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FCMPASS Protocol Collection

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1 Works for me dx.doi.org/10.17504/protocols.io.bhv3j68n

Translational Nanobiology Section

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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

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PROTOCOL CITATION

Joshua A Welsh, Jennifer Jones 2020. FCMPASS Protocol Collection. **protocols.io** dx.doi.org/10.17504/protocols.io.bhv3j68n

KEYWORDS

fcmpass, flow cytometry, calibration, EVs

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PROTOCOL INTEGER ID

38555

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”

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Cataloguing Reference Beads

1

FCMPASS - Cataloguing light scatter reference materials

by Joshua Welsh,

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PREVIEW RUN

Citation: Joshua A Welsh, Jennifer Jones (08/03/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bhv3j68n

1.1 Open FCM_{PASS}.

FCMPASS 3

1.2 Click ‘Catalogue’ in the top menu bar

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

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

1.3.2 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.

1.3.3 ‘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.

1.3.4 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.

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

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

1.5 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.

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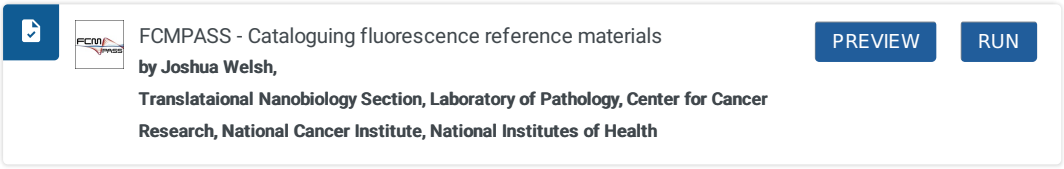
1.5.1 In the ‘Selection’ column of the table, check all the bead populations to be included within a bead set.

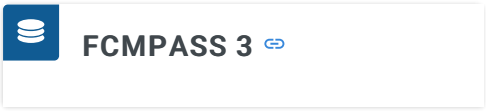
1.5.2 Click ‘Create Set’, enter a unique Set name, and click ‘OK’.

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

2



2.1 Open FCMPASS



2.2 Click ‘Catalogue’ in the top menu bar

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

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

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

2.3.3 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.

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

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2.3.5 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.

Creating Cytometer Database and Datasets

3

FCMPASS - Creating a cytometer database and datasets
by Joshua Welsh,
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PREVIEW RUN

3.1 Open FCM$_{PASS}$.

FCMPASS 3

3.2 Click the ‘+’ icon next to ‘Cytometer IDs’ list and enter a unique name to identify a instrument.

3.3 Select the relevant cytometer ID to add the dataset to

3.4 Click the ‘+’ icon next to the ‘Datasets’ list.

3.4.1 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.

4

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

4.1 Once a dataset has been created click the ‘Begin Calibration’ button.

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

4.2.1 In the new window navigate to the folder containing the fcs files you wish to calibrate and select 'OK'.

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

4.3.1 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.

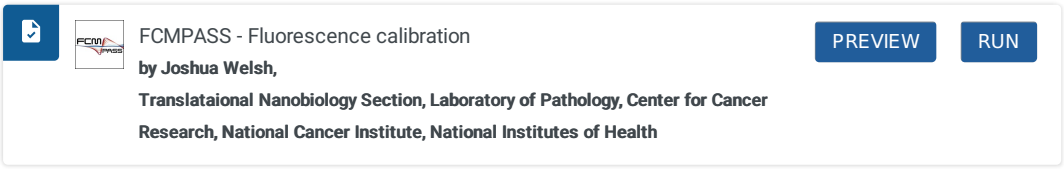
4.3.2 The parameters e.g. SSC-H, SSC-A that are available in further steps of the software are those that are common to all the loaded fcs files. If files that are loaded do not have any common parameter names a selection will not be available in these steps.

4.4 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.

4.5 Once completed select 'Next'.

Fluorescence and Light Scatter Calibration

5



5.1 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'.

5.1.1 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.

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5.2 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.

Citation: Joshua A Welsh, Jennifer Jones (08/03/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bhv3j68n

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

5.4 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.

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

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

5.7 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.

5.8 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.

6

FCMPASS - Light scatter calibration

PREVIEW RUN

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6.1 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'.

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

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

Citation: Joshua A Welsh, Jennifer Jones (08/03/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bhv3j68n

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

6.4 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.

6.5 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.

6.6 The ‘Sheath RI’ field automatically accounts for ‘Scatter Wavelength’ but can be updated manually by double clicking the field.

6.7 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.

6.8 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.

7

FCMPASS - Performing and reporting calibration

by Joshua Welsh,
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PREVIEW RUN

7.1 Upon completing fluorescence and/or light scatter calibration steps click the ‘Calibrate’ button.

7.2 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.

Citation: Joshua A Welsh, Jennifer Jones (08/03/2020). FCMPASS Protocol Collection. https://dx.doi.org/10.17504/protocols.io.bhv3j68n

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

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