

Time Delay Calibration

Purpose: The second laser on the FACSCalibur generates independent data from the first laser and the machine must associate the two datasets accurately in order to provide correct profiles for each cell. This is done by calibrating the time it takes for a cell to pass from the first laser to the second laser. If the machine knows exactly how long this takes, it will be able to associate the data from the first laser with the second laser, thus the name "time delay calibration". There is no doubt that the machine can drift and it may be out of calibration. Experience has shown that this will generate artifactual data. In addition, if the machine cannot calibrate, this is an indication of a serious problem, which should be corrected before you acquire data. **Therefore, it is recommended you perform this every time you intend to use the FL4 channel.**

Description: The machine uses beads that fluoresce in channel 4 (e.g. Calibrite APC Beads). When they pass by the first laser, they generate forward scatter signals and when they pass by the second laser they generate fluorescence signals. The machine adjusts its circuitry until these two signals are correlated.

Materials:

APC Calibrite beads
Sheath fluid or PBS
FACS tubes

Procedure

1. Shake the tube containing beads well- this is essential as beads settle rapidly.

2. Add 0.5ml of sheath/PBS to a FACS tube and then add one drop of beads and mix.
3. On the FACS machine, open the file "Time Delay Calibration" from the desktop.
4. Make sure the cytometer is connected to Cellquest (Acquire-Connect to Cytometer).
5. Open the following windows: Detectors/Amps, Threshold, Compensation and Counters (Hold the Apple key down and then press 1, 2, 3, and 4).
6. Make sure Four Color checkbox is selected at the bottom of the Detectors/Amps window. The P7 parameter should change to FL4 in the Detector column. P7 should be set to "Log" mode not "Lin". You will need approx. 700-800 on the voltage setting.
7. In the Threshold window, make sure Primary Parameter is FSC-H (using "radio button"). Set the threshold for 200 using the slider pop up.
8. Start the APC beads running, first on low pressure. In the Acquisition panel, Setup should be checked: click Acquire. You may not see anything at first. In the Detectors/Amps window, set the FSC gain up to around 6-7 or whatever it takes to see events on the histogram. Note on the Time Delay Calibration document window that there is a Histogram Statistic box. Adjust FSC gain until the entire peak is above 400.
9. Now increase the pressure to medium and then high. Note the shape of the histogram. It should not change much and should not spread its width on high power. If it does, check for pressure leaks at the buffer supply area or at the tube attachment to the cytometer. If you are only getting a narrow spread on low or medium, you will have to complete the Calibration on this pressure and will have to run your experiment only at the lower pressure. The event rate (in the Counters window) must be at least 400 under the

pressure you are using. If not, mix the beads well again and add another drop or remix the beads in the tube.

10. Scroll down the Time Delay Calibration window to see the second histogram clearly. It is of the FL4 channel. Now, adjust the gain so that the mean peak in the M1 gate is at the "target value" of the beads +/- 5. This can be seen on the label that is attached to the box. It is likely that a value of 800 +/- will be sufficient.
11. From the Cytometer Menu, choose Time Delay Calibration. After a few seconds, the computer will beep and there will be no dialog box. It only gives you feedback when there is a problem (i.e. signal out of range).
12. If you didn't get an error, congratulations. You can now go on to set up the rest of the machine using either beads, cells or both.

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