.

1.5.4 On some instruments that utilize peristaltic pumps there can appear to be an increase and decrease of the baseline corresponding to the turnover of the pump. This is a result of the threshold being set close to (but above) the electronic noise, resulting in the 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 increasing the threshold and therefore decreasing the instrument's limit of sensitivity.

1.6 Analyze each bead sample at the same acquisition settings until >5000 bead events are recorded.

1.6.1 It is preferable to analyze and store bead populations individually. This will minimize population overlap, aggregates, background noise, and artifacts.

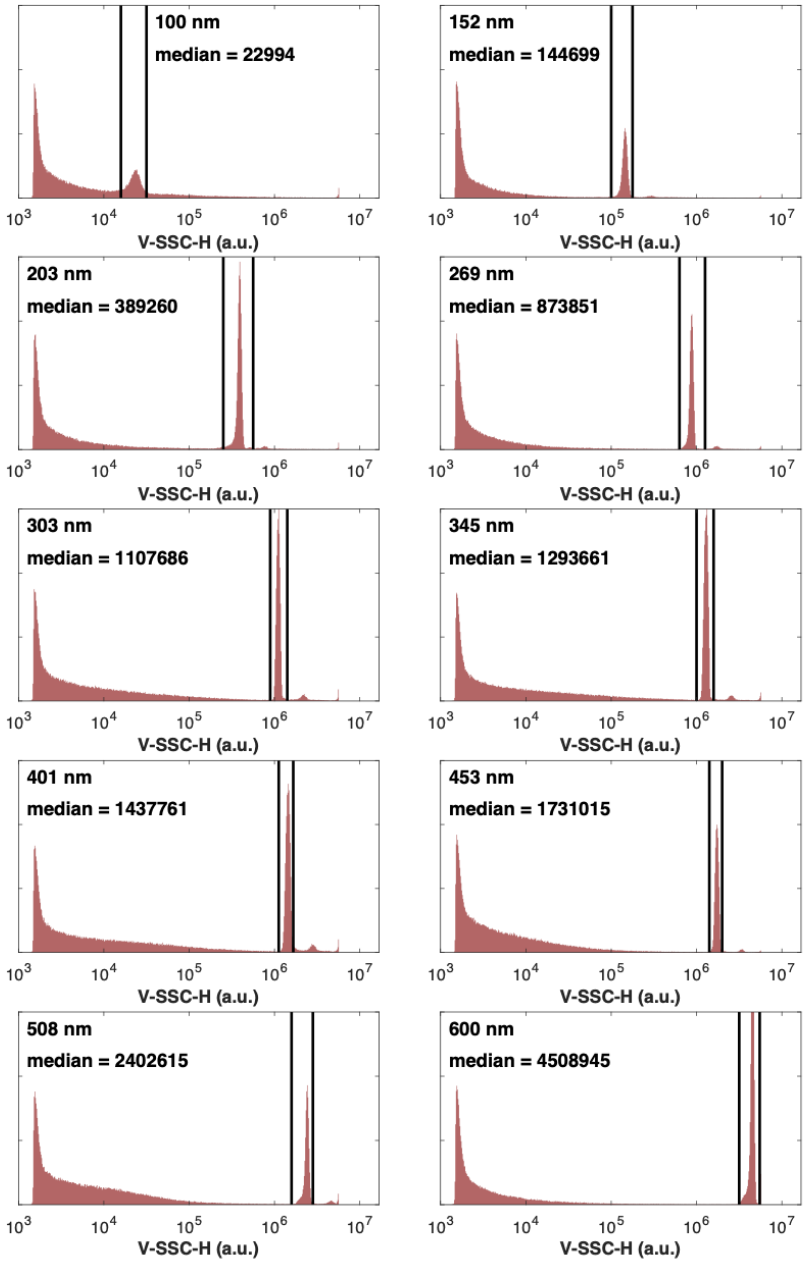


Panel A demonstrates the cumulative distribution of the gated populations when mixed together. While some populations are clearly distinguished some are not. The areas where bunching of populations occurs is dependent upon the cytometer and is useful in determining the collection angle. Panel B illustrates overlaid and colored gated bead population from Oanel A.

