*quanti*Flash

User Manual

*quanti*Flash®
User manual

Inhaltsverzeichnis

1. Introduction to the *quanti*Flash®

In a flow cytometer, fluorescently labeled cells or beads are first and foremost a source of light. The light is emitted from a cell as it passes through the laser beam in the focus of the cytometers' flow cell. As it quickly flows with high speed through the flow cell its emitted light is recognized by the detectors as short light pulses.

By replacing those bead-based light pulses with highly stable and precise light pulses generated artificially, reproducible calibration routines become available and open the door for accurate detector setup, calibrated intensity readings and possible cross-instrument standardization.

The *quanti*Flash® is a precision LED based light source that is ideal for instrument independent quality control in flow cytometry. It enables the user to calibrate the intensity scale of each channel in a flow cytometer to a meaningful absolute unit which is "number of detected photo electrons". By this a direct quantitative comparison between different devices and different manufacturers becomes possible. Using a calibrated intensity scale makes it easier to transfer a measurement from one flow cytometer to another one and it improves the comparability of the results obtained from different flow cytometers.

The *quanti*Flash® generates simulated high precision pulsed light signals that are collected by the instrument in a manner similar to the light from fluorescent events emanating from the flow cell. As the light pulses delivered by the *quanti*Flash® are independent of the presence of fluorescent particles this device can be used to directly measure the noise generated within the instrument when there are no specific fluorescent particles in the laser beam.

The light pulses provided by the *quanti*Flash® are fiber coupled making it easy to direct them into the flow cytometer. The light pulses are directed to either one or

simultaneously many of the fluorescence detectors. This can be done by simply injecting the light pulses at the collection optics of the flow cytometer e.g. at the flow cell, into a filter block directly, or directly into a fluorescence detector via the respective filter holder.

The variable amplitude light pulses are controlled to within 0.1% of their set amplitude, markedly outperforming all currently available calibration particles. They have a dynamic range of over 5 decades and can be precisely tuned. To extend the dynamic range even further a separate set of fixed optical attenuator is available as accessories.

Further the *quanti*Flash® provides a second independent light channel that can be used for triggering.

2. Connecting the *quanti*Flash to your flow cytometer

The high quality light pulses are delivered from the *quanti*Flash to the flow cytometer by means of two optical fibers. This provides easy handling and reproducible measurement conditions. These light pulses emitted from the trigger and fluorescence LEDs now have to be directed to the various photodetectors in the flow cytometer. The trigger light pulses can be directed into either the forward scatter or the side-scatter detectors, or alternatively to one of the fluorescence detectors. The fluorescence light pulses are directed to either one or simultaneously to the many fluorescence detectors by injecting the light pulses at the collection optics of the flow cytometer e.g. at the flow cell, into a detector block, or directly into a fluorescence detector via the respective filter holder.
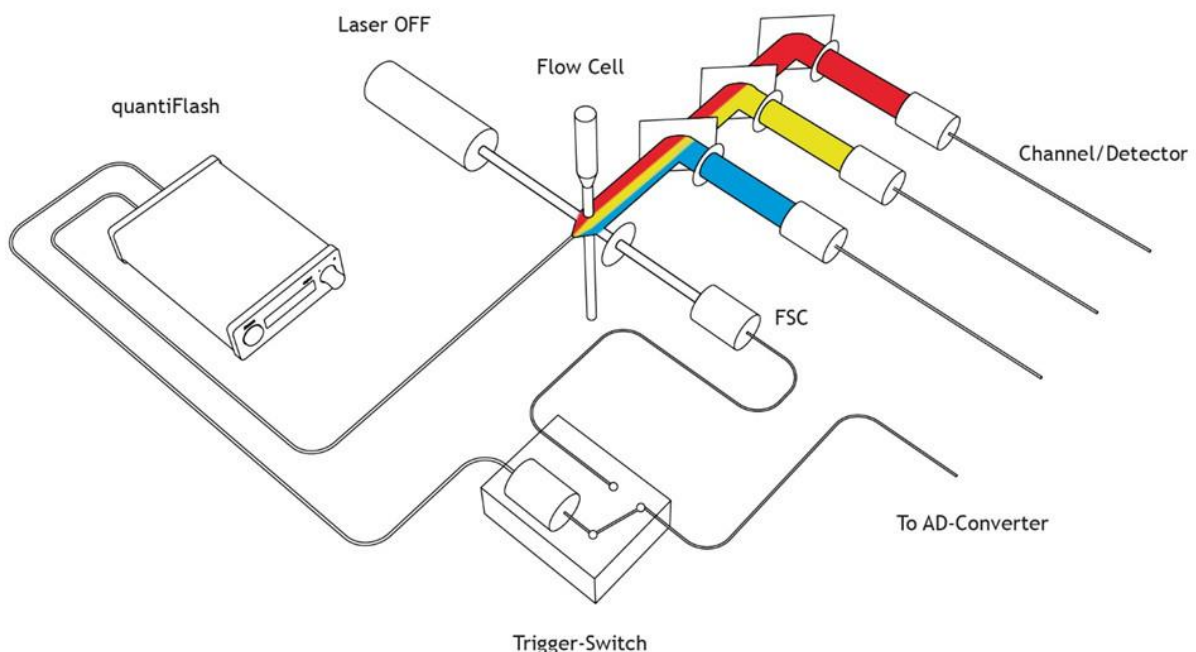
For ease of use a set of two adapters is available to connect the fibers in a stable way to the flow cytometer.

*quanti*Flash®
User manual

3. Setting up your *quanti*Flash

Cells passing through the excitation laser in the flow cell will emit short pulses of light, either scattered laser light while the cell is in the laser of fluorescence signals from the markers/labels on the cell. The *quanti*Flash® is a perfect tool to mimic these light pulses in a well-controlled and highly stable manner.

The *quanti*Flash® is equipped with a digital waveform generator, enabling the user to fully control various parameters of the emitted light pulses. These parameters are the following:

- Pulse amplitude
- Pulse duration
- Repetition rate of the light pulses
- Pulse shape

The first two parameters are the most basic and most important parameters. They are accessible for every *quanti*Flash® by default. The repetition rate of the light pulses defines the event rate of light pulses measured with your cytometer.

The basic version of the *quanti*Flash® allows a limited set of pulse shapes. To also program individual user defined pulses an upgrade to the PC software *quanti*Flash® Pro is required.

3.1. Pulse amplitude and pulse duration

Typically the pulse amplitude is defined as the height of the pulse above the baseline, which is depicted in figure 1. Instead of amplitude of the light pulses one could also speak of the brightness of the light pulses or the amount of light emitted. This definition is identical to the height signal given by any flow-cytometer.

For the *quanti*Flash® the pulse duration is always calculated as the "full width at half maximum" (FWHM).
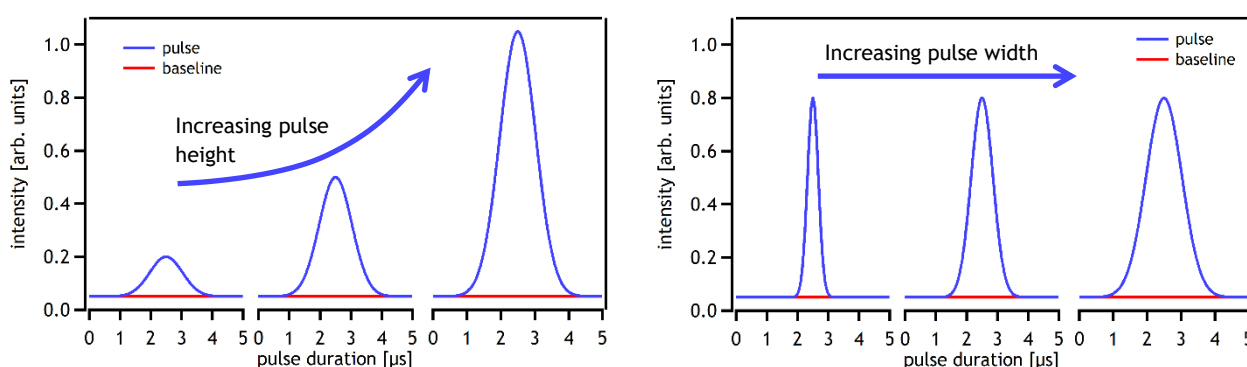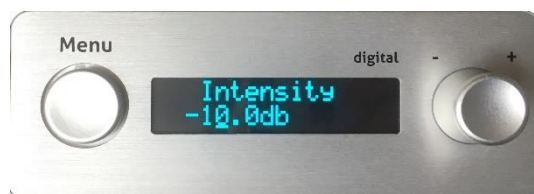

Figure 1: Examples of pulses with varying pulse height and width

To change or set any of the parameters defining the output of the *quanti*Flash® such as:

- amplitude or height of the light pulses

- repetition rate or event rate

simply choose the corresponding menu with the "Menu" button on the front panel and set the desired value by turning the dial on the *quanti*Flash®.

