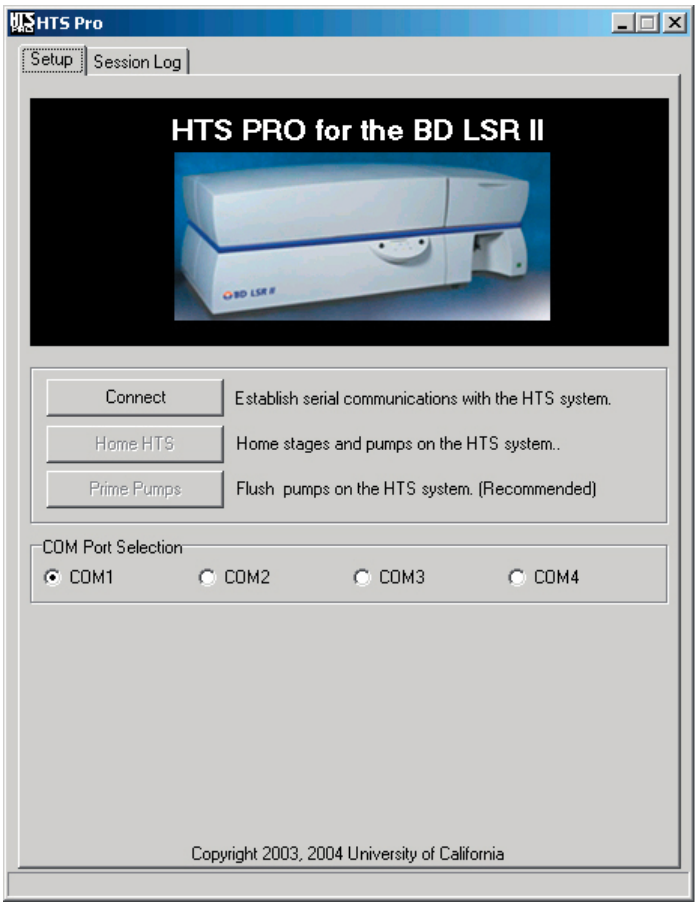
Instructions for operating the HTS-Pro v1.0

April 22nd, 2004

Introduction

HTS-Pro permits thousands of samples to be delivered from 96-well or 384-well plates located on HTS devices to LSR-II flow cytometers. It functions by controlling HTS devices directly, and by interfacing with the FACSDiva software used to run LSR-II cytometers.

The HTS software has four main panes (**Setup**, **Timed Run**, **Counts Run**, **Session Log**) and each pane performs distinct functions. **Setup** establishes a connection between a controlling computer and the HTS device, permits the HTS to home its axes, and primes the HTS pumps.The **Timed** and **Counts Run** panes are used to set parameters that will control the collection of data.The **Session Log** pane reports on actions that have been performed, and permits these actions to be saved to a text file.



Setup

Overview

The **Setup** pane permits three operations: *Connect*, *Home* and *Prime Pump*. The results from these operations are displayed at the bottom of the **Setup** pane. Note that during the initial start-up only the *Connect* button is active. Once a connection has been established the *Home* button is activated. After homing the HTS axes the *Prime Pump* button can be used.

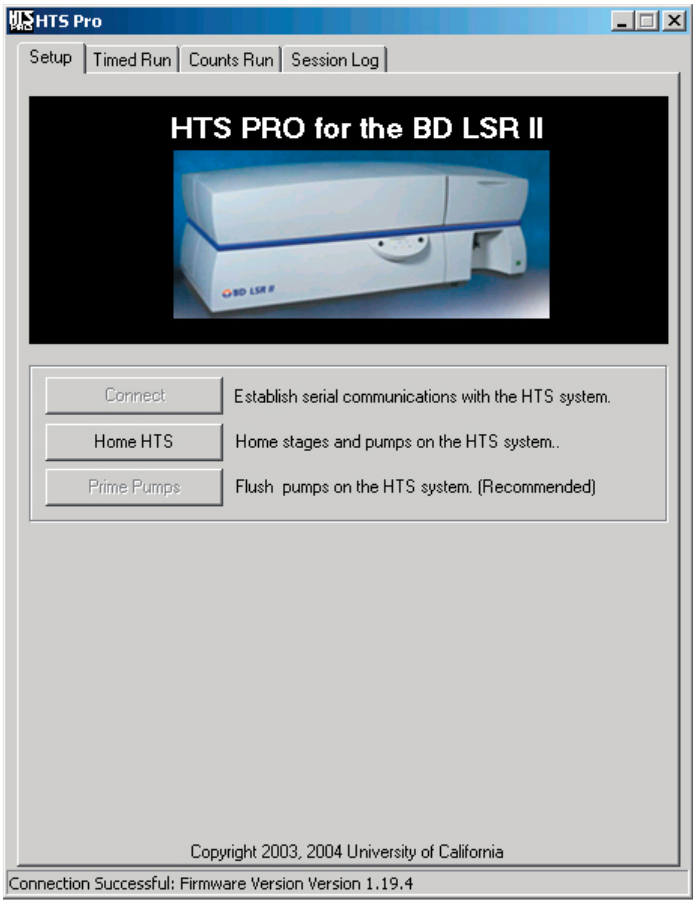
At the beginning of a set of experiments all three operations should be performed. The HTS device should be re-homed if the stage is bumped. It will automatically home if an emergency stop is executed. Priming may be necessary if air is introduced into the sheath flow lines during instrument operation.