1.7 Gate each bead population using the parameter Height vs. Area in a dot-plot to remove doublets/aggregates and then use a histogram on the light scatter parameter (Height) to obtain statistics for each population. The light scatter parameter should use log scaling.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



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

1.8 Obtain the median statistic for each of the bead populations.

1.8.1 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 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 therefore a more reliable method for extrapolation.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

2

FCMPASS - Acquisition and gating of fluorescence reference materials
PREVIEW RUN

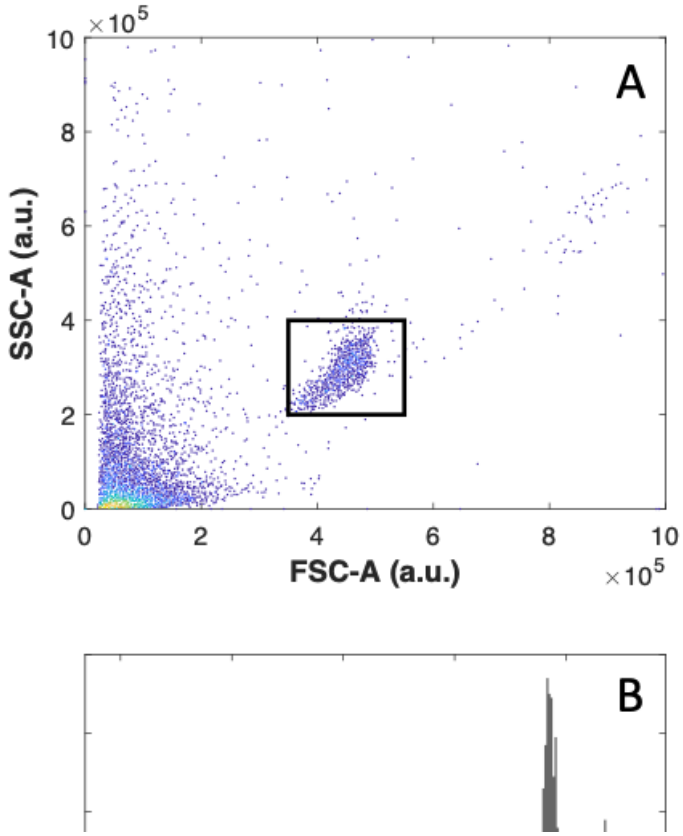
by Joshua Welsh,
Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