Turn dial to set Value of the activated parameter

Press dial to change between toggle mode and curser mode of ◀▶ (relevant for Pulse duration menu)

Use navigation keys left/right to move the cursor to the desired position (increasing or decreasing the increment of the dial) to toggle through the options of the submenu

Use navigation keys up/down to select the desired setup menu

Intensity — Use dial to change the output intensity of the LED pulses

Count rate — Use dial to change the event rate (Default is 1000 events/s)

Pulse duration — Use ◀▶ to change pulse duration 2us ◀▶ 5us ◀▶ custom

Pulse shape — Use ◀▶ to change pulse shape Standard ◀▶ Gaussian ◀▶ Flat top

Trigger intensity — Use dial to change trigger intensity

Intesity mode — Use ◀▶ to toggle between logarithmic and linear mode

Battery status — Information on battery status

Reset timer — After indicated time without interaction the device will return to intensity menu

Alternatively if one is using the PC software set the desired intensity in the "Home" menu.
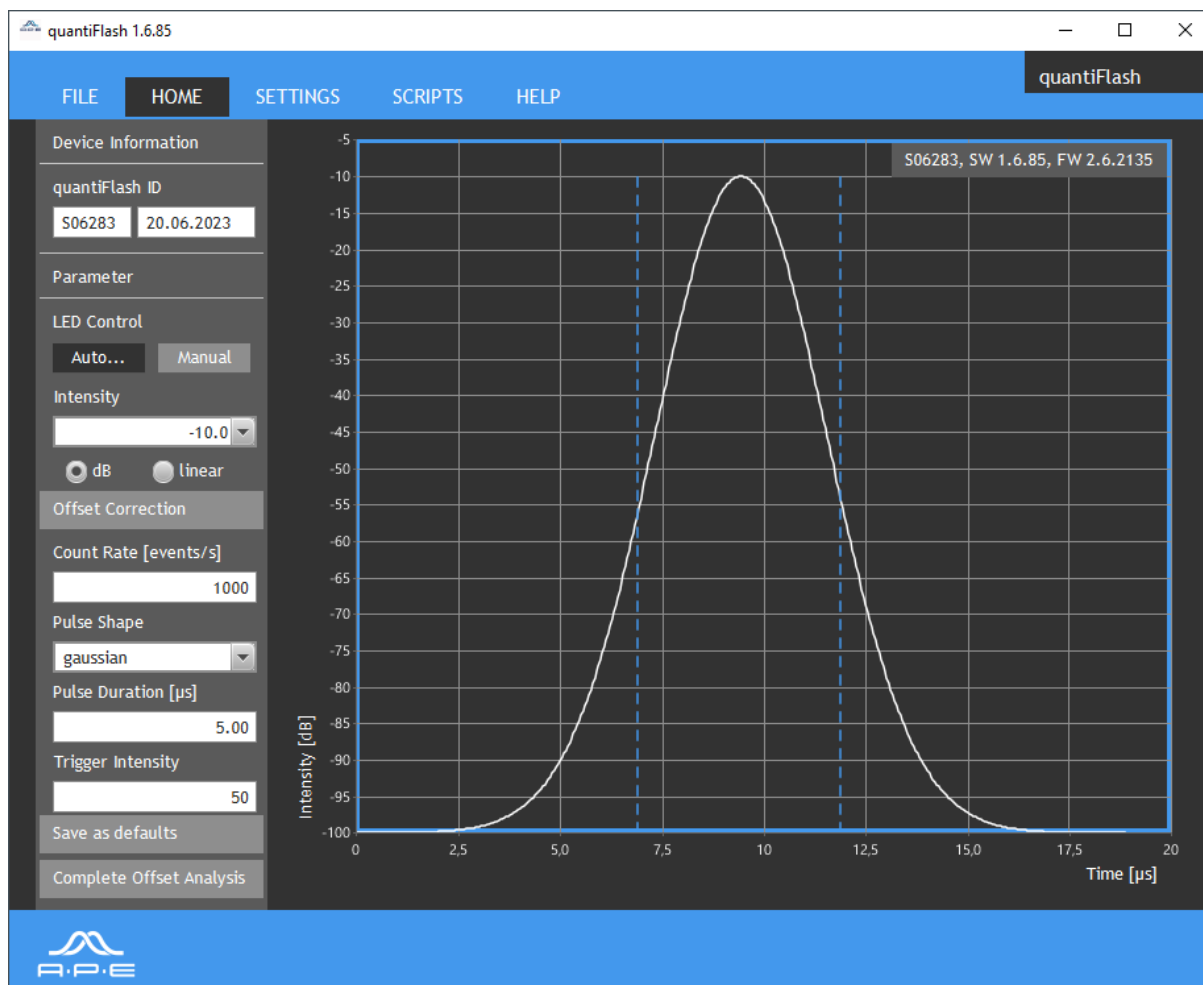
quantiFlash®
User manual

Figure 2: GUI of the quantiFlash user software

Typically the quantiFlash will be used in automatic mode. To set the intensity this mode one can choose between a logarithmic scale in dB or a linear scale showing the relative attenuation as a fraction of the maximum intensity.

Note: the attenuation in dB is calculated using the formula

$$I_{dB} = 10 \log(I / I_0)$$

Earlier versions of the quantiFlash where using a different formula. The intensity range didn't change, just the displayed numbers

	Linear scale	dB scale	historic dB scale
Max. intensity	1	0 dB	0 dB
	10^{-3}	-30 dB	-60 dB
Min. intensity	~1.58 10^{-5}	-48 dB	-96 dB

3.2. *Pulse duration and repetition rate*

The duration of the LED light pulses as well as the repetition rate defining the event rate measured by the cytometer can be chosen freely up within certain limits.

The product of the two number has to stay below 0.1 to prevent any damage to the LEDs. The product of the two numbers describes the ratio of the time when the LED is "on" to the total time.

The firmware of the quantiFlash prevents the setup of any parameter combination potentially dangerous to the LEDs. If changing either one of the parameters and the new value would exceed the safe range it is not accepted, the parameter change will be discarded.

If setting the pulse duration first then the maximum possible event rate is given by the following table:

Pulse Duration [µs]	Max. Event rate [events/s]	ratio LED "on" [%]
2	50000	10%
5	20000	10%
10	10000	10%

If setting the event rate first then the maximum possible pulse duration is given by the following table:

Event rate [events/s]	Max. Pulse Duration [events/s]	ratio LED "on" [%]
50000	2	10%
20000	5	10%
10000	10	10%
Only relevant if using the "advance pulse feature" option		
5000	20	10%
2000	50	10%
1000	100	10%

4. Accessories

4.1. Attenuator set

To measure PMTs directly further appropriate attenuation is necessary.

Experienced users, direct fiber coupling to the detector block. (Beware not to touch the fiber surface!)

