### General remarks

The NanoFCM software cannot display two fluorescent channels in combination with the particle size based on SSC signal. Here, we present a work-around solution for FlowJo.

I.) Generate the standard curve based on the S16M/S17M bead recording

1) Open the recording of S16M/17M beads in the NanoFCM software

2) Change to **SS-A** in the x-axis of the dot plot (default is SS-H but you have usually better resolution with the area signal)

3) Click on **Auto Threshold** button and select the "Small Signal" option

4) Click on the **Size MESF** button

5) In the new window select **Standard** (S16M or S17M) and click on **Find peak** button. "Width Min" and "Threshold" options can be adapted if peaks are not recognized correctly

6) Do a quick check of the standard curve for correct appearance. Next, copy the displayed formula and save it in a txt file

   In this example: *y = 7.6347E-7*x^(5.03099)+0*
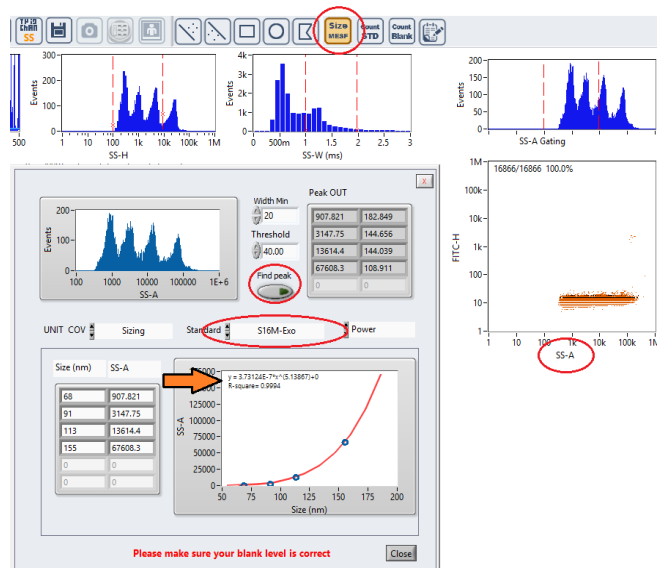
II.) Export data as FCS files

1) **Select NFA** data file to be exported

2) **Adjust threshold** (note that only data above threshold will be exported to the FCS file!)

3) Click **Save** button and select FCS 3.0 as format

➢ Repeat previous steps for all NFA files you want to export

III.) Generate size (nm) parameter in FlowJo

1) Open FlowJo and **import the FCS** files by drag & drop

2) Select the first file

3) Go to **Tools → Derive Parameters** to open the transform window

4) Change the parameter name in the transform window

5) Insert following formula in the formula field

   (<Param name="SS-A" /> /(X.XXX*10^-7))^(1/Y.YYY)

   Replace the colored section with the numbers from the formula you got from the NanoFCM standard curve.

   In our example: **y = 3.73124E-7*x^(5.13867)+0**

6) Decrease minimum & maximum range of the data to optimize the plot scaling

7) Apply the transformation to all files by dragging

   SS-A in nm   onto   {⫶} All Samples

IV.) What extra functionality do we get from this?

➢ All 3 available channels can be displayed (SSC, FITC, PC5) in any order.

➢ No more limitations on the number of gates.

➢ You can display subgates as shown here for an EV example labelled with CD63-GFP and stained for CD81. Note that the size distribution of EVs can be displayed for each gate by the generated "SS-A in nm" parameter. Interestingly, the GFP/CD81+ population seems to have an increase in the median EV size compared to the CD81+ only population…

Median:	64.5 nm	69.2 nm	60.4nm