2.1 Vortex each fluorescence reference bead bottle before use.

2.2 Add 1 drop (~50 µL) of each bead population to separate FACS tubes containing 250 µL of DPBS.

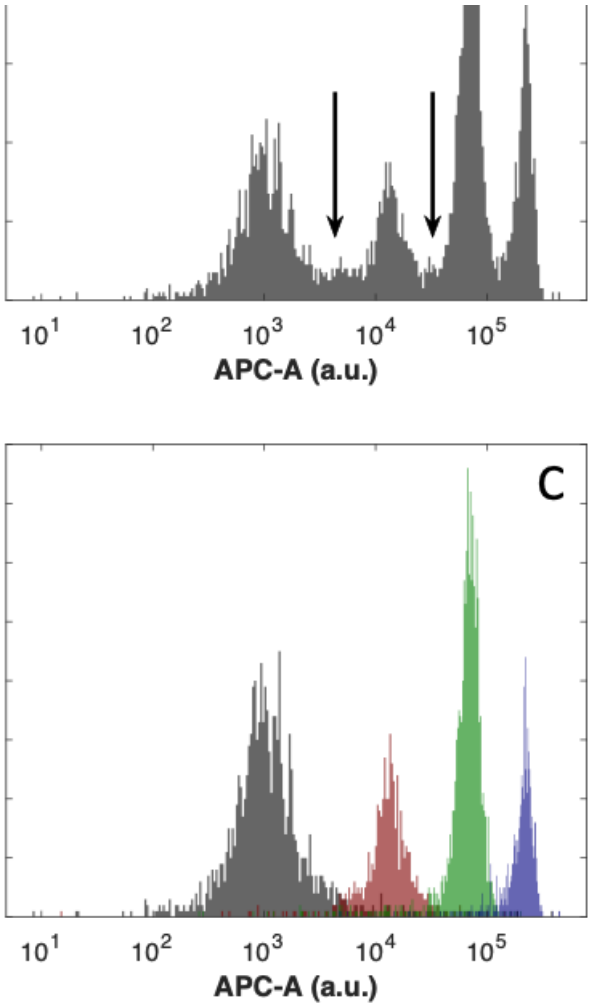
2.2.1 Due to the high autofluorescence of the 'Blank' beads, their use is not recommended to use as 0 MESF.

2.2.2 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 a number of factors including the gating of the populations. While less ergonomic, it is preferable to analyze 1 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.



Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



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

2.3 Ensure cytometer fluorescence settings are those used for small particle analysis.

2.4 If the beads are >1 µm in diameter the use of a forward-scatter trigger threshold will likely yield optimal detection and reduced background.

2.5 Analyze each bead sample at the same acquisition settings until >5000 bead events are recorded.

2.6 Gate each bead population on FSC-A vs. SSC-A and obtain the median area statistic for the fluorescence parameter being calibrated to move on to the 'FCMPASS Fluorescence Calibration' protocol.

2.6.1 By default, flow cytometers trigger the acquisition of an event using the pulse height parameter. In

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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 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.

2.7 Vortex the rainbow multi-peak reference bead and MESF bead bottles before use.

2.8 Add 1 drop (~50 µL) of each MESF bead population to separate FACS tubes containing 250 µL of DPBS.

2.9 Add 1 drop (~50 µL) of the 8-peak bead population to separate FACS tubes containing 250 µL of DPBS.

2.10 Ensure cytometer fluorescence settings are those used for small particle analysis

2.11 If the beads are >1 µm in diameter the use of a forward-scatter trigger threshold will likely yield optimal detection and reduced background.

2.12 Analyze each bead sample at the same acquisition settings until >2000 bead events are recorded. For the 8-peak beads this will be >16,000 events.

2.13 Gate each MESF bead population on FSC-A vs. SSC-A and obtain the median statistic for the parameter and perform calibration of the MESF and 8-peak bead files.