Set of attenuator comprises two fixed attenuators, additional -20dB and -40dB. Maybe small deviations, attenuators not perfectly calibrated, but perfectly stable.

Mounting the attenuator: screw directly on detector output of the *quanti*Flash, then mount fiber on attenuator.

Figure 3 (left to right): screw attenuator directly on detector output, mount fiber on attenuator

4.2. Flow cell adapter

The first adapter is an adjustable fiber extension that can be easily mounted on BD machines where the user has access to the flow cell. Currently these are

1. LSR
2. FACSAria
3. FACSCanto
4. FACSCalibur
5. LSRFortessa

A complete list is still under development (for others, please contact us).

The flow cell adapter provides a stable light coupling for reproducible, high quality measurements. It is attached to the flow cytometer using two of the threads next to the flow cell that are originally have been designed as a mounting fixture for the BD alignment microscope used to service the machines as shown in figure 3.
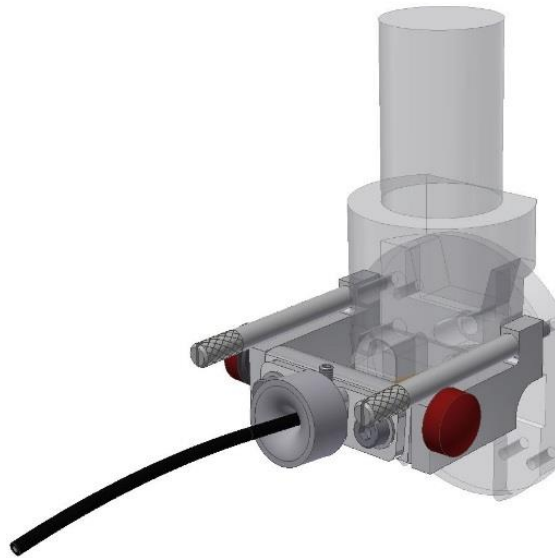
Figure 3: Schematic drawing of the flow cell adapter mounted to a BD cytometer.

The adapter can be quickly installed and offers easy realignment possibilities to accommodate for slight differences in the optical path if used on different machines. For frequent routine checks it can stay on the machine without any interference in the daily operation of the flow cytometer.

4.3.   Trigger switch

The second output of the *quanti*Flash® are trigger pulses that are perfectly in sync with the fluorescence output. If these pulse are used to trigger the flow cytometer on the forward scatter channel it gives the user additional power and flexibility for various different characterization methods.

In most BD flow cytometers the forward scatter (FCS) detector is a photo diode connected with a BNC cable to the signal processing electronics of the machine. The trigger switch is a small box (shown in figure 4) comprising a photo diode and a fiber mount as well as a high quality BNC terminated signal bypass. It is plugged directly in between the FCS detector and the BNC cable delivering the trigger signals to the signal processing electronics. Once it is in place a simple switch allows the user to easily decide whether to use the trigger signals from the FCS detector or to trigger the flow cytometer with the *quanti*Flash®.
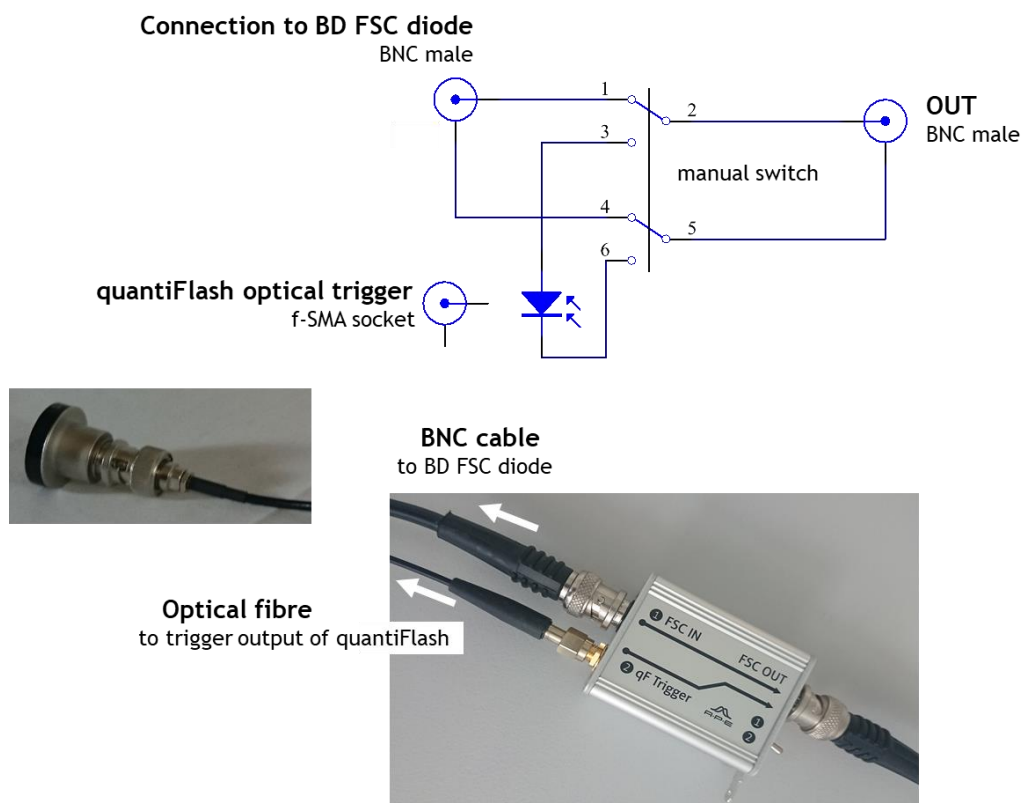
Figure 4: Schematic mounting of the trigger switch

4.4. *Installing the flow cell adapter and the trigger switch*

The flow cell of the above mentioned cytometers from BD is rather easily accessible by opening the hood of your cytometer. Around the flow cell you will find 4 thread holes that can be used to mount an alignment microscope typically used by BD service engineers. A schematic drawing of the flow cell is shown in figure 4.

Before installing the flow cell adapter please retract the fiber all the way using the Allen keys supplied with the flow cell adapter.
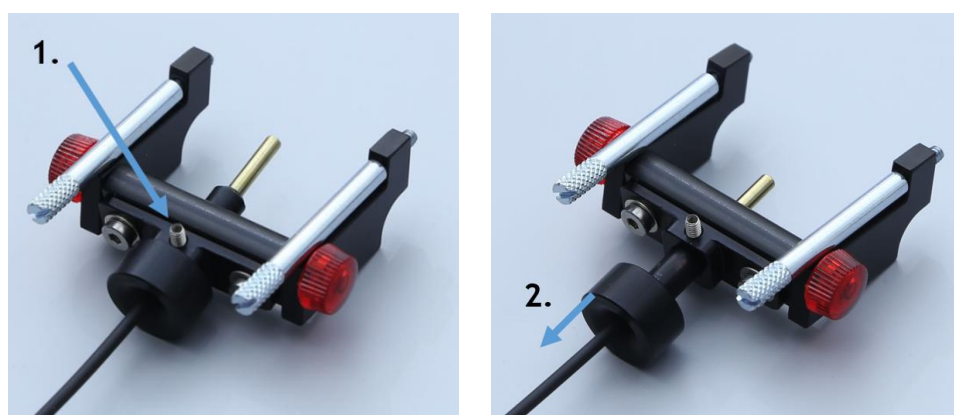