Detailed Information

Connect establishes a serial connection between the computer running HTS-Pro and FACSDiva, and the HTS device. If a successful connection is established the firmware version of the HTS is returned. This is noted in the status bar at the bottom of the **Setup** pane and in the **Session Log** pane. If no connection can be established the software reports 'Failed' (see e.g., the **Setup** status bar). Failure to communicate might occur if the HTS device is not physically connected to the controlling computer or if the HTS device is not powered on. Additionally, if an attempt is made to connect too soon after the HTS device is powered on it may not be possible to establish a link between HTS-Pro and the auto-sampler. In any event, if an attempt to communicate with the HTS fails, the user should check physical connections, check that correct COM port on the controlling computer is selected, restart the auto-sampler, wait a few seconds and press *Connect*.

Home causes the HTS device to home its various axes. The cursor will appear as an hourglass during this operation and notification of successful homing will appear at the bottom of the application window. This operation is needed for the auto-sampler to operate correctly. Homing is required only once, provided that the sample stage is not bumped and that the HTS device is not turned off. Additionally, if it is necessary to stop in the middle of a run the HTS-Pro software will attempt to home the auto-sampler. Occasionally, if pumps are running during a home operation they will continue to run after homing is complete. Beginning another run will reset the pumps, as will turning the HTS device off and on, as will priming the pumps.

Prime Pumps empties and fills the forward and aft syringes on the HTS device. Also, during priming air is expelled from the sheath-fluid lines entering the instrument. For typical instrument operation it will be necessary to prime the HTS device only once at the start of a set of experiments.

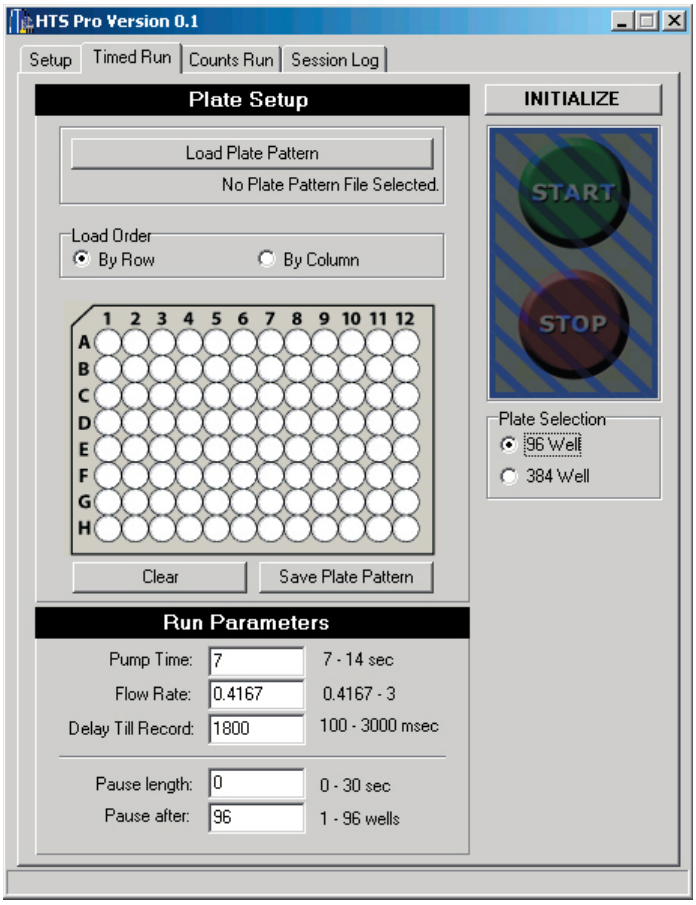


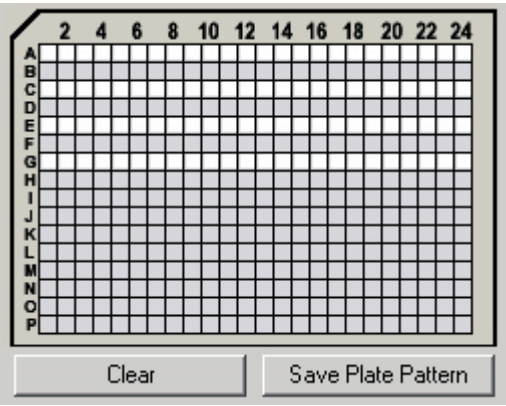
Timed Run

Overview

The **Timed Run** pane is the core of the HTS-Pro software. It allows the user to specify the type of plate to run (96- or 384-well), which wells to sample, and the order in which these wells will be sampled. The **Timed Run** pane also establishes the amount of sample to be delivered to the flow cytometer (set by choosing *flow rate* and *pump time*), and a delay that avoids collecting data before the sample stream has had a chance to settle down. Users can also program a pause if the FACSDiva software cannot manage the amount of data being generated by the cytometer, thus allowing FACSDiva to "catch up" with the HTS.

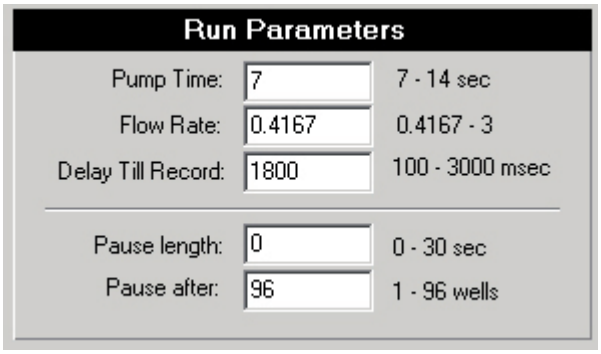
Once the user has set the requisite experimental parameters, the FACSDiva software and the HTS device are initialized (via the *Initialize* button). Finally, the experiment is begun using the *Start* button. In the case of an emergency the run can be terminated using the *Stop* button. Note that the *Start* button will be unavailable until the *Initialize* button is clicked.



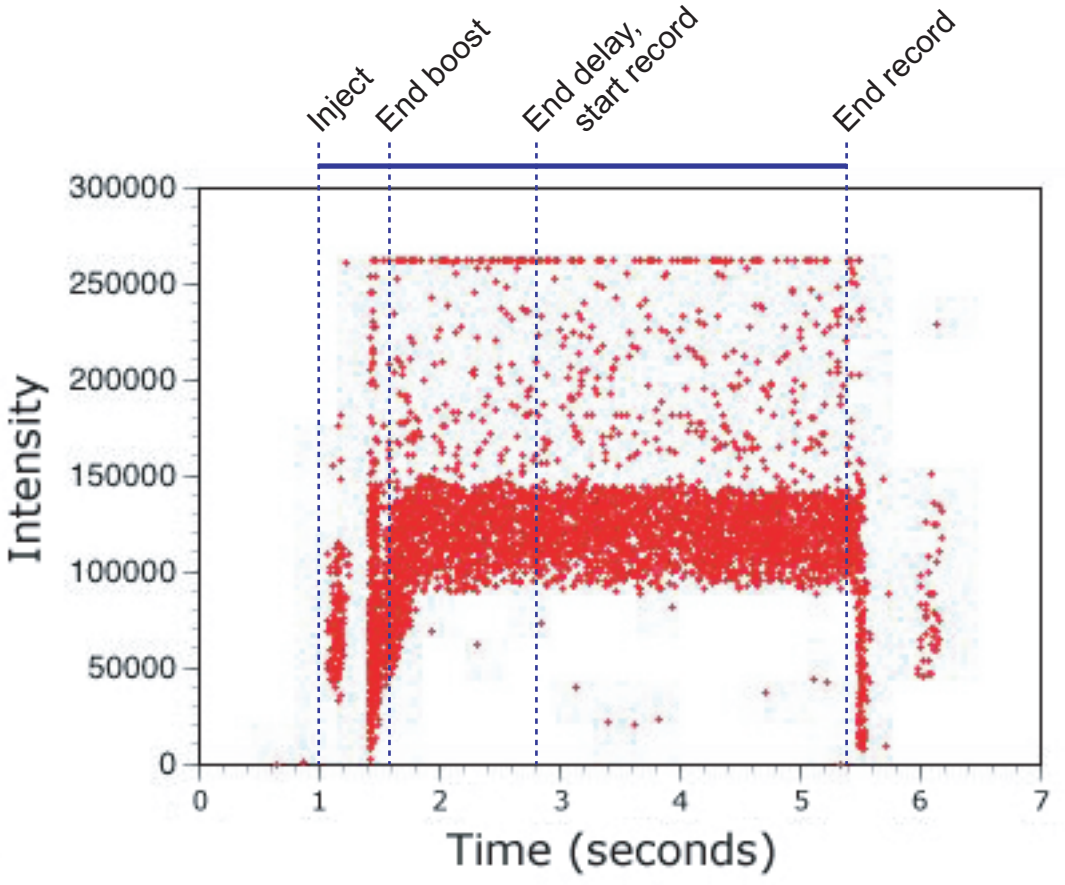


Detailed Information

The HTS device can run in 96- or 384-well format in the **Timed Run** mode. The mode is selected using the radio buttons under the *Stop* button. Next, the number of wells to be run must be entered. By default, HTS-Pro assumes all wells will be run. If this is incorrect, the wells can be re-defined by importing a text file containing row and column information[1]. This is done using the *Load Plate Pattern* button. Alternately, wells can be cleared using the *Clear* button and then entered manually by clicking on each well. Dragging the mouse from left to right and top to bottom will select a rectangular range of wells. Clicking on the row letters or column numbers outside the graphical representation of the plate will select all wells in a defined row or column, respectively. In the current version of HTS-Pro individual wells cannot be deselected. However, once a pattern has been defined it can be saved using the *Save Plate Pattern* button. Once wells have been selected, the run order is defined using the two radio buttons above the plate graphic.



The *Run Parameters* determine how each sample is delivered to the LSR-II and how the resulting data are collected. The *Pump Time* reflects the amount of time the HTS device will send sample to the LSR-II. Note that choosing sample delivery times much below 6 seconds does not decrease the amount of time it takes to measure a plate, but only decreases the amount of data collected for a given well. This is because the housekeeping functions of the HTS device (movement to and from plate, mixing, pumping) become the rate-limiting step at small *Pump Time* values. The *Flow Rate* determines how fast the sample will be delivered to the instrument. Fast flow rates will increase counts per second, but at the expense of increasing the coefficient of variation (CV) of the sample. The *Delay Time* permits sample flow through the cytometer to settle down prior to the start of data acquisition. (See the graph below) Note that the sensitivity of the LSR-II to perturbations in sample flow is determined also by laser alignment and by the tightness of the coupling between the HTS device and the SIP (a tight connection is highly desirable). The *Delay Time* should be adjusted by each operator following a series of control experiments: monitor the relevant cytometry parameters as a function of time and increase the *Delay Time* until choppiness is eliminated (or greatly reduced) at the start of data files.

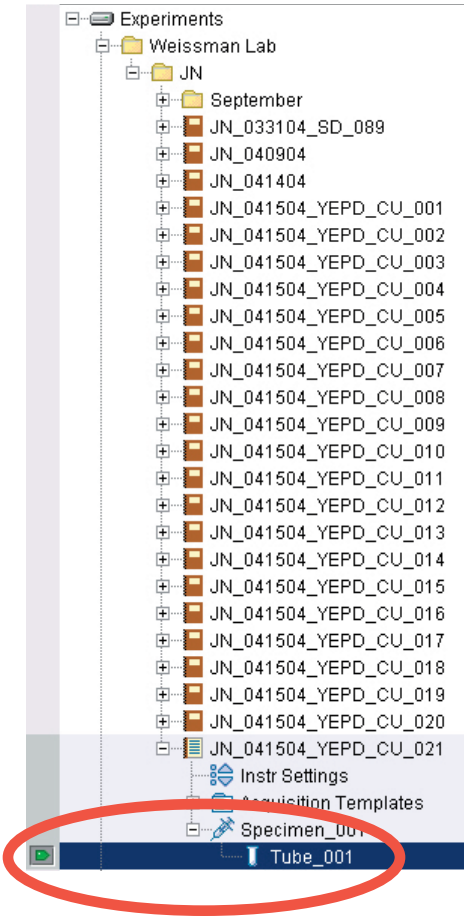


Graph of counts per second. Note the non uniformity of the data during the first 1.5 seconds which is due to the initial injection and boost. Occasionally uneven flow is observed beyond 1.5 seconds. External programs can be useful in eliminating uneven data.

If the FACSDiva software becomes unresponsive during the collection of data it is possible that this is due to the generation of too much data for FACSDiva to process efficiently. The exact timing of this slowdown will likely vary depending on the users specific setup, including processor speed and memory. Pausing data collection after 24-48 wells for 10-30 seconds will eliminate this problem.

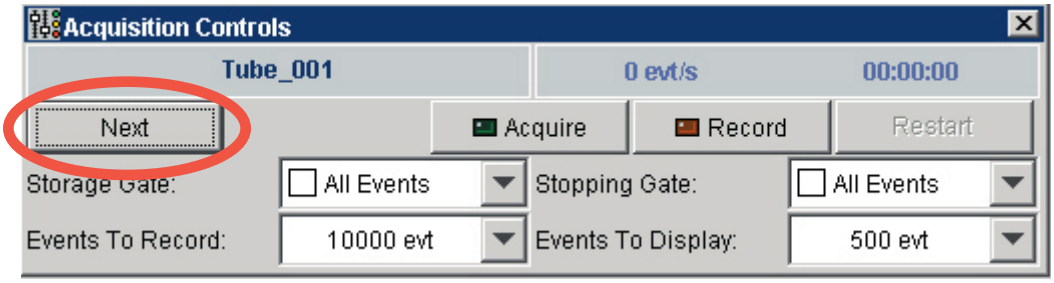
Once the run parameters have been chosen it is necessary to transfer them to the HTS device and to the LSR-II. This is done at the same time using the *Initialize* button. First, however, the FACSDiva software must be launched, and an experiment created.

Double-click on the Diva software, navigate to the appropriate directory and either open or create an experiment. Note that the performance of the Diva software decreases the larger the number of files in an experiment, so if many wells are to be sampled, it is best to create a new experiment for each plate (96 per experiment is recommended currently). Now, highlight the first <u>empty</u> tube to be used for collecting data (a green arrow should be positioned at the left of the tube name).



When this is done click on the *Acquisition Control Panel* to make it active (if needed make the panel visible using the **View** menu in the Diva software). If none of the buttons are highlighted press the TAB key once. The *Next* button should now be highlighted (see figure above). If one of the other buttons is already highlighted, or if the *Next* button is not highlighted, press the TAB key to move the focus to the *Next* button.

The NEXT button has the focus:



HTS-Pro utilizes keyboard events to control the recording of data via FACSDiva. Therefore, it is essential that the current keyboard focus be on the *Next* button prior to pressing *Initialize*.

Returning to the HTS-Pro software press *Initialize* once. Notice that the focus has moved from *Next* to *Record*. Also, the number of cells to count has been set at 2,500,000. The Diva software may also beep during initialization. If these changes have occurred the experiment can be started by putting the LSR II instrument into RUN mode, and then pressing *Start* on HTS-Pro. Otherwise, click on the *Acquisition Control Panel* header bar to make this panel active, check the position of the focus (it should be on *Next*!), and press *Initialize* once more on the HTS-Pro **Timed Run** pane.

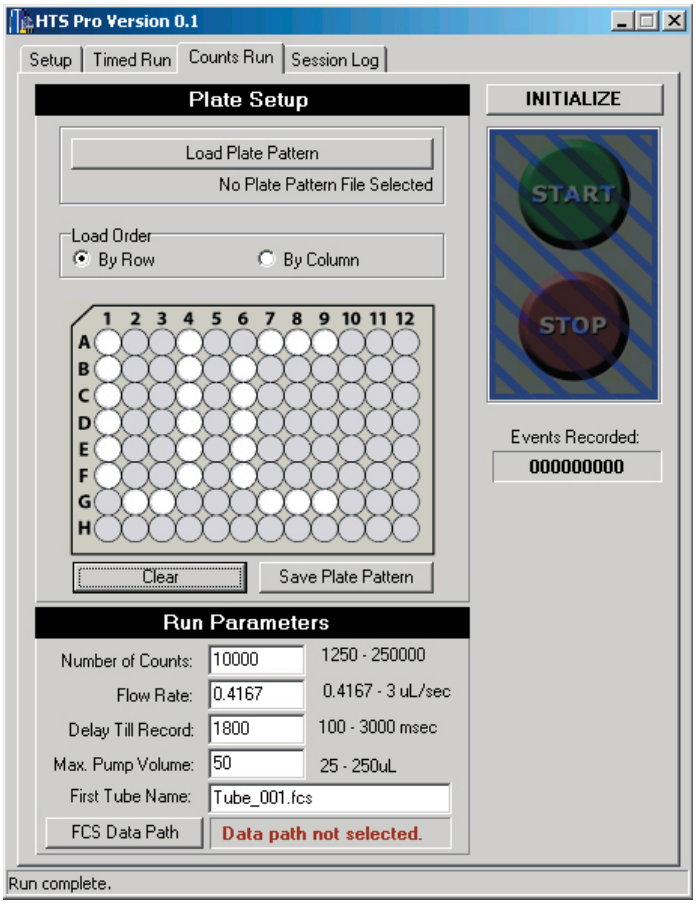
Before initialization.

After initialization.

NOTE: Once a run has been started, it is imperative that the keyboard be left undisturbed during the run. Because HTS-Pro uses keyboard events to control FACSDiva, user input will disrupt normal operation of the program.

If a run must be stopped, the *Stop* button can be used. If the *Stop* button is used, HTS-Pro will attempt to home the auto-sampler axes. If information is needed about which samples were run prior to the stop, this can be found by clicking on the tab that accesses the **Session Log** pane.

Counts Run



Overview

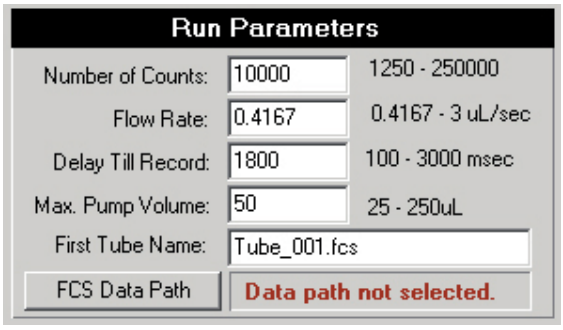
The **Counts Run** pane controls the delivery to sample such that at most the specified number of cells is collected. After collecting the specified number of cells the HTS Pro program directs the auto-sampler to empty any remaining sample and move to the next well. If the injection sample is used up before the desired number of counts is reached, the HTS Pro program closes the current data acquisition file and moves to the next well.

Note that the **Counts Run** mode is inherently slower than the **Timed Run** mode: the former uses only one syringe to control sample delivery, while the latter uses two. Consequently, it is recommended that most high-throughput experiments use **Time Run** mode.

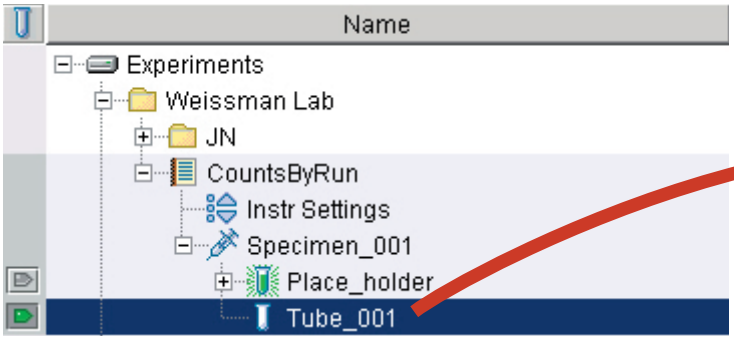
Detailed Information

The HTS device can run only in 96-well format in the **Counts Run** mode. The number of wells to be run is entered using the interface under the *Plate Setup* banner. By default, HTS-Pro assumes all wells will be run. If this is incorrect, the wells can be re-defined by importing a text file containing row and column information (see also **Timed Run** for file format). This is done using the *Load Plate Pattern* button. Alternately, wells can be cleared using the *Clear* button and then entered manually by clicking on each well. Clicking on the row letters or column numbers outside the graphical representation of the plate will select all wells in a defined row or column, respectively. In the current version of HTS-Pro individual wells cannot be deselected. However, once a pattern has been defined it can be saved using the *Save Plate Pattern* button. Once wells have been selected, the run order is defined using the two radio buttons above the plate graphic.

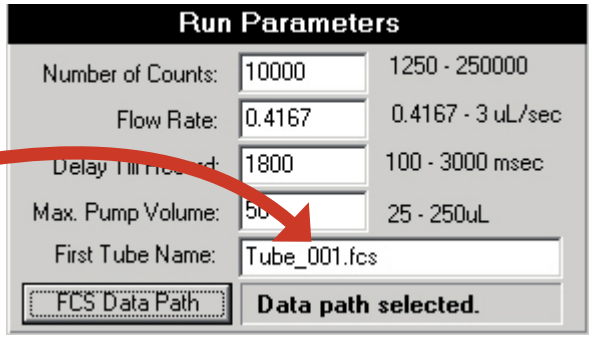
The *Run Parameters* determine how each sample is delivered to the LSR-II and how the resulting data are collected. The *Number of* Counts obviously determines the target number of events to be recorded, with the caveat that the requisite number of events must be processed before the delivery syringe runs out of sample. The *Flow Rate* determines how fast the sample will be delivered to the instrument. Fast flow rates will increase counts per second, but at the expense of increasing the coefficient of variation (CV) of the sample. The *Delay Till Record* time permits sample flow through the cytometer to settle down prior to the start of data acquisition. Note that the sensitivity of the LSR-II to perturbations in sample flow is determined also by laser alignment and by the tightness of the coupling between the HTS device and the SIP (a tight connection is highly desirable). The *Delay Time* should be adjusted by each operator following a series of control experiments: monitor the relevant cytometry parameters as a function of time and increase the *Delay Till Record* time until choppiness is eliminated (or greatly reduced) at the start of data files. *Max. Pump Volume* determines how much sample the injection syringe will take up.



NOTE THAT HTS-PRO REQUIRES THAT THE EXPERIMENT PATH MUST ALSO BE SELECTED. However, the folder that collects data for any given experiment is only created by FACSDiva after at least one tube with non-zero counts is present in the experiment. Therefore, either collect a few events into a tube, or copy and paste-with-data a preexisting tube into the folder that will contain data from a counts run. Next, create a new tube. The name of this empty tube will be entered into the *First Tube Name* record field in HTS-Pro. Once this has been done, select the FCS path of the experiment that will be run. The experiment path usually starts D:\BDDatabase\BDdata\…



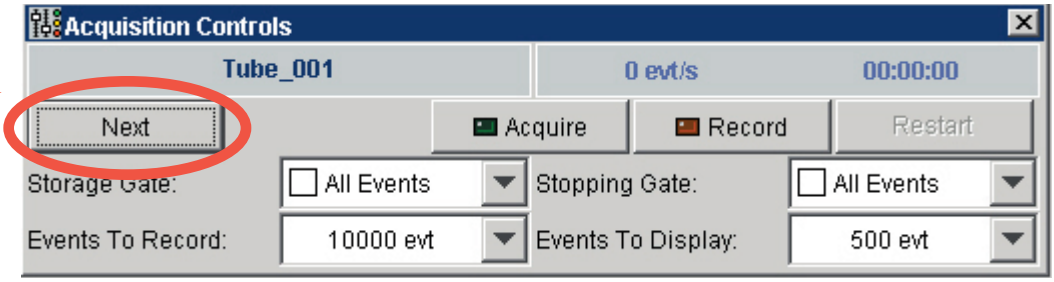
Correct setup for a Counts Run. A placeholder tube with data (marked by the green tube) must be present in addition to the first empty tube for which data will be collected.



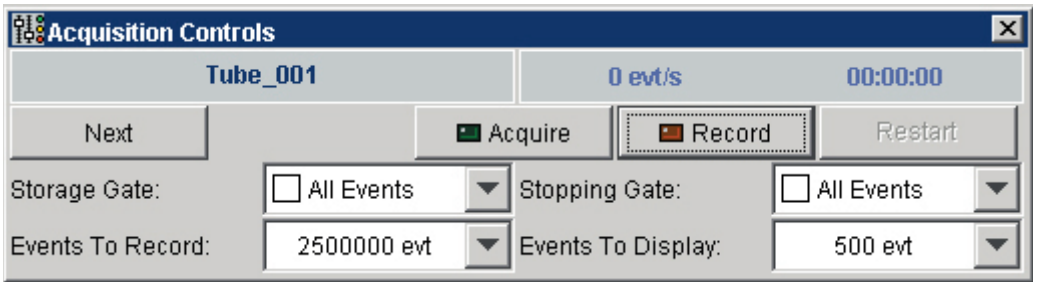
Run Parameter box after datapath has been selected. Note that tube filename must include the ".fcs" extension.

Once the *Run Parameters* have been chosen it is necessary to transfer them to the HTS device and to the LSR-II. This is done at the same time using the *Initialize* button. First, click on the *Acquisition Control Panel* in the Diva software to make it active (if needed make the panel visible using the **View** menu in the Diva software). If none of the *Acquisition* buttons are highlighted press the TAB key once. The *Next* button should now be highlighted on the *Acquisition Control Panel*. If one of the other buttons is already highlighted, or if the *Next* button is not highlighted, press the TAB key to move the focus to the *Next* button.

The NEXT button has the focus:



Returning to the HTS-Pro software, press *Initialize* once. Notice that the focus has moved from *Next* to *Record*. Also, the number of cells to count has been set at the value entered into the field in the HTS-Pro software. The Diva software may also beep during initialization. If these changes have occurred the experiment can be started by pressing *Start*. Otherwise, click on the *Acquisition Control Panel* header bar to make this panel active, check the position of the focus (it should be on *Next*!), and press *Initialize* once more on the HTS-Pro **Counts Run** pane.



After initialization, the focus should be on the *Record* button of the Acquistion Control panel. At this point, check that the LSR II instrument is in run mode. Now the Counts Run may begin by pressing the start button.

NOTE: Once a run has been started, it is imperative that the keyboard be left undisturbed during the run. Because HTS-Pro uses keyboard events to control FACSDiva, user input will disrupt normal operation of the program.