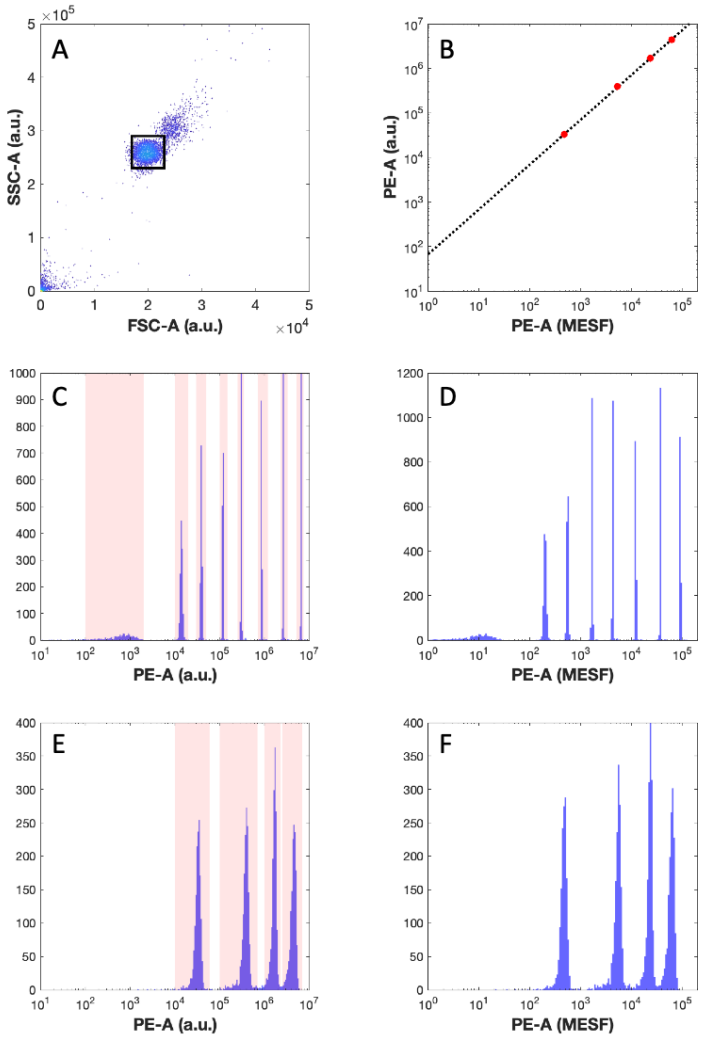
2.14 Once the 8-peak rainbow beads are calibrated in PE MESF units, gate the population on FSC-A vs. SSC-A to obtain singlets. Using the singlet population gate each of the 8-peak populations.

2.14.1 The gating the individual fluorescent bead populations can be done in the parameter which best separates each population. This may be a different fluorescence detector than the calibrated parameter. While the gating of each population does not have to be on the MESF parameter itself, the MESF parameter should be checked to ensure all populations are on scale. In some 3rdparty software the scale limits (minimum and maximum value) will influence the outputted statistic due to how the data is binned.

2.15 Once each of the 8-peak populations has been gated, obtain the 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.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



Gating and cross-calibration of fluorescence reference beads. *A) Gating of 8-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 (Panel A) 8-peak reference beads and gating of each population (red). D) Histogram of gated (Panel A) 8-peak reference beads converted to PE MESF units using regression (Panel B). E) Histogram of gated PE MESF beads and gating of each population (red). F) Histogram of gated PE MESF beads converted to PE MESF units using regression (Panel B).*

2.15.1 If an instrument is re-aligned or filters are changed these values will no longer be valid and will require cross-calibration to be performed again. In general, it is good practice to regularly cross-calibrate 8-peak bead reference values, e.g. once a month.

Cataloguing Reference Beads

3

FCMPASS - Cataloguing light scatter reference materials

by Joshua Welsh,

Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

PREVIEW RUN

3.1 Open FCM$_{PASS}$.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

FCMPASS 3

3.2 Click ‘Catalogue’ in the top menu bar

3.3 Under the ‘Light Scatter’ tab entry fields exist for each of the pertinent metadata for reporting with light scatter calibration.

3.3.1 Diameter CV should be the percent coefficient of variation of the mean diameter provided on the certificate of analysis

3.3.2 Refractive Index should be the provided refractive index of the bead population on certificate of analysis

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

3.3.3 ‘RI Measurement Wavelength’ is the wavelength at which the refractive index was measured and should be provided on the certificate of analysis. This tends to be 589 nm.

3.3.4 Composition can be selected as polystyrene, silica, or other. If polystyrene or silica are 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 propositionally to the sheath refractive index.

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

3.4 Once the fields have been completed for a bead population click ‘Add Bead’. The population should then appear in the table below.

3.5 Once the relevant beads have been added ‘Bead Sets’ can be created. A bead set are the bead populations that are used for calibration. Any number of bead sets and combinations can be made.