Figure 5: Preparations before installing

Loosen the screw as indicated in figure 5.1 and fully retract the fiber. Use the top two thread holes as indicated in figure 6 to install the flow cell adapter. Please be gentle and take care that nothing is jammed.
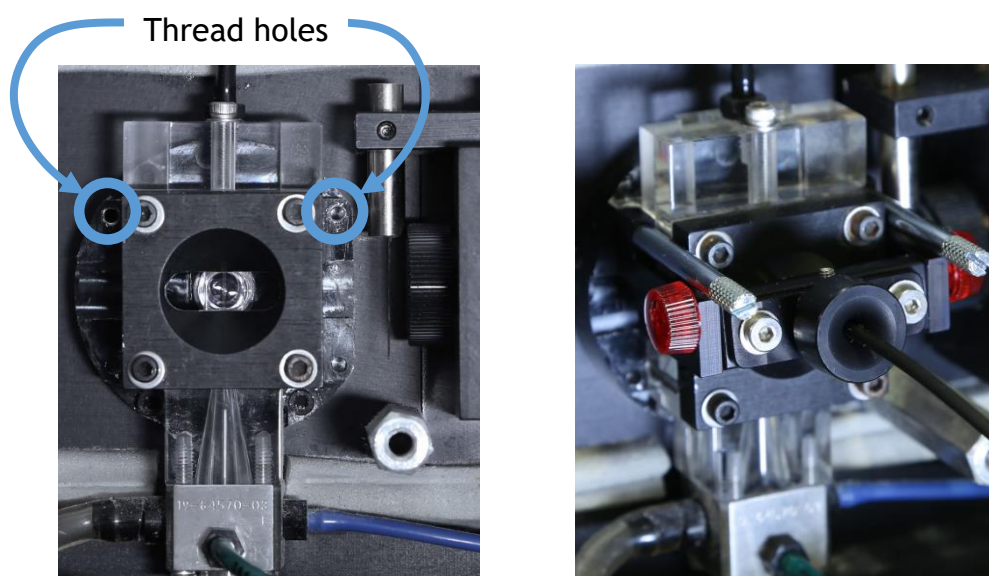
Thread holes

Figure 6: Details on the mounting thread holes and how the mounted flow cell adapter will look like

The next step will be installing the trigger switch. For this you will have to unplug the BNC cable connecting the FSC diode to the cytometer electronics. If you have never done such a thing it might be wise to practice once using the APE trigger switch and the supplied cable as shown in figure 7.
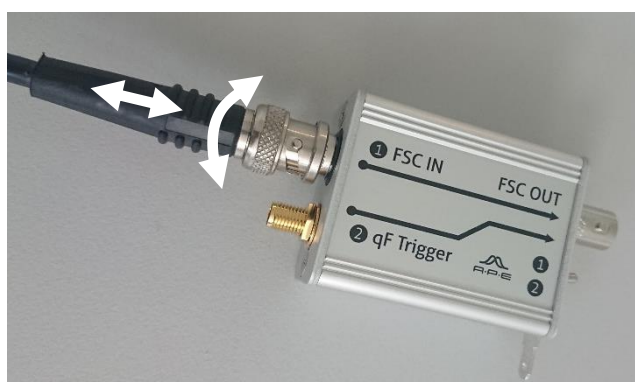

Figure 7: Test connecting BNC cables

To unplug the BNC cable from the FCS diode it is important to make sure **not** to apply excess force on the FCS diode itself. To prevent damage to the FCS diode hold it firmly in place using one hand while unlocking the BNC cable and gently pulling it off the socket.
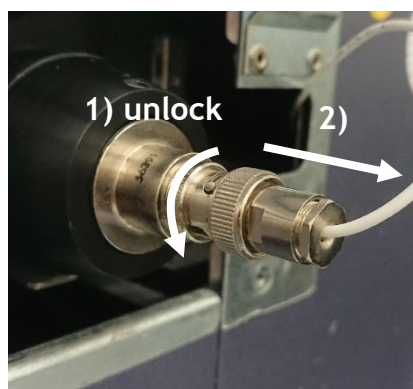
Figure 8: Disconnecting the BNC cables from the FSC diode

Mount the BNC cable that you have just pulled off to the trigger switch and mount the supplied cable to the FCS diode. Finally mount the free end of the supplied cable to the trigger switch.

This procedure only has to be done once. Once installed the trigger switch can stay in place. Just flip the switch on the back side to "FSC" and the signal from the FCS diode will pass completely unchanged through the trigger switch to the BD electronics as if the trigger switch wouldn't be present.

4.5. *Triggering a flow cytometer with the quantiFlash®*

To trigger your cytometer with the quantiFlash you have to connect one of the supplied fibers to the "Trigger qF" of the trigger switch.

Connect the other end to the trigger output of the *quanti*Flash®. Start a new measurement on the flow cytometer and open a histogram with the FCS signal.

If the FCS signal is off scale or you will have to adjust the trigger level. To adjust the trigger level to the desired value use the trigger menu in the FACS Diva software. Alternatively one can also change the brightness of the trigger pulses delivered by the *quanti*Flash®.

4.6. *Aligning the flow cell adapter*

The easiest way to couple the light pulses from the *quanti*Flash® into the flow cytometer is to shine directly through the flow cell into the collection optics. For best results and to achieve a good coupling efficiency it is of interest to carefully align the fiber end such that the light emitted is optimally collected and directed to the detection optics.

If using the *quanti*Flash® on various flow cytometers or if comparing to other groups inevitably there will be some differences in alignment or in coupling efficiency. This difference is not detrimental and will not harm any of the results obtained by the *quanti*Flash® nor will it alter any of the protocols presented in this manual. So don't worry if a colleague from another lab has achieved an even better alignment – one can easily compensate this by changing the output intensity of the *quanti*Flash® slightly.

Nevertheless some alignment is necessary, so please follow the next steps carefully.

*quanti*Flash®
User manual

The following steps are written for BD FACSDiva software (BD)

1. Prepare a worksheet in the BD FACSDiva software (BD) with histograms of the FSC signal and all fluorescence detection channels of interest (henceforth referred to as detector channels). Set the events to display in the Acquisition Dashboard to 1000 events.

2. Make sure that for each detector channel to be recorded the Height (H) parameter is selected. Note: Saturation can be directly measured and detected only by the Height signal

3. If the instrument of interest uses more than one excitation laser the laser delay should be set to zero for all lasers. The laser delay has to be set to zero in order to also detect the light pulses of the *quanti*Flash® in fluorescence channels in which the read out is delayed due to the spatial gap of the excitation lasers. Before setting the laser delays to zero please write them down and store the note in a safe place to be able to reset the laser delay to its original value later. The laser delays are also stored in the CS&T settings and set when "use CST settings" is selected when opening an experiment.

4. Install a sample tube with aqua destillata onto the cytometer and acquire at flow rate "low".

5. Turn on the *quanti*Flash® and use the following settings:
    - Count rate: 3000/s
    - Pulse duration: 5µs
    - Pulse shape: Gaussian
    - Intensity: 0dB

6. Flip the trigger switch to "Trigger qF"

7. Check the event rate on the Acquisition Dashboard. If the triggering works correctly it should be identical to the count rate set in the *quanti*Flash.
    - If no events are visible, lower the trigger threshold of the FSC detector
    - If the event rate is higher than the count rate on the quantiFlash lower the FSC gain
    - If the FSC signal is off scale, lower the trigger intensity of the quantiFlash

8. Keep the FSC gain fixed for all following measurements.

9. Set the PMT voltage to 500V for each channel. The mean channel peak position for all histograms should be somewhere between 10^2 and 10^5
    - If the mean channel is <10^2: re-align the fiber in front of the flow cell by changing the position as well as the angle in relation to the optical axis.
    - If the mean channel is >10^5: lower light input (dB) from *quanti*Flash®

10. To align fiber position in front of the flow cell one has to iteratively
    - Align the tilt of the fiber by slightly loosening the red screws at the side of the flow cell adapter
    - Align the fiber in the plane perpendicular to the flow cell using the Allen keys supplied
    - Carefully and gently try if the fiber can be placed even closer to the flow cell. Do not use force as this might damage the surface of the flow cell!