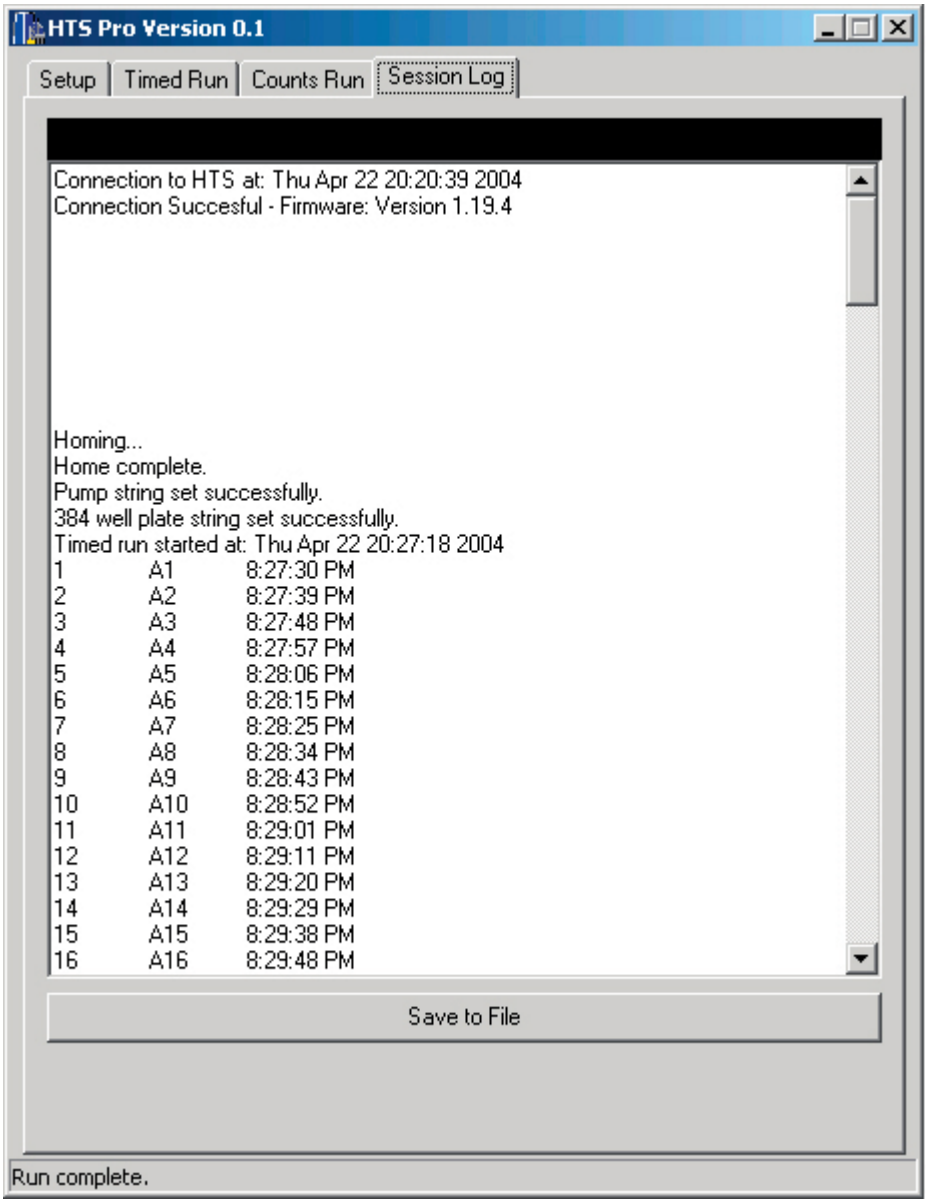
If a run must be stopped, the *Stop* button can be used. If the *Stop* button is used, HTS-Pro will attempt to home the auto-sampler axes. If information is needed about which samples were run prior to the stop, this can be found by clicking on the tab that accesses the **Status** pane.

A detailed account of the Counts Run session will appear in the **Session Log**, including filenames and times associated with each well of the plate.

Session Log

Overview

The **Session Log** pane provides a record of the actions performed by the HTS-Pro software. For example, it can be consulted to confirm that the HTS device has been correctly initialized. Importantly, the **Session Log** contains information about when a particular well has been run. This information can be compared to the time that a file was saved by the Diva software, and in this way critical information about the relationship between a file and a sample is preserved. The **Session Log** is not cleared after each plate is run, therefore information about many experiments can be saved to a single file.



Detailed Information

The **Session Log** is active from the launch of the HTS Pro software and records most actions performed by the software and the outcomes of these actions. It is possible to save the contents of the **Session Log** window to a text file by pressing the *Save to File* button at the bottom of the **Log** window. The **Log** is only cleared when the HTS Pro program is closed.

NOTES

[1] The format for each line in a text file is: RowColumn [0/1]. Acceptable row values are A-H or A-P (96-384-well plates, respectively). Acceptable column values are 1-12 or 1-24 (*ibid*). A tab separates RowColumn information from inclusion information. A 1 indicates the well is to be included in a run, a 0 that the well is to be excluded. A file contains information about all wells on a plate and is ordered by row then by column.