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

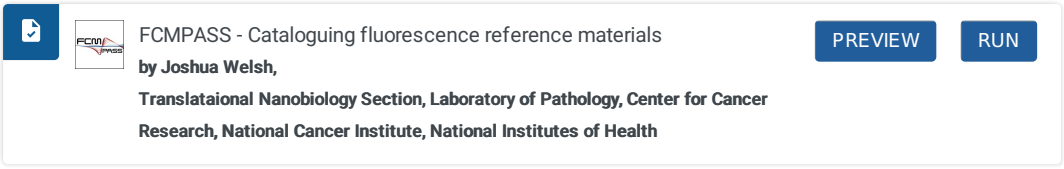
Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

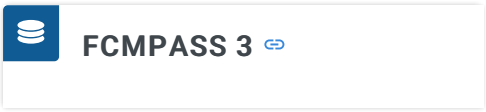
3.5.2 Click ‘Create Set’, enter a unique Set name, and click ‘OK’.

3.6 Once your bead set has been defined you will be able to perform light scatter calibration.

4



4.1 Open FCMPASS



4.2 Click ‘Catalogue’ in the top menu bar

4.3 Under the ‘Fluorescence’ tab entry fields exist for each of the pertinent metadata for reporting with fluorescence calibration.

4.3.1 Enter the name of the fluorophore on the fluorescence reference beads.

4.3.2 Enter the manufacturer, catalogue number, and lot number fields appropriately.

4.3.3 In the ‘Bead Ref Values’ field enter each fluorescence beads reference values. This may be in molecules of equivalent soluble fluorophore, equivalent reference fluorophore, or antibody binding capacity.

4.3.4 After each reference value click the ‘+’ button.

4.3.5 Once all fields and reference values have been added click ‘Create Set’. The beads will then appear on the table below and will be available for selection when performing fluorescence calibration.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Creating Cytometer Database and Datasets

5

FCMPASS - Creating a cytometer database and datasets
by Joshua Welsh,
Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

PREVIEW RUN

5.1 Open FCM_{PASS}.

FCMPASS 3

5.2 Click the '+' icon next to 'Cytometer IDs' list and enter a unique name to identify a instrument.

5.3 Select the relevant cytometer ID to add the dataset to

5.4 Click the '+' icon next to the 'Datasets' list.

5.4.1 In the window enter the acquisition date of the calibration data and the dataset/experiment name. If there are any notes related to the experiment that are beneficial, they can be entered in the 'Dataset Notes' field.

6

FCMPASS - Importing fcs files
by Joshua Welsh,
Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

PREVIEW RUN

6.1 Once a dataset has been created click the 'Begin Calibration' button.

6.2 Import fcs files by selecting the '+' icon next to the 'Files to calibrate' table.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

6.2.1 In the new window navigate to the folder containing the fcs files you wish to calibrate and select ‘OK’.

6.3 The fcs files and related metadata will now be imported.

6.3.1 If the folder contains fcs files that you do not wish to be calibrated, select them and click the ‘–‘icon. The metadata related to the remaining files will then be reprocessed.

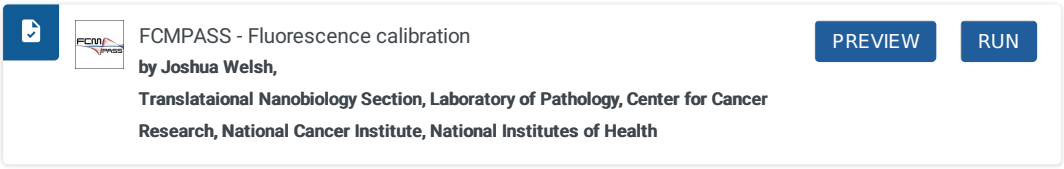
6.3.2 The parameters e.g. SSC-H, SSC-A that are available in further steps of the software are those that are common to all the loaded fcs files. If files that are loaded do not have any common parameter names a selection will not be available in these steps.

6.4 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.