- If you suspect that you have touched the surface of the flow cell retract the fiber a tiny little bit
- Align the tilt of the fiber
- …

For all steps observe the signal in your detection channel and choose the position where it reaches maximum. It is often sufficient to maximize the signals in the long wavelength channels (e.g. PE-Cy7, APC-Cy7). The signals in all other channels are then automatically maximized.
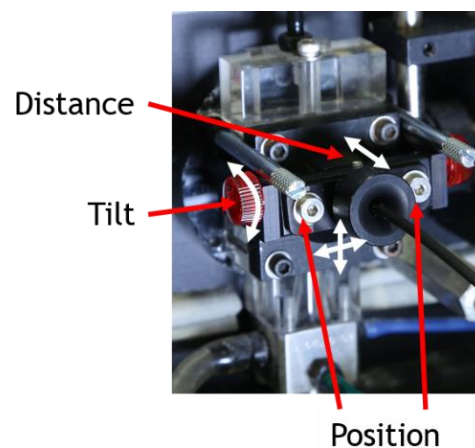
Typically two to three iterations are more than enough one will very quickly see, that no further improvements regarding the signal intensity on the histogram of the long wavelength channels. If the signal goes off scale, reduce the output intensity of the *quanti*Flash® by a few dB.

11. Close the lid without crippling the fiber

5. Advanced features

5.1. Install feature license

To enable the "Advanced pulse feature options" a license file has to be installed on the quantiFlash itself. On the device the different features will be unlocked. Further it will act as key for the software – the moment you connect a quantiFlash to a PC with the user software running it will identify itself and the software will display all additional control to setup and operate the advanced pulse features.

Step-by-step instruction to install a feature license

1. Download the license file (mail). If ordered togher with your device you can also find it on the USB stick supplied with the device. If you need to order: sales@ape-berlin.de

2. Connect quantiFlash to PC, turn it on and start user software.

3. To install the feature license on the quantiFlash select the FILE menu.

4. Within the FILE menu select "Feature upgrade …", browse to the license file on your PC and hit the "Open" button.

5. The file will undergo a security check by the user software and after that the corresponding features will automatically be installed on the quantiFlash
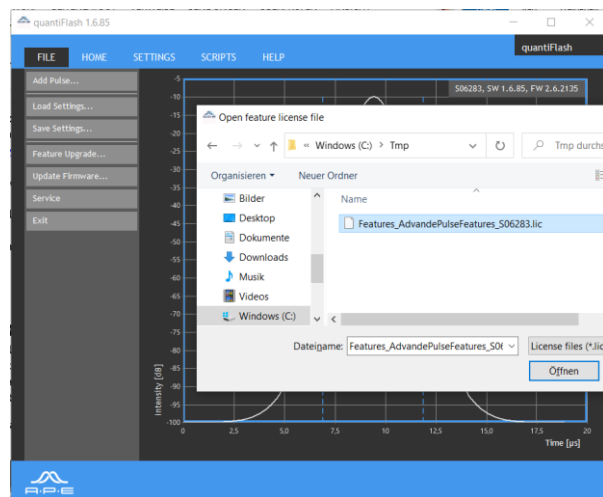

5.2. Manual control of the LED settings

For specific applications sometimes the manual control of the LED settings within the *quanti*Flash® is the preferred way. In this mode one can directly choose which LED bank should be used and access the raw intensity values of the LEDs without any layer of calibration function in between.

To access the manual control the "Advanced pulse feature option" is needed. Select the HOME menu and switch the LED control to Manual
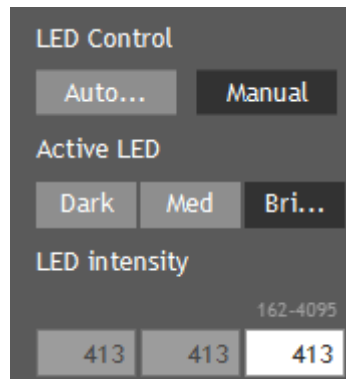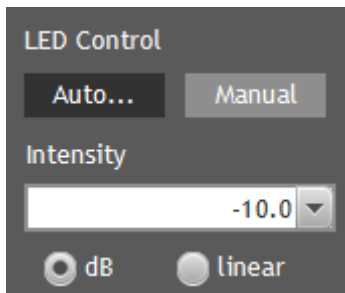
Choose the desired LED bank and enter LED intensity by hand. The range of possible values is marked in light grey small letters above each of the entry fields for the LED intensity.

5.3.   *User defined individual pulse shapes*

Individual user defined pulse shapes can be programed into the *quanti*Flash® with the PC software *quanti*Flash® Pro. The pulse has to be provided as an external file with the following structure:

- filetype: *.csv or *.txt
- one column, no header
- up to 4096 sample points, typically a few hundred points are more than enough
- every sample point has to be given as a 14-bit integer number (0 … 16383)
- the first 14 characters of the filename will define the name of the pulse displayed either in the *quanti*Flash® display or in the software

The amount of sample points does not change the duration of the pulse. Once a file containing the pulse data is opened with the PC software, the FWHM of this pulse will be calculated by the software as an additional parameter. Depending on the pulse duration set in the *quanti*Flash® the pulse will be stretched or compressed accordingly. This is done automatically, so one has the full possibility to set and change the pulse duration in the same way as for the predefined pulse shapes.
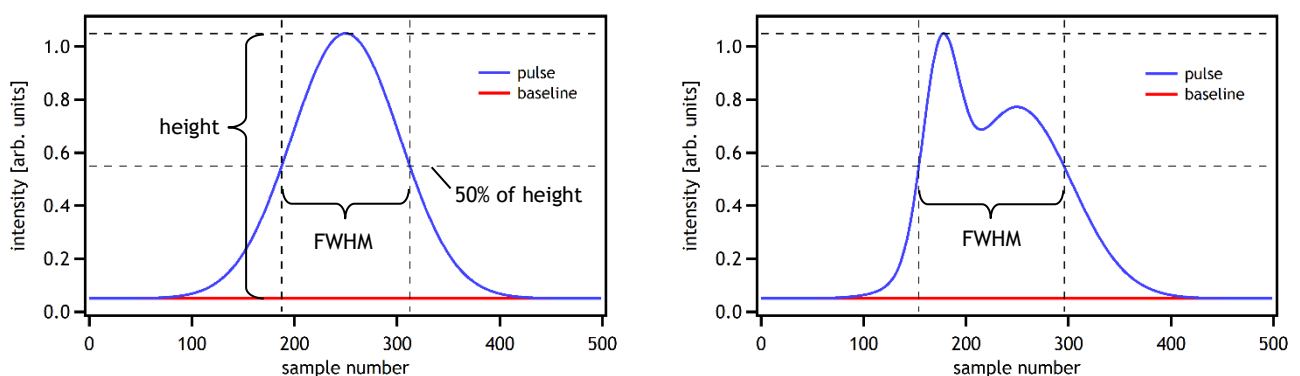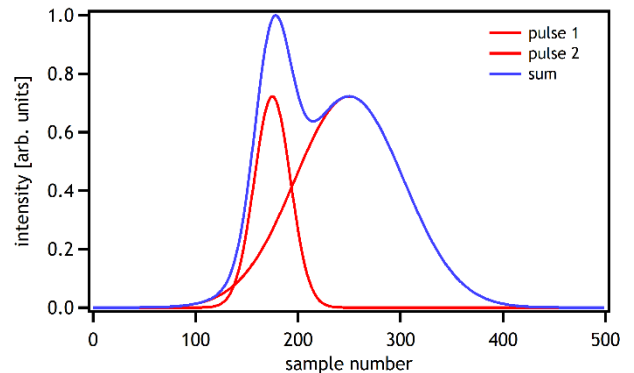

Figure 2: Two examples of pulses and how the pulse height and width is calculated by the *quanti*Flash® user software.

Step-by-step instructions

1. Generate the desired pulse shape

   For this illustration a double pulse, generated as a sum of two gaussian pulses was chosen. (Excel can do the job quite well)

Step 1: generated double pulse

2. Convert the sample points to a 14 bit integer. The easiest way would be to normalize the generated pulse shape, multiply it by $2^{14}=16383$ and round the numbers to integer values
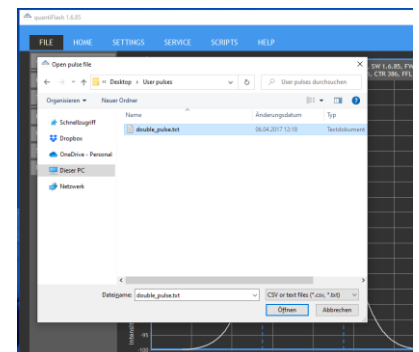
3. Export the pulse as plain text file (*.txt) or as a CSV-file

4. Open the quantiFlash PC software and connect your quantiFlash

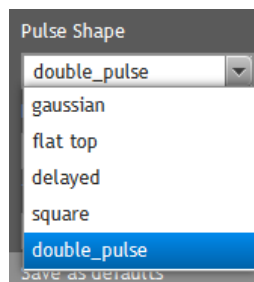5. Go to "File" -> "Add pulse ..." and select the file you just exported

6. Now you can select the new pulse shape through the drop-down menu

   "Home" -> "Pulse Shape"

Step 5: import pulse file

7. Adust the pulse duration and the pulse height to the desired levels as you would do for the standard pulses

Step 7: select pulse
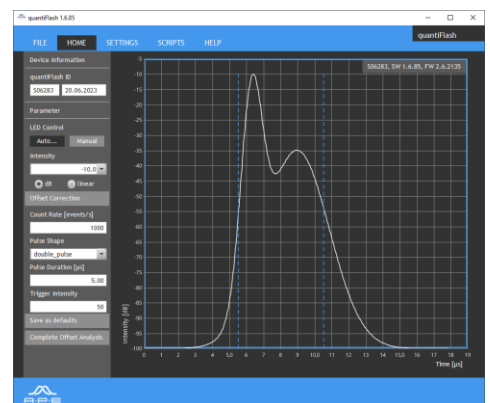
5.4.   *Remote control of the quantiFlash using the API*

The user software supplied with the *quanti*Flash® offers an application programming interface (API) based on TCP/IP. To be able to use this API an upgrade to the PC software *quanti*Flash® Pro is required.

At startup to the *quanti*Flash® user software the TCP/IP interface is deactivated. To activate the interface open the "SETTINGS" menu and press the "Start TCP/IP Server" button. Now the build-in TCP/IP server is activated and listening, in the example on the right on port 51019.
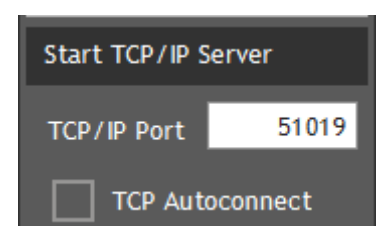
If the TCP/IP server should start automatically when the quantiFlash user software is opened, check "TCP Autoconnect".

The command structure of the *quanti*Flash® API is mostly in agreement with the SCPI-standard. However, A·P·E does not state compliance nor conformance to the standard, since some standard commands are not yet implemented in the present version. Detailed information about the SCPI is found at: www.ivifoundation.org

Complete command set see Appendix (page 27)

5.5.   Script interface

Some or the proposed applications for testing detectors on a flow cytometer are rather labor intensive. However most of the work can be automated which greatly reduces the time needed for the data acquisition, the data evaluation and leads to only little manual interaction.

To ease the implementation of these testing protocols a series of open source scripts are supplied with the software (version 1.2.53 or higher) and make use of the API. Please make sure that TCP/IP interface is activated.

**Preparations**

The scripts are written in Python 3.7. For all users that do not have Python already installed on their PC a ready-to-use software bundle with a portable Python environment is available for download at

https://www.ape-berlin.de/dl/quantiFlashPortablePythonBundle_1.6.85.zip

Unzip the downloaded file to a folder of your choice and start the quantiFlash software from that folder.

quantiFlash®
User manual

Script execution

All scripts are executed from the "SCRIPT" menu. If you use the script interface the first time click the browse button and select the "scripts" subfolder within the folder structure of the quantiFlash user software.
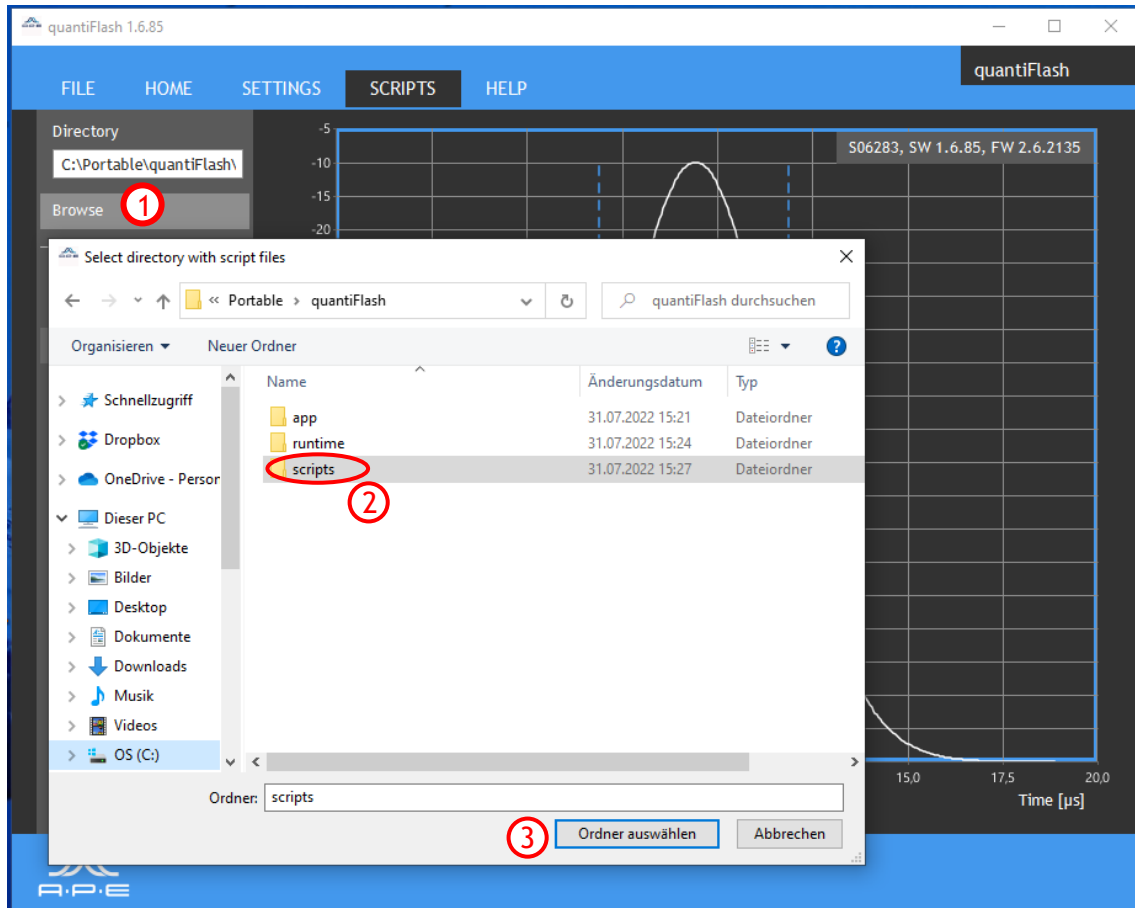
The scripts, contained within the corresponding folder can be selected through the dropdown menu.
Currently 4 scripts are available:

1. Signal ramping (data acquisition)
2. Data consistency check
3. Data evaluation SNR / DNR
4. Data evaluation Beads
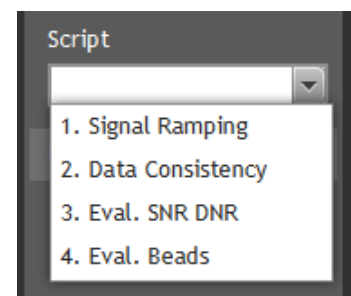
For further information on the corresponding script and protocols see section **Fehler! Verweisquelle konnte nicht gefunden werden.** (page **Fehler! Textmarke nicht definiert.**)

*quanti*Flash®
User manual

6. Firmware updates

APE is constantly working on new features, improve the stability of our devices or fixing bugs. This means that every once in a while it is advisable to update the firmware of your *quanti*Flash®. To make the process of updating the firmware as easy and as safe as possible the *quanti*Flash® software takes care of almost everything:

- Check for firmware updates
- Download the firmware
- Taking safety precautions
- Installing the firmware on your *quanti*Flash®
- Rebooting of the *quanti*Flash® after successful firmware update

6.1.   *Official firmware releases - stable update channel*

Before releasing a new firmware APE will rigorously test it. Then the new firmware version will become the new production standard and only after some time in serial production this firmware will become available as firmware update. If you want to benefit from early feature updates or latest bug fixes please check the next paragraph on our "Beta (Unstable) update channel".

The *quanti*Flash® software will check at regular intervals if there is a firmware update available for your device. For this check you the *quanti*Flash® has to be connected via USB to a computer connected to the internet, the *quanti*Flash® has to be turned on and the *quanti*Flash® software has to run.

If there is an update, you will get a notification through the *quanti*Flash® software (fig. 1). To update your device, click on "Update", which will start the update process. It is very important that during the update process neither the computer nor the *quanti*Flash® is turned off!
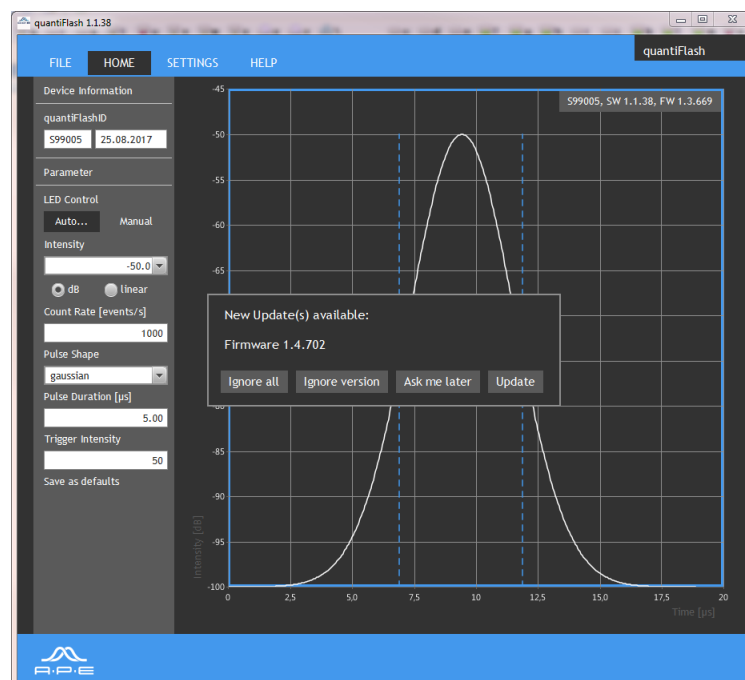
Figure 1: Notification for a firmware update in the *quanti*Flash® software.

The firmware update might take a while, so please be patient and check the progress bar in the *quanti*Flash® software (fig. 2). During the update progress it might happen that your *quanti*Flash® will reboot several times. This is completely normal.

Figure 2: the status of the *quanti*Flash® firmware update is indicated with a progress bar.

In many cases not only the firmware has been improved but also the *quanti*Flash® software as well. After successfully updating your *quanti*Flash® it might happen that you get a second notification stating that there is a software update as well (fig 3).
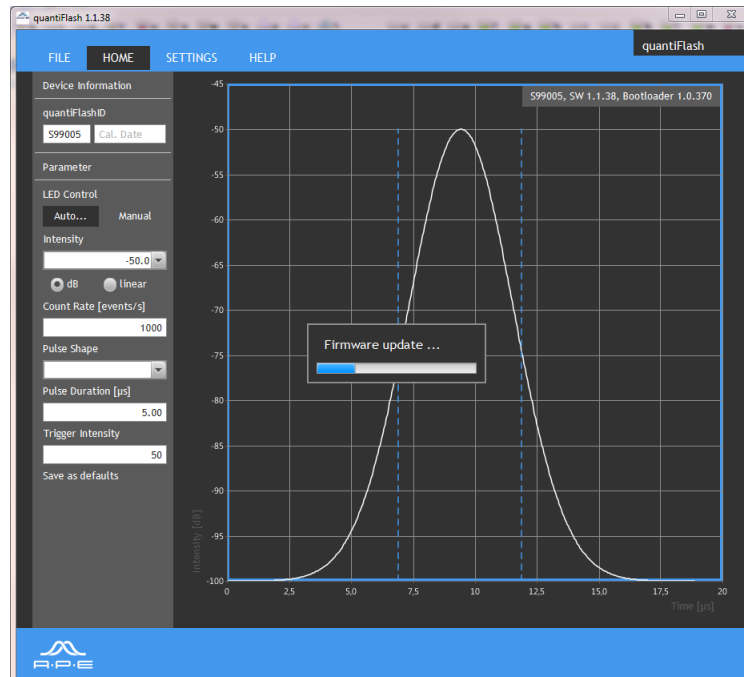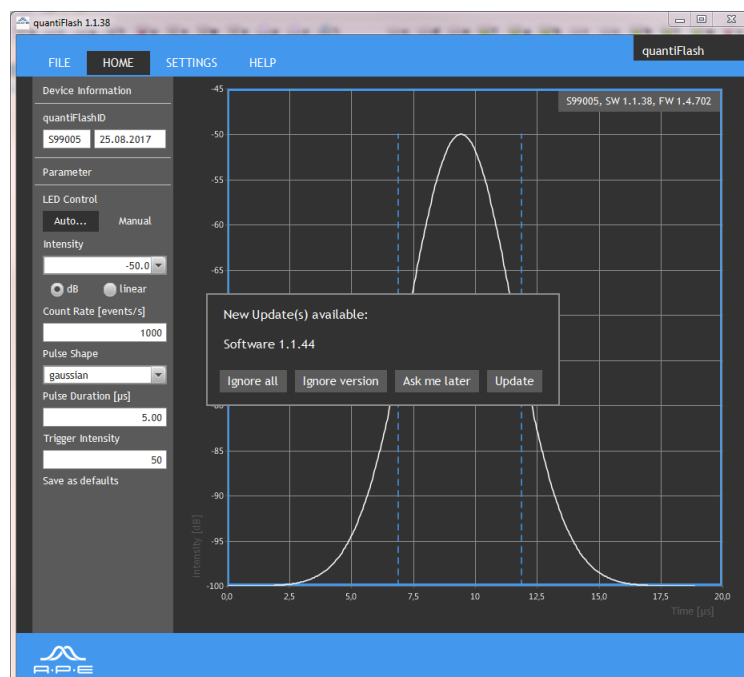
Figure 3: Notification for a software update in the *quanti*Flash® software.

After the software has been updated successfully it will restart automatically.

The current firmware and software versions used are shown in the info string on the top right corner of the main graph in the *quanti*Flash® software or in the device information summary, available through "Help" -> "About quantiFlash".

6.2.   *Activating the Beta (Unstable) update channel*

To activate the Beta (Unstabel) update channel you have to edit a file stored on your computer where you have installed or used the *quanti*Flash® software.

First you have to close the *quanti*Flash® software if it has been running.

In the folder with the name "C:\Users\{usermane}\AppData\Local\APE\quantiFlash" you will find the file "user.properties" (please replace {username} with your individual username). Open the file "user.properties" with a text editor of your choice or if there is no such file create on.

To activate the Beta (Unstabel) update channel this file has to contain the following entries (fig. 4):

    updateUnstableVersion = true
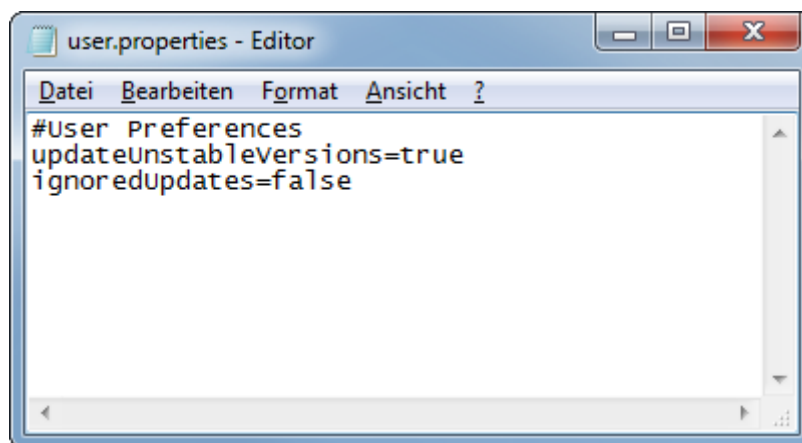   ignoredUpdates = false

Figure 4: Screenshot of the file "user.properties" with the entries to activate the Beta (Unstabel) update channel

After editing the file as indicated above once you restart the *quanti*Flash® software it will check for beta (unstable) firmware releases as well.

7. Frequently asked questions

Sometimes things go wrong or seem not to be working as they should. A few issues however can be easily solved by yourself:

7.1.   Installing license file doesn't work

Problem: You have received a license file for your device but the installation using the software fails.

Solution: Connect your quantiFlash to the PC where you are working on, switch it on and then start the quantiFlash user software. The license has to be installed within the device itself.

7.2.   After a factory reset the advanced features are missing

Problem: A factory reset of the quantiFlash had been activated. Now the advanced features such as long pulses, custom pulse shapes or external triggering are not working anymore.

Explanation: The Advanced pulse feature license has been erased by the factory reset of the quantiFlash.

Solution: Reinstall the Advanced pulse feature license following the step described in section 5.1 on page 16.

7.3.   Pulse shape looks ugly

Problem: When measuring the emitted light pulse with a fast photodiode the pulse shape looks distorted.

Solution: The pulse offset may have drifted away. Click on the "Offset Correction" button in the HOME menu. If the pulse shape looks nice again after that it is advisable to run an complete offset analyses by clicking on the corresponding button in the HOME menu. This way the new offset value will be stored in the quantiFlash.
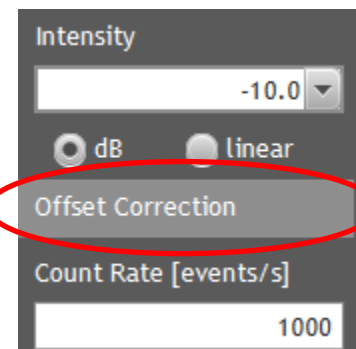
8. Calibration methods

Characterization, calibration and monitoring a flow cytometer are essential for high quality data acquisition. Especially for long term studies or large comparison studies involving various clinics or flow cytometry core facilities distributed over several places a high degree of coherence within the data and the results derived from it is absolutely essential. Typically the characterization of a flow cytometer is performed using various kinds of calibration particles. With the quantiFlash® some relevant parameters defining the quality of a flow cytometry measurement become accessible in an easier and more direct way than ever. Among these parameters are:

- Calibration of intensity scales to absolute physical units
- Laser induced background in a given detection channel
- Signal-to-noise ratio (SNR) of a given detection channel
- Dynamic range of (DNR) of a given detection channel

The relevance of these parameters is quite obvious. Measuring intensities in absolute physical units makes it easier compare flow cytometers amongst each other. The same holds true for determining SNR and DNR of flow cytometers. All of these three parameters might be needed to choose the right device with the highest sensitivity in a particular channel to ensure that dim population in a specific sample are measured correctly. Or one has to choose the right cytometer with the best dynamic range in specific channel to guarantee that both dim and bright signals are on scale.

If comparing data of different instruments from different facilities calibrated results will prove a highly valuable tool.

To determine the SNR, DNR and instrument's background the *quanti*Flash® must be coupled to the flow cytometer's optical detector pathway and the FSC trigger.

After optimizing the coupling efficiency the fiber has to be kept in the same position for all measurements to ensure comparability of the results within one machine. It should be mentioned, that once the coupling efficiency is optimized, only the stability of the light pulses during the experiment is important. When the *quanti*Flash® is used on different instruments the calculated number of photoelectrons N_{PE} on both instruments can differ in relation to the given dB intensity of the *quanti*Flash®. The coupling efficiency will stay unknown and will vary between experiments and machines. However, scale calibration relies on Poisson statistics and therefore on the CV of photoelectrons, and thus the only relevant and most important prerequisite is stability and low CV of the light pulses.

For the *quanti*Flash® the light pulses have a CV < 0.1%.

8.1. Theoretical basis: Poisson statistics

The absolute output power of the quantiFlash is not a specified parameter. Still there is a way to access a precise number that directly relates to the intensity of the emitted light pulses.

This alternative way that goes back to a publication by H.B.Steen (Cytometry 13, 822, https://doi.org/10.1002/cyto.990130804). It can be applied for PMTs or APDs and makes use of the intrinsic properties of the detection process of light within such a photo detectors.

For APDs and PMTs the detection process is governed by Poisson statistics and in this case it is enough to measure the statistical intensity distribution of many single events. If have this then the following simple formula can be applied:

$$CV = 1/\sqrt{N}$$

which leads to

$$N = 1/CV^2$$

where N is the number of detected photo electrons. This direct relation can be used for the calibration of intensity scales.

9. Appendix: Command set

The *quanti*Flash® API will execute the following commands:

*IDN?

 Get device identification

 <idn>   Device Information (APE GmbH, Device name, Serial number, Software version, Firmware version) as string

 Example:

 *idn?

:sys:device?

 Return device name

 <name> Device Name as string

 Example:

 :sys:device?

:sys:snumber?

 Return serial number

 <snr>   Serial number as string

 Example:

 :sys:snumber?

:sys:software?

 Return software version

 <sv>   Software version as string

 Example:

 :sys:software?

:led:control <value>

 Set LED control mode

 <value> Control mode as string (value = AUTOMATIC | MANUALLY)

 Example:

 :led:control=AUTOMATIC

:led:control?

 Return LED control mode

 <value> Control mode as string (value = AUTOMATIC | MANUALLY)

 Example:

 :led:control?

:led:mode <value>

Set LED mode

<value>   Control mode as string (value = BRIGHT | MEDIUM | DARK)

Example:

:led:mode=BRIGHT

:led:mode?

Return LED mode

<value>   Control mode as string (value = BRIGHT | MEDIUM | DARK)

Example:

:led:mode?

:pulse:intensity <value>

Set pulse intensity

<value>   Pulse intensity as integer

Example:

:pulse:intensity=10

:pulse:intensity?

Return pulse intensity

<value>   Pulse intensity as integer

Example:

:pulse intensity?

:pulse:shape <value>

Set pulse shape

<value>   Pulse shape as integer (0=standard, 1=Gaussian, 2=Flat top, 3=user defined …)

Example:

:pulse:shape=0

:pulse:shape?

Return pulse shape

<value>   Pulse shape as integer (0=standard, 1=Gaussian, 2=Flat top, 3=user defined …)

Example:

:pulse:shape?

:trigger:intensity <value>

      Set trigger intensity

      <value>   Trigger intensity as integer (between 0 … 100)

      Example:

      :trigger:intensity=50

:trigger:intensity?

      Return trigger intensity

      <value>   Trigger intensity as integer

      Example:

      :trigger:intensity?