6.5 Once completed select ‘Next’.

Fluorescence and Light Scatter Calibration

7



7.1 If fluorescence calibration is being performed click the '+' button to add a calibration parameter to the table. If fluorescence calibration is not required, click 'Next'.

7.1.1 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 as per the protocol.

.

7.2 Once a parameter is added double click the ‘Reference Fluorophore’ item and select the bead set used for calibration. The displayed sets are those that have previously been added to the Catalogue.

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

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

7.5 The reference bead values for the selected parameter should appear in the 'Regression Values' table.

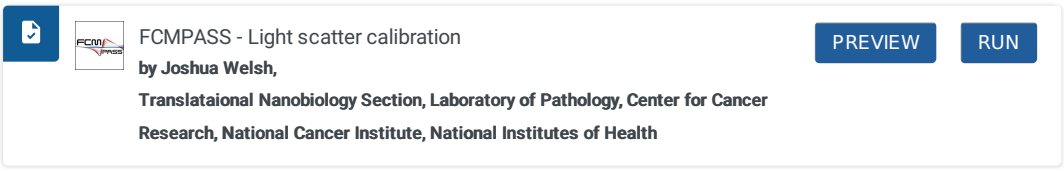
7.6 Click in the 'Acquired Value' box next to each bead reference value and input the acquired statistic

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

7.8 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 an fluorophore:protein ratio or alter the regression method between linear, log, weighted linear, weighted log.

8



8.1 If light scatter calibration is being performed click the '+' button to add a calibration parameter to the table. If light scatter calibration is not required, click 'Next'.

8.1.1 If you have not yet defined the light scatter bead sets in Catalogue', click 'Catalogue' and complete as outlined in the protocol.

8.2 Double click the 'Scatter Parameter' field to change which parameter is being used for light scatter calibration.

8.3 Alter the '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

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

reference bead, core-shell model, and homogenous sphere model refractive indices will all also be updated.

8.4 If the selected 'Scatter Parameter' was used as a triggering threshold then the 'Scatter Threshold' field will automatically update to show the values used as thresholds in the .fcs files loaded. Select a 'Scatter Threshold' by double clicking the field and selecting and option from the dropdown menu. A custom entry can also be inputted.

8.5 Load the light scatter reference beads used by double clicking the 'Bead Set' field. Once loaded the beads within the set will populate the bottom table.

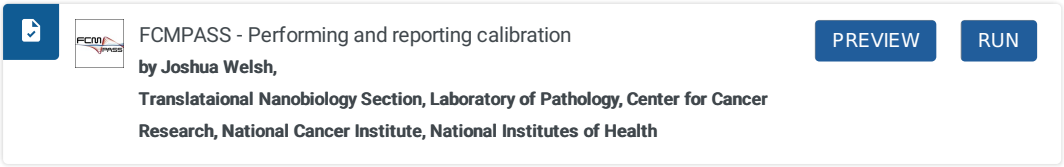
8.6 The 'Sheath RI' field automatically accounts for 'Scatter Wavelength' but can be updated manually by double clicking the field.

8.7 In the bottom table enter the median scatter parameter statistic for each population. The acquired CV can optionally also be completed, its use will, however, only be used for plotting purposes and not alter the model calculations.

8.8 Once complete click 'Next'.

Custom core-shell models, solid sphere models, plot data points, modelling 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



9.1 Upon completing fluorescence and/or light scatter calibration steps click the 'Calibrate' button.

9.2 The FCMPASS software will now perform fluorescence and light scatter calibration. An FCMPASS 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 complete and supplementary sheets for reproducing the calibration. A calibration output report file will also be generated that contains the relevant figures to support the fluorescence calibration and light scatter calibration that was performed. All of these files should be kept together when shared.

9.3 The remaining fields within the MIFlowCyt-EV report should be completed as recommended in the associated position paper.

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Citation: Joshua A Welsh, Jennifer Jones (08/05/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bjcqkivw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited