Highway1 User Guide

V1.1

Approvals

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Reviewer *Tim Atkins*	R**ole** *PQO*	25 January 2024

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1. Document History

Version	Updates	Date
1.0	First release	01 December 2023
1.1	Added specifications and compliance information	25 January 2024

2. Specification and Compliance Information

2.1. Technical specification

Cytometry	▪ Lasers: 488 nm (200 mW), 638 nm (180mW) (blue and red) ▪ Detection: 4 x SiPM (fluorescence), 3 x PD (FSC, SSC, RFSC) ▪ Filters: 525/40 (FITC), 585/29 (PE), 684/24 (PerCP / PE -Cy5), 665/12 (APC) ▪ DSP: 24 bit, 80 MHz, 200,000 events per second ▪ 5-decade log scale, linear scale and logicle scale ▪ Peak measurement: height, area, width (FWHM) ▪ Sensitivity: MESF 50 (FITC), 70 (PE) ▪ Daily QC function uses Highway1 DailyQC Beads, tests cytometry and sorting functions. ▪ Cartridge-to-cartridge reproducibility
Fluidics and Cartridge	▪ Highway1 Cartridge + Highway1 Cartridge Loader (ETO sterilised) ▪ 30-µm Cell Strainers (cartridge loader / in-line) (ETO sterilised) ▪ Traceable NFC-tagged cartridges ▪ No contamination, no cross contamination ▪ No sheath; non-diluting flow; no waste ▪ Centrifuge tube outputs ▪ Cell size range 5 – 25 µm ▪ Maximum input 22 mL ▪ Volumetric flow rate 260 µL/min ▪ Low hydrodynamic stress (peak energy dissipation rate 2 W/mL) makes high-viability serial sorting practical ▪ Gentle magnetic stirring ▪ Absolute volumetric flow calibrated for 20 mL and 3 mL syringes ▪ Optional cold pack (cartridge input/outputs refrigerated) ▪ Continuous auto-alignment, flow control, chip cooling ▪ *Gunk Be Gone ™*

Sorting	▪ 42 kHz sort envelope rate (23 µs sort envelope, 30 µs sort mask purity mode) ▪ >99% purity and >80% yield depending on process parameters ▪ 4 kHz sustained deflection rate ▪ Automated actuation timing and amplitude ▪ Automated sort verification ▪ Walk-away operation; audible notifications of process completion and process errors
Host PC and User Software	▪ HighwayR software package runs on MS Windows 11 on Dell host PC supplied; 27" 1440p monitor ▪ Connectivity: Ethernet, WiFi, USB ports ▪ Automated workflows for cytometry, sorting and dailyQC ▪ FCS 3.1 export, supported by FlowJo ▪ Automatic and manual full matrix compensation ▪ English language only
Installation	▪ Instrument size 210 mm x 570 mm x 318 mm (W x H x D) ▪ Instrument weight 20 kg ▪ Environmental temperature 18-25°C ▪ Electrical power 100 – 240 V, up to 250 W, cable available for all regional consumer sockets

2.2. Name and address of manufacturer

Cellular Highways

TTP Campus

Melbourn Science Park

Melbourn, Cambridgeshire

SG8 6HQ

UK

2.3. Equipment maintenance details

- Change fan filters every 12 months.
- Do not replace mains power cords with any replacement not specified by the manufacturer.
- Do not open instrument covers.
- Only accessories specified by the manufacturer should be used.
- Take care in lifting or carrying.

2.4. Environmental information:

- Indoor use only
- Altitude up to 2000 m
- 18 – 25 °C ambient temperature range
- Relative humidity up to 60%

2.5. Equipment Electrical Rating Information:

- Input voltage range: 90 – 264 Vac
- Input frequency: 47 – 63 Hz
- Maximum power: 250W
- Mains AC/DC power supply to connect to instrument

2.6. FCC Compliance

This equipment has been tested and found to comply with the limits for a Class B digital device, pursuant to part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference in a residential installation. This equipment generates, uses and can radiate radio frequency energy and, if not installed and used in accordance with the instructions, may cause harmful interference to radio communications. However, there is no guarantee that interference will not occur in a particular installation.

If this equipment does cause harmful interference to radio or television reception, which can be determined by turning the equipment off and on, the user is encouraged to try to correct the interference by one or more of the following measures:

- Reorient or relocate the receiving antenna.
- Increase the separation between the equipment and receiver.
- Connect the equipment into an outlet on a circuit different from that to which the receiver is connected.
- Consult the dealer or an experienced radio/TV technician for help.

Changes or modifications not expressly approved by Cellular Highways Ltd could void the user's authority to operate the equipment.

3. Safety

3.1. Intended Use

3.1.1. The Highway1 Cell Sorting System is for Research Use Only. It is not sold as a medical device. Any clinical application of the cells (i.e. the use of cells in humans) is exclusively the responsibility of the user of the Highway1 Cell Sorting System. This use is not intended by Cellular Highways. Therefore, the safety and regulatory compliance of any clinical application is exclusively the responsibility of the user responsibility.

3.2. General safety

3.2.1. Do not operate the Highway1 without training from an authorised representative of Cellular Highways.

3.2.2. Do not open the casework or alter the Highway1 in any way.

3.3. Laser safety

3.3.1. The instrument contains class 3B lasers, which emit intense, coherent electromagnetic radiation that can cause irreparable damage to skin and eyes.

3.3.2. 488nm wavelength laser with maximum power of 200mW

3.3.3. 632nm wavelength laser with maximum power of 180mW

3.3.4. Do not remove casework or alter the instrument in any way.

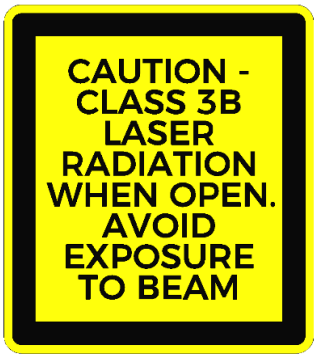
3.3.5. Only authorised Cellular Highways service personnel are allowed to remove the casework to adjust any components.

3.3.6. Caution – Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.

3.3.7. The Highway1 has been classified as a class 1 laser product in accordance with BS EN / IEC 60825-1:2014.

3.3.8. There are no apertures through which laser radiation in excess of class 1 limits is emitted when the Highway1 and cartridge are used according to instructions.

3.3.9. The labels below have been affixed to the rear panel of the Highway1 product.



3.4. Biosafety - Instrument cleaning

3.4.1. Cleaning of the Highway1 external surfaces may be required to decontaminate the Highway1 before entry into controlled lab areas or after use with biological material as part of standard lab practices. This procedure is intended to be used for these purposes.

3.4.2. Cleaning of the Highway1 optical window glass (behind the door) should be conducted only when required such as when dust or liquid or stains are observed on the window surface. Any attempts to clean the optical window glass of a new Highway1 are likely to reduce cleanliness. Experience and a careful approach are necessary to obtain a good result and avoid damage to the optical surface.

3.4.3. If cleaning is required, only the following materials have been verified for use with Highway1:

Purpose	Material
External Highway1 surfaces	Standard biology disinfectant (e.g., 70% ethanol solution)
Optical window glass	Highway1 Optics Wipes, A10000-1101
Gloves	Powder-free and oil-free gloves

3.4.4. Highway1 external surface cleaning procedure:

1. Wear gloves and select suitable lab disinfectant (if in doubt, contact Cellular Highways).
2. Unplug power supply and insert attached blanking plug into power socket.
3. Unplug USB cable.
4. Close Highway1 door.
5. Spray lab disinfectant over each Highway1 surface, avoiding overspray onto the optical window behind the door and avoiding direct spray into the back fan ducts.
6. Wipe down each Highway1 surface with lab wipes, avoiding contact with the optical window behind the door.

3.4.5. Highway1 optical window cleaning procedure:

Note: This procedure should only be performed if necessary to improve cytometry measurements. The procedure should not be performed routinely: it is best not to touch the optical window unless it is contaminated. Contact Cellular Highways support if in doubt.

1. Put on a new pair of gloves (powder-free and oil-free).
2. Open the Highway1 door and inspect the optical window.
3. If there are any particles, care should be taken with the first wipe to avoid scratching the surface.
4. Using the specified cleanroom wipe (part number 10000-3257 supplied by Cellular Highways), apply light pressure and wipe from the top of the window to the bottom in one continuous motion, minimising the contact with the metal surround as this may introduce more contamination.
5. Rotate the wipe to the next unused section and repeat the above step, avoiding using the same section of the wipe more than once.
6. Continue to repeat the above step until the window appears free of dust or stains.
7. As a last step, draw an unused section of the wipe slowly with light pressure from the top of the window to the bottom, aiming for the motion to take 10 – 20 seconds (to minimise streaks from solvent residue).
8. If this process is not effective, then contact Cellular Highways for further advice.

3.5. Biosafety - Cartridge disposal

3.5.1. Cartridges can be disposed of via conventional biohazard disposal routes (including incineration and autoclaving).

3.6. Biosafety - Sample containment

3.6.1. The use of the Highway1 cartridges with hazardous samples is entirely within the responsibility of the user. Risks must be assessed, and safety measures applied by the user.

3.6.2. The following advice is provided to the user to aid the risk assessment process:

- Do not overtighten the syringe luer connection to the cartridge as this may result in breakage of the luer fitting and leakage of sample.
- Do not re-use the cartridge multiple times as this may increase the likelihood of sample leakage.
- Ensure the correct syringe type is selected in the Highway1 software as an incorrect selection may result in sample leakage.
- Ensure that the cartridge is kept in the vertical position after use to minimise the risk of sample leakage.
- Avoid overfilling the cartridge output tubes (for example by pre-filling with additional liquid) to minimise the risk of cartridge leakage.

3.7. Trapping hazard

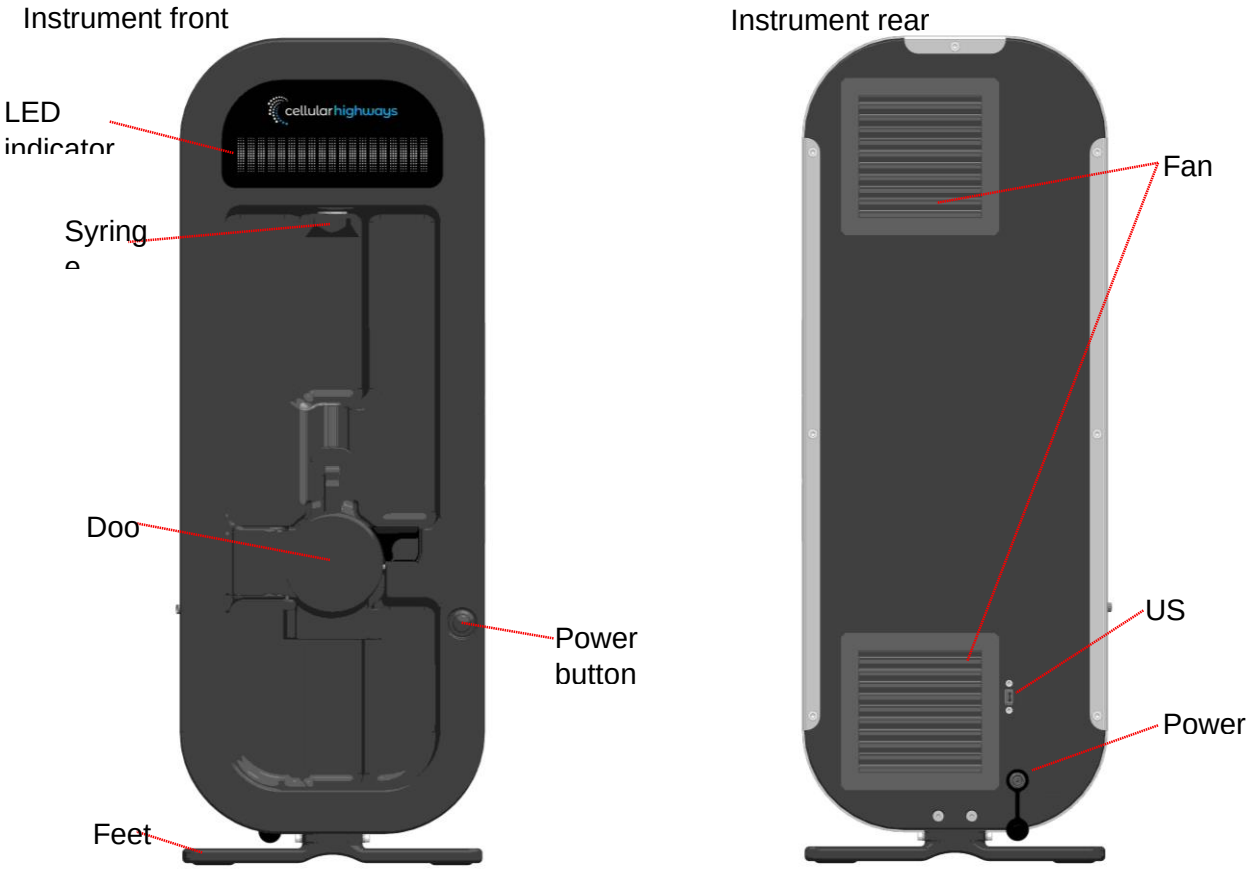
3.7.1. Only trained users should operate the Highway1 instrument.

3.7.2. Avoid touching any moving parts during operation as there is a risk of finger trapping and crushing.

4. Highway1 Cell Sorting System Overview

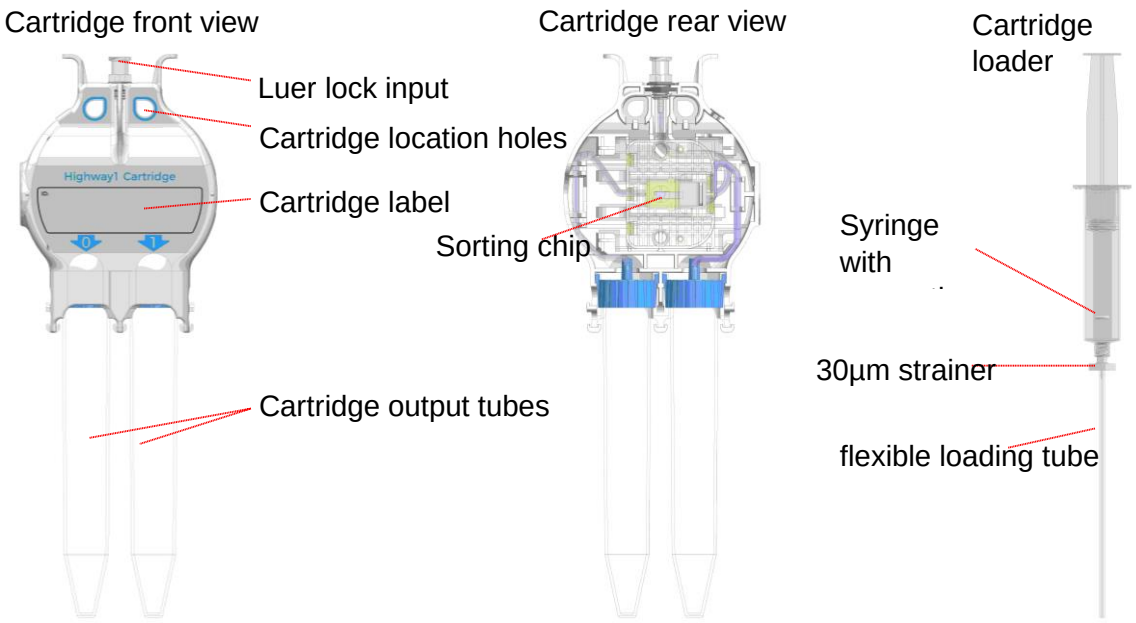
4.1. Highway1 Instrument

The instrument front and rear views are shown below.



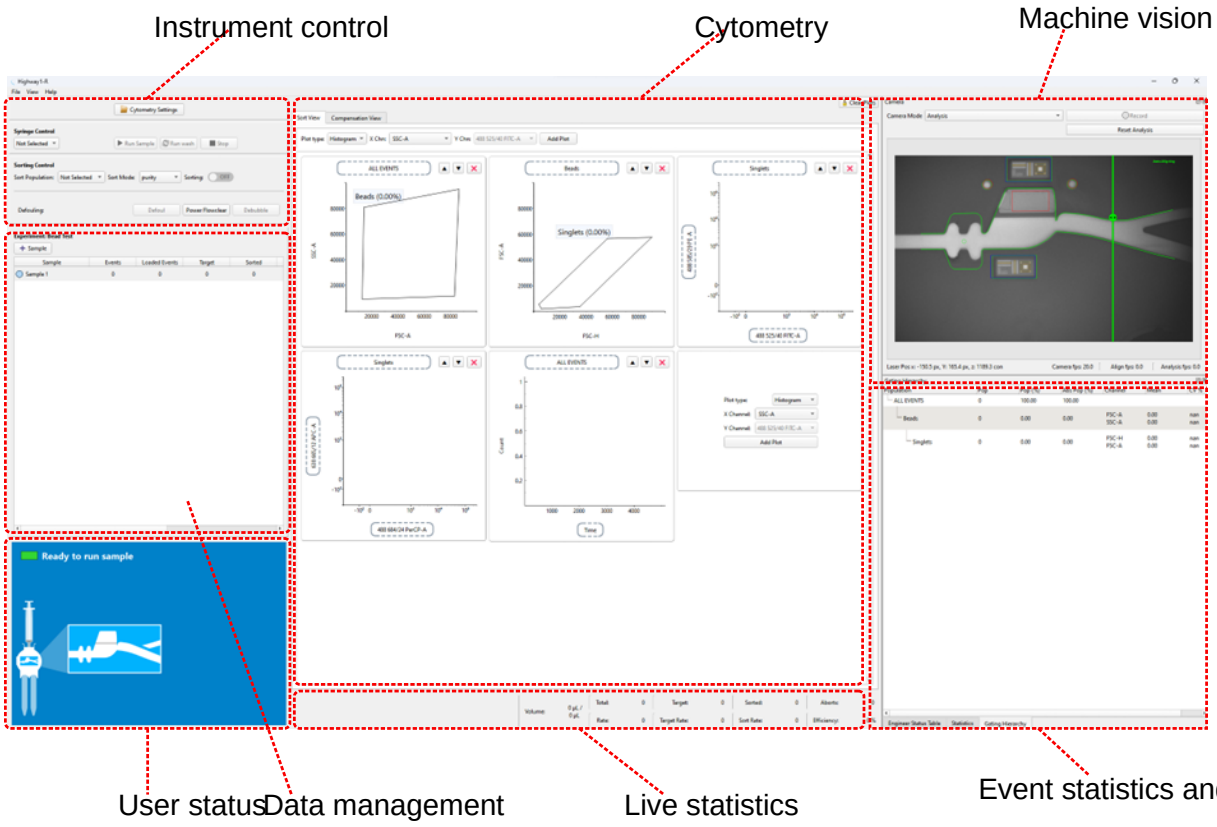
4.2. Highway1 RUO Cartridge and Cartridge Loader

The front and rear views of the RUO cartridge and cartridge loader are shown below.

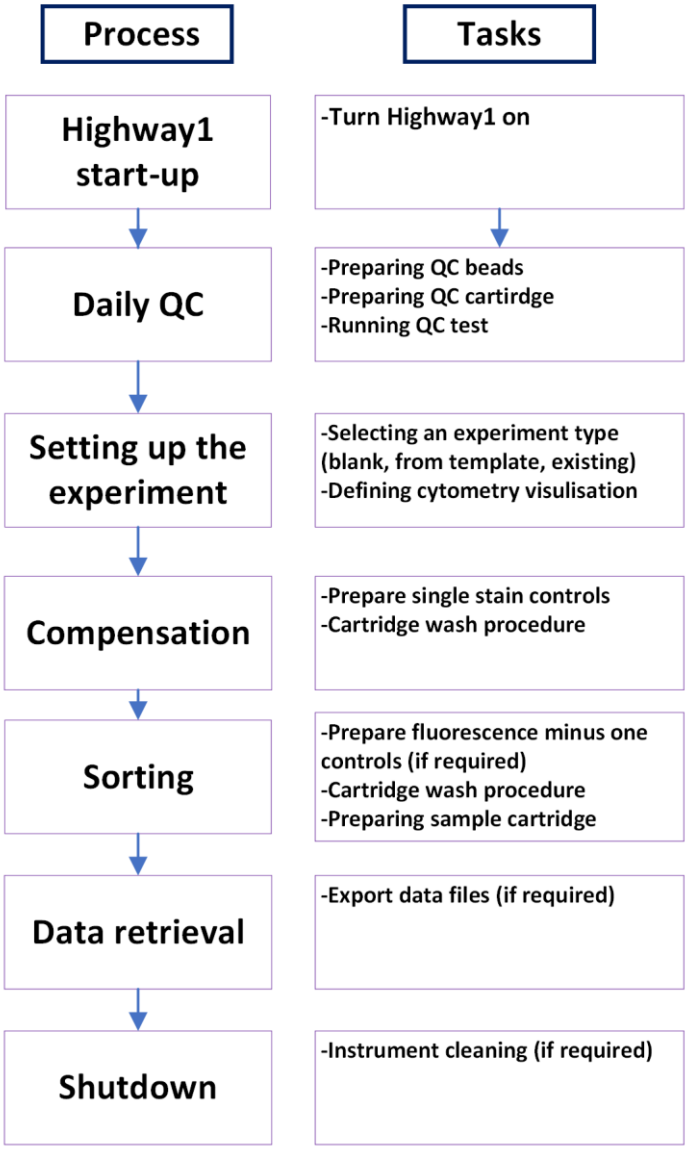


4.3. HighwayR Software

The Highway1 instrument is controlled by the HighwayR software, which is pre-loaded onto the host PC supplied by Cellular Highways. The operation and use of the software is described further in section 6 below.



3.4 Generic workflow diagram



5. Materials

Product code	Product Name	Pack Size
A10000-1001	Highway1 Instrument	1
A10000-1029	Highway1 Syringe Adapter 3mL	1
A10000-1069	Highway1 In-line Strainer	25
A10000-1073	3mL Syringe	25
A10000-1076	3mL Syringe Blunt Needle	100
A10000-1077	3mL Syringe Strainer	25
A10000-1078	Highway1 Fan Filter	5
A10000-1079	Daily QC Beads Kit	1
A10000-1081	Highway1 Cold Pack Kit	1
A10000-1082	Highway1 Cartridge Rack	1
A10000-1083	Highway1 Cartridge	25
A10000-1084	Highway1 Cartridge Loader	25
A10000-1101	Highway1 Optics Wipes	30

Notes:

Only the syringes listed above and included in the Highway1 Cartridge Loader are validated for use with Highway1. Highway1 Cartridge Loader's syringe contains a magnetic stirrer which is crucial for maintaining a homogenous sample and good sort performance during sorting.

Each cartridge is only validated for a single use.

6. Operating Highway1

6.1. Start-up procedure

6.1.1. To start the Highway1 cell sorter, the following procedure should be followed:

1. Turn on Highway1 using the power button.
2. Switch on the PC.
3. On the desktop, double click the HighwayR icon. The software user interface (UI) will initialise.
4. Once loaded, check the user status panel at the bottom left of the UI to make sure the Highway1 and computer are connected. A red warning will say *Instrument disconnected* if there is any connection problem.
5. Highway1 warm-up will be initiated and requires up to 30 minutes.
6. Once the warm-up is complete the user status states, *Instrument Ready*.

6.2. Highway1 cartridge loading procedure.

6.2.1. Three types of Cellular Highways cartridge are compatible with the Highway1:

- Highway1 RUO cartridge.
- Highway1 GMP cartridge OS.
- Highway1 Pro GMP cartridge CS (separate instructions).

6.2.2. Highway1 RUO cartridge and Highway1 GMP cartridge (basic) are loaded with the following instructions. The cell suspension is drawn into a syringe through an in-line 30µm strainer. This syringe is then placed on the cartridge, which is then closed to airborne microbes.

1. Unwrap Highway1 cartridge (consistent with aseptic handling techniques, e.g., in a biosafety hood).
2. Place the cartridge upright in the Highway1 Cartridge Rack.
3. Open the sample for loading (consistent with aseptic handling techniques).
4. Unwrap the Highway1 cartridge loader (consistent with aseptic handling techniques).
5. Using the syringe, draw the sample into the cartridge loader, up to a maximum volume of 22 mL in the nominally 20 mL syringe.
6. With the end of the cartridge loader tube still in the sample, invert the syringe without detaching it from the loader tube.
7. By pushing on the syringe, expunge the air bubble so that no more than around 0.5 mL of air remains in the syringe.
8. Detach the syringe from the cartridge loader.
9. Place the syringe on the cartridge luer lock and apply very gentle torque with the fingertips to seal. The cartridge is now ready for sorting. If using a biosafety hood, the cartridge can now be taken out of the hood.

Note: DO NOT OVERTIGHTEN THE LUER LOCK! A luer lock needs only very gentle pressure to seal as can be applied with the fingertips. Overtightening risks cracking the port.

6.3. Daily QC procedure.

6.3.1. The performance of the Highway1 should be confirmed prior to use by running the Daily QC test. This checks both cytometry and sorting functions on a separate cartridge before sorting cells on a new sterile cartridge.

6.3.2. To prepare the Daily QC beads for running on Highway1:

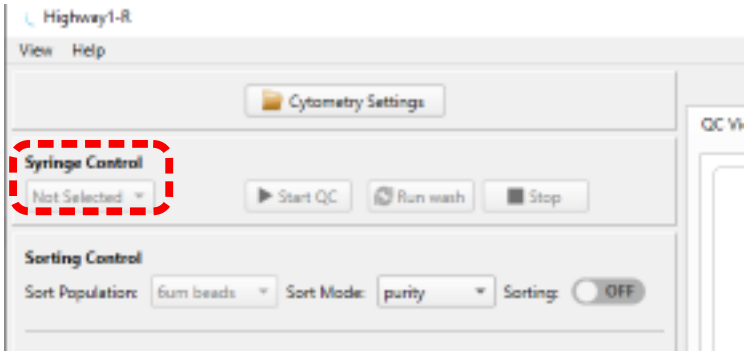
1. Vortex the Daily QC bead bottle for 10 seconds, then add 2 drops of beads to 2 mL of deionised water in a 12x75mm polypropylene tube.
2. Draw up the solution in a 3 mL syringe using a blunt needle.
3. Remove the blunt needle then attach the syringe to a Highway1 cartridge.

6.3.3. Performing the Daily QC test.

1. Open HighwayR and select *Daily QC* as shown below.

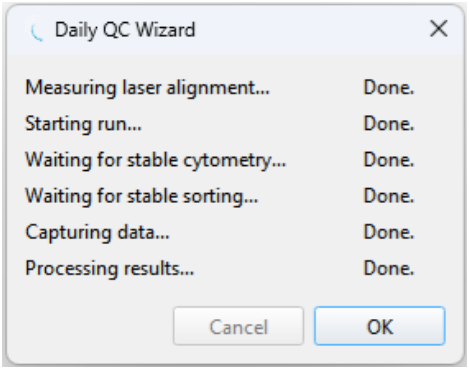
1. *Figure 6-1 Start up window*

2. Select 3 mL syringe from *Syringe Control* dropdown menu. A cartridge loader (A10000-1084) may be used if needed.



1. *Figure 6-2 Sorting Control panel*

3. Install the 3 mL adapter into Highway1.
4. Open the door and insert the prepared cartridge.
5. Close the door (ensure the latch engages). Highway1 will now start auto-alignment.
6. Wait until the status box states, *Cartridge Aligned*.
7. Select *Start QC*. The QC will go through several steps, checking laser alignment, checking cytometry acquisition, starting sorting, and checking the stability of sorting.



1. *Figure 6-3 Daily QC Wizard*

8. On completion, a .pdf report will be generated, with either a pass or fail result, Figure 6-4 Daily QC report example-. The test criteria and test results are reported in detail to allow remote debugging by Cellular Highways support in case of a failure.
9. If a failure is observed on the Daily QC test, inspect the report, and repeat the Daily QC once with a new cartridge. If the error recurs, contact Cellular Highways support.

Note: The Daily QC procedure tests both cytometry and the sorting process. This is our recommended test to make sure that the Highway1 is working correctly before running a real sample, to check that the sample shall be sorted properly.

cellularhighways

Daily QC Report - 2023-11-23 10:24:40

Date: 2023-11-23 10:24:40
Instrument Uptime: 01:08:31
Instrument: SPLINTER
Board Serial: YYYY-MMDD-XXX

FPGA Version: HW1B.1.11.1138
MCU Version: HW1B.1.12.6
Software Version: HW1B.1.12.0

Signal Readings - Pass

Channel	Median	Target Median	Median Diff (%)	rCV (%)	Target rCV (%)	Result
BFSC (6um)	7810.2	7675.9	1.7	3.9	10.0	Pass
BFSC (10um)	21295.6	21008.7	1.4	1.6	10.0	Pass
RFSC (6um)	8233.9	7992.2	3.0	4.2	15.0	Pass
RFSC (10um)	24197.9	23546.9	2.8	4.7	15.0	Pass
SSC	11700.9	11228.6	4.2	14.8	20.0	Pass
FL1+	42086.3	41003.8	2.6	6.2	10.0	Pass
FL2+	41769.5	41068.3	1.7	6.4	10.0	Pass
FL3+	20639.3	20350.5	1.4	5.8	10.0	Pass
FL4+	1732.8	1640.9	5.6	6.7	10.0	Pass
FL1-	39.2	39.4	0.6	28.5	500.0	Pass
FL2-	15.6	16.0	2.6	54.7	500.0	Pass
FL3-	14.7	15.6	5.7	104.8	500.0	Pass
FL4-	3.7	3.8	3.5	396.1	500.0	Pass

Stability Readings - Pass

Parameter	Value	Target	Warning Threshold	Error Threshold	Result
Instrument					
Warmup (%)	100.0	-	≥ 100.0	≥ 100.0	Pass
Temperature (°C)	30.1	30.0	± 1.0	± 1.5	Pass
Cartridge					
Chip Resistance (Ω)	25.1	25.5	± 5.0	± 7.0	Pass
Flow					
Pressure Stability (mbar)	1.0	-	≤ 50.0	≤ 100.0	Pass
Flow Rate (µL/min)	277.2	265.0	± 50.0	± 100.0	Pass
Laser Pocket Bubble (%)	0.0	-	≤ 4.0	≤ 8.0	Pass
Actuation X Position Jitter (µm)	1.3	-	≤ 6.0	≤ 10.0	Pass
Inertial Focusing (µm)	0.3	0.0	± 2.0	± 4.0	Pass
Cytometry					
Laser Delay Stability (µs)	0.0	-	≤ 1.0	≤ 3.0	Pass
Splice Quality (%)	98.0	-	≥ 80.0	≥ 70.0	Pass
Stream Correction Split (%)	50.2	50.0	± 20.0	± 40.0	Pass
Sorting					
Image Actuation Presence (%)	100.0	-	≥ 85.0	≥ 70.0	Pass
Verification Deflection (µm)	28.1	28.0	± 8.0	± 14.0	Pass
Verification Declension (µm)	143.4	130.0	± 20.0	± 40.0	Pass
Bubble Coverage (%)	31.1	-	≥ 10.0	≥ 5.0	Pass
Sort Fidelity (%)	99.4	-	≥ 90.0	≥ 80.0	Pass
Focusing Error Rate (Hz)	0.0	-	≤ 100.0	≤ 200.0	Pass
DSP					
Channel 0 (BFSC) Baseline	-35446.0	-34897.3	± 3000.0	± 6000.0	Pass
Channel 0 (BFSC) Baseline Stability	120.7	-	≤ 600.0	≤ 1000.0	Pass
Channel 1 (RFSC) Baseline	-41509.1	-42115.6	± 5000.0	± 10000.0	Pass
Channel 1 (RFSC) Baseline Stability	1126.2	-	≤ 1200.0	≤ 2000.0	Pass
Channel 3 (SSC) Baseline	-101221.7	-101180.0	± 800.0	± 1500.0	Pass
Channel 3 (SSC) Baseline Stability	28.1	-	≤ 200.0	≤ 500.0	Pass

Figure 6-4 Daily QC report example

7. Performing a sort

7.1.1. The first step to perform a sort is to prepare an experiment in the software in which the cytometry visualisation and the instrument settings have been defined to allow proper identification of the target population.

7.1.2. We recommend that a diluted aliquot of the fully stained sample should be run first on Highway1 to set voltages prior to any controls.

7.1.3. Once the experiment workspace has been defined and the appropriate controls (either single stain compensation controls, fluorescence minus one controls or both) have been acquired, the sample of interest will be loaded, and the sort initiated. The sort will then run to completion.

7.2. Setting up an experiment

7.2.1. There are two options to run an experiment on the Highway1,

1. Load experiment,
2. Create new experiment blank experiment or from template.

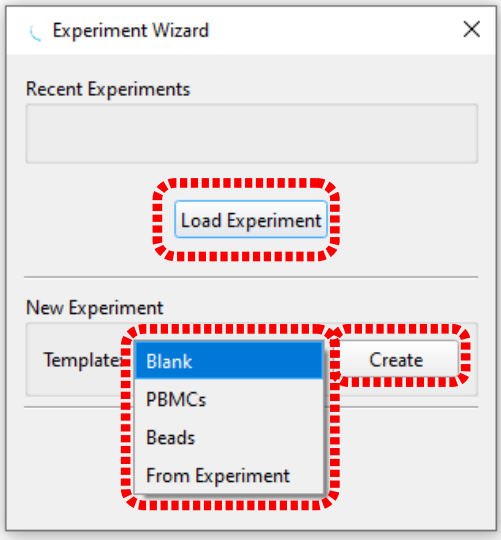


Figure 7-1 Experiment Wizard

7.2.2. Three template options are provided for new experiments that set plot ranges appropriately:

- PBMCs – ranges appropriate to cells found in blood, such as B cells and T cells.
- Beads – ranges appropriate to polystyrene microspheres up to around 10µm diameter.
- From Experiment – ranges, gains, plots, gate names and gating hierarchy will be copied from an existing experiment file as selected.

7.2.3. If a new experiment is selected, file explorer will launch and prompt the user to name the experiment file.

7.3. Defining the cytometry data visualisation

7.3.1. To enable the user to visualise the cytometry data, the user can display event parameters (for example FSC-A) in either 1D or 2D histograms. Subsets of data can be further interrogated by using a hierarchical gating structure. This section will outline how to create, modify, and delete these plots to allow the user to set-up the experiment as they require.

7.3.2. The default layout is shown here:

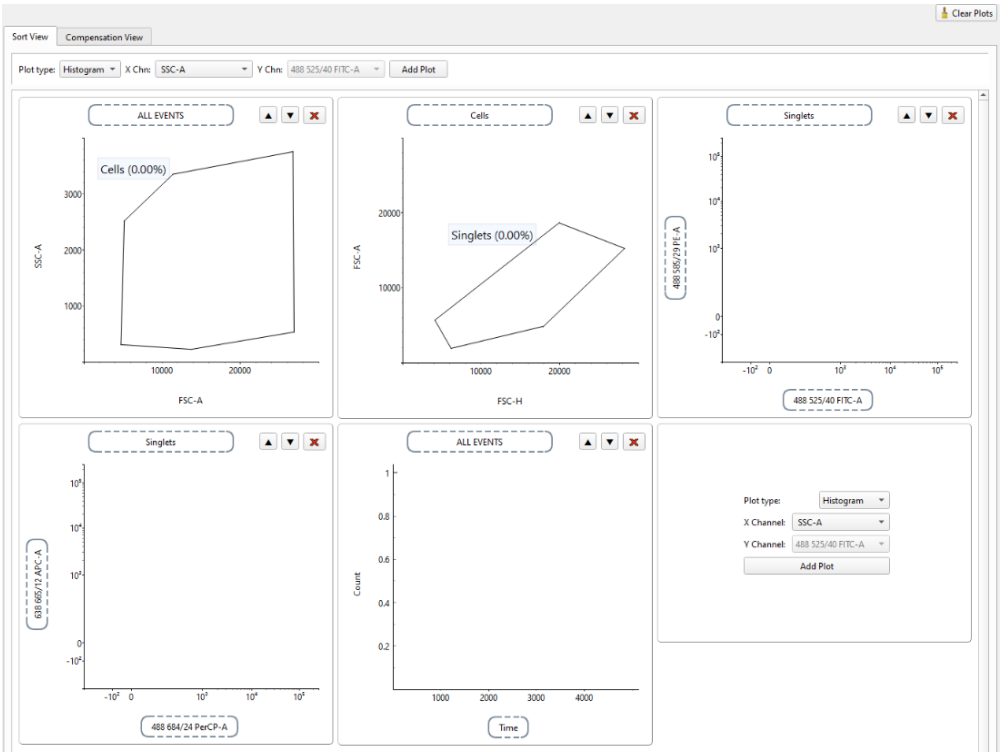


Figure 7-2 Default Plot layout and gates for PBMCs template.

The following operations can be performed to change the visualisation of the cytometry data.

a) Add or remove plots.

b) Change the ordering of the plots.

c) Change the axis parameters.

d) Change the scaling of the axis.

e) Change the parameter labels.

7.3.3. Add or remove plots.
To add a plot, at either the top or bottom of the *Sort View*, select Plot type (Density = 2D histogram, Histogram = 1D histogram), X and Y parameters, and press *Add Plot.*

Figure 7-3 Top Add plot menu

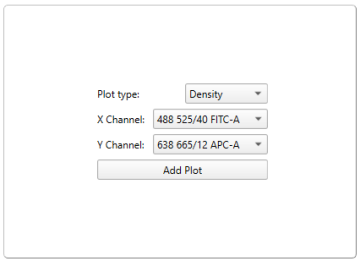
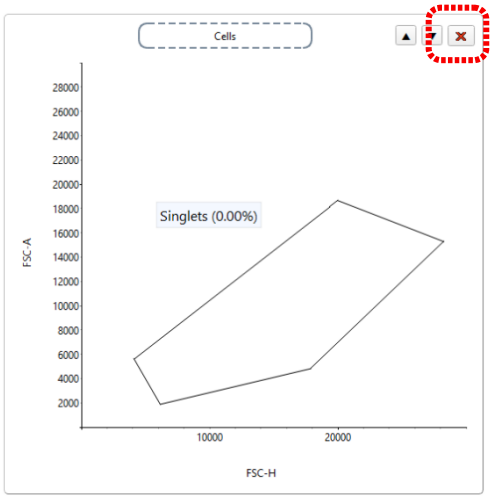
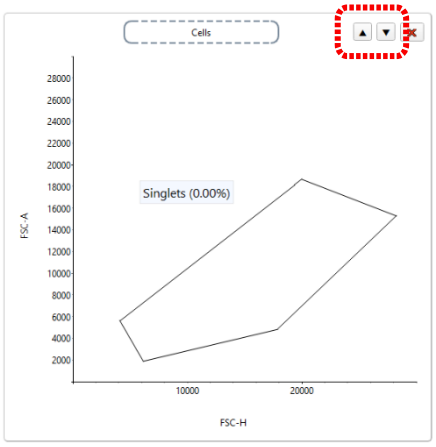


Figure 7-4 Bottom Add plot menu

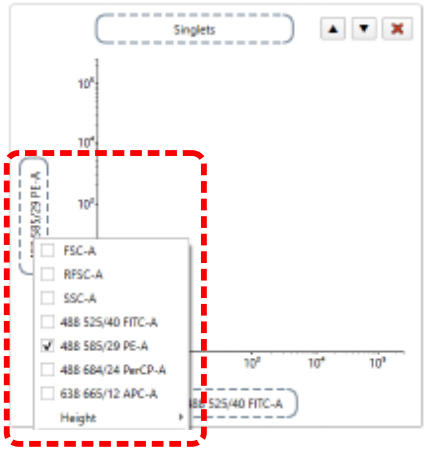
To remove a plot, press the red cross in the top right corner of the plot to be deleted (Note: this will also delete the gating structure).



Graph ordering: to change the ordering of the plot on the screen, press the up and down arrows in the top right corner.



7.3.4. Axis Parameters: to change graph axis parameters, left click on the axis label and select the chosen parameter from the dropdown menu.



7.3.5. Axis Scaling: the scaling of a plot can be changed by changing the values in *Cytometry Settings* > *Plot Ranges* (Note we have provided defaults to ease usage). The type of scaling can also be changed by right-clicking on a plot axis and selecting a different type of scaling options: linear, log or logicle).

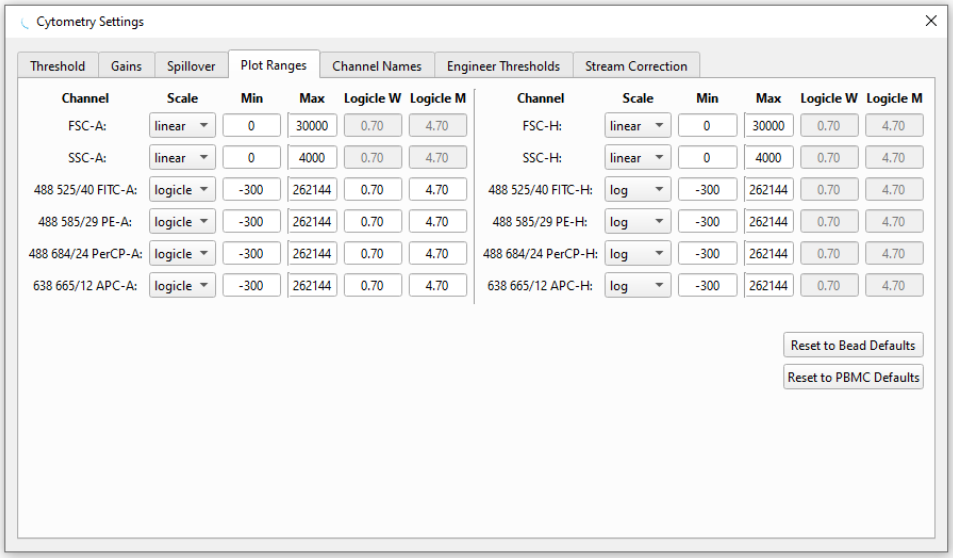


Figure 7-5 Plot Ranges window

7.3.6. Parameter labels: to change the channel names, navigate to: *Cytometry Settings* > *Channel Names.*

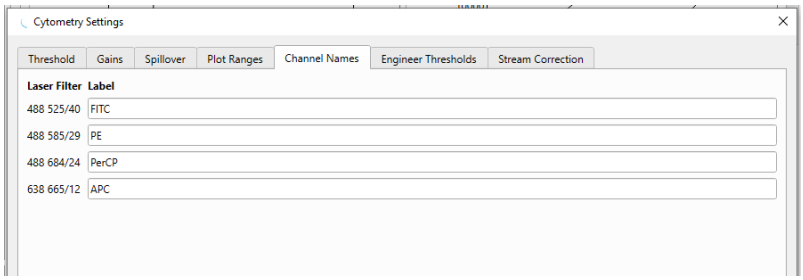
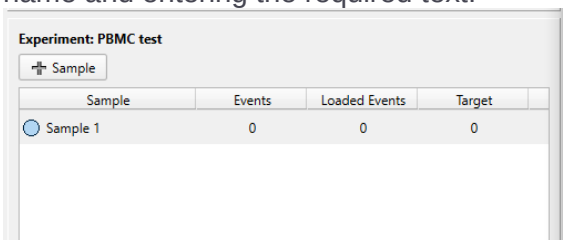


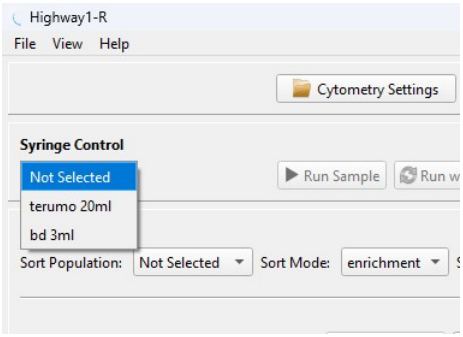
Figure 7-6 Channel Names window

7.3.7. Acquiring samples

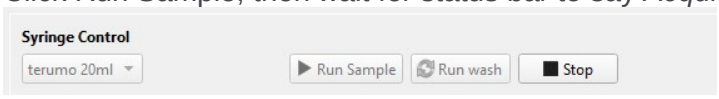
1. Press + *Sample* to add a new sample. Name sample by double clicking on sample name and entering the required text.



2. In *Syringe Control*, select chosen syringe from the dropdown menu. Ensure the correct adapter is in the machine.



3. Load syringe with a small amount of fully stained sample and attach to setup cartridge.
4. Wait until the status bar says *Cartridge Aligned*.
5. Click Run Sample, then wait for status bar to say *Acquisition Stable*.



6. Click Clear Logged Data to clear event data acquired during the stabilisation stage.

7.3.8. User Threshold

Once data acquisition is stable adjust user threshold so that it sits just below the cells in the FSC. Anything below this threshold will not be displayed or considered in sorting. If threshold is set too high, some cell data will be missed in analysis or sorting. If it is too low debris will be included in coincidence rejection, which will reduce the efficiency and recovery of sorting.

7.3.9. Stream Correction (Optional)

To ensure the Highway1 is sorting optimally, the stream correction plot may be checked within the cytometry settings.

Note: Stream correction is an automatic feature in the Highway1 Cell Sorting System that allows us to display conventional cytometry data to the user. Since the cells are focussed with inertial focussing rather than sheath flow, instead of a single focussed particle stream passing through the laser foci, there are two streams, which generate different measurements. Stream correction automatically detects which stream a cell is in and automatically maps the measurements from both streams onto a single corrected scale for each measurement channel.

To check and/or update the stream correction settings, follow these steps:

1. Begin acquiring sample.
2. Open Cytometry Settings, and navigate to the Engineer Thresholds tab.
3. Adjust the stream correction gate (dotted line) so that it diagonally divides the two populations, or press the *Estimate Stream Split* button, which recalculates the stream correction gate and all stream correction factors automatically.

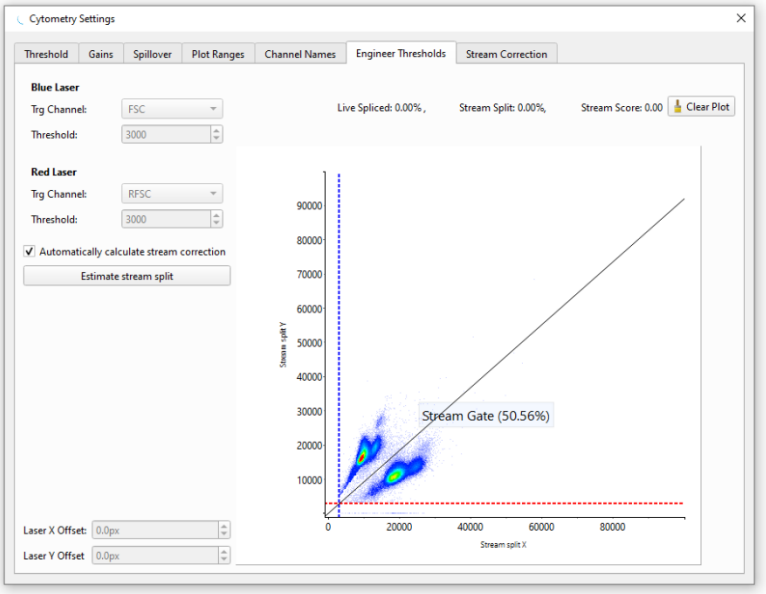


Figure 7-7 Engineering Thresholds window (Stream Gate)

7.3.10. Adjusting Forward and Side Scatter

To improve visualisation of the FSC (Forward Scatter) and SSC (Side Scatter), go to *Cytometry Settings* > *Plot Ranges*, as described in 7.3.5 above.

7.3.11. Setting gains

To set gains go to C*ytometry Settings* > *SIPM* (Silicon Photomultiplier). Alter SiPM fluorescent voltages so that populations are well-resolved on the cytometry plots.

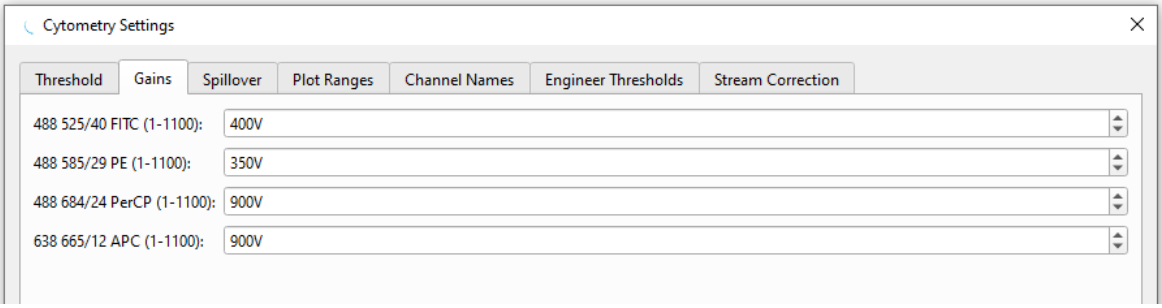


Figure 7-8 Gains window

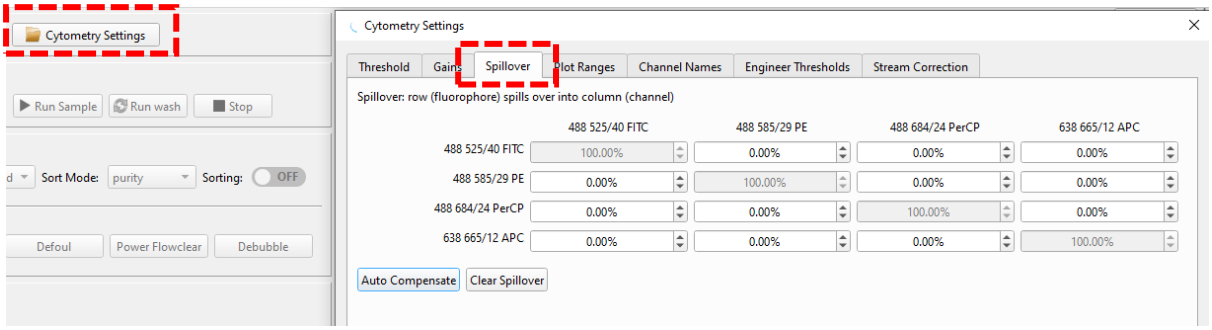
7.3.12. Running a wash (Optional)

1. Load a syringe with buffer or a cleaning solution and attach it to the cartridge.
2. Press Run Wash to clear out the cartridge.

Note: it is not recommended to re-use cartridges for valuable or sterile samples. However, in most circumstances, it is acceptable to re-use cartridges for DailyQC and for cytometry set-up and checking of outputs on the same gates. If it is desirable to wash out a sample, it is recommended to flow either 2 mL of the wash solution or load the cartridge with an excess of the wash solution and flow it until the event rate reaches zero. For a wash solution, either use plain saline (PBS/DPBS plus 0.1% P188) or use a commercial cytometry cleaning solution.

7.3.13. Compensation

1. Once the voltages have been set with the fully stained control, click on the *Compensation View* tab to see if any compensation is required.
2. To compensate, first prepare your compensation samples (see note below). Flow each control and record at least 2000 positive and at least 2000 negative events. Name each sample with the fluorochrome being used. Make sure to wash between each control to prevent sample spillover.
3. Once the samples have been acquired, go to: *Cytometry Settings* > *Spillover* > *Auto Compensate* to automatically compensate each sample.
4. Alternatively, the compensation values can be altered manually in the table.
5. To clear, select Clear Spillover.



Note: compensation can be done with either compensation beads, antibody-capture beads, or cells. It is possible to use all the usual methods of compensation that cytometrists employ:

- Compensation from the individual single-colour controls. Note that these controls may be fluorescent beads, antibody-capture beads, or stained cells. HighwayR's automatic compensation normally works well on all these sample types.
- Compensation from the mix of individual single-colour controls. HighwayR's automatic compensation normally works well on the mix fluorescent beads or antibody-capture beads. This normally saves time since the compensation can be done on a single sample with no washing.
- Compensation of the fully stained sample, by eye. This is typically performed when there is a strong expectation of what the compensated cytometry should look like.

7.3.14. Drawing gates

a. To draw gates, right click on a cytometry plot and select *Add Rectangle/Ellipse/Polygon/Bisection.*

b. To adjust a gate, select a handle so that it turns blue and drag it to the desired location.

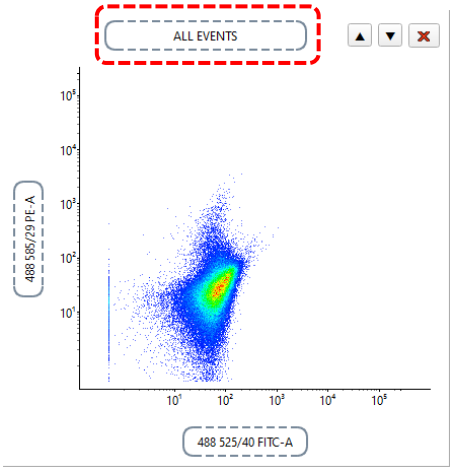
c. To move an entire gate, hover the mouse over the centre of the gate until all the dotted lines turn blue, then drag the gate.

d. To add another handle (polygons only), hover over one of the edges until it turns to a dashed blue line, then left click. To delete a handle, right click on the unwanted handle and select *Remove handle*.

e. To convert to a polygon (rectangles only), right click on the gate and select *Convert to Polygon*.

f. To delete a gate, either right click on the plot containing the gate and go to *Delete Gate* in the dropdown and select the desired gate. Or right click on the gate and select *Delete Gate.*

g. To change gating hierarchy, left click on the plot title (default: *ALL EVENTS*) at the top of a plot and select a gate from the dropdown menu.



7.4. Sorting

Selecting a population to sort:

1. Once compensation has been completed, return to *Sort View,* and set gates for fluorescent parameters. Fluorescence minus one (FMO) control samples may be required for this if there are not well separated positive and negative populations.
2. Set gating strategy for sorting and select *Sort Population.*
3. Select the *Sort Mode.* There are three options: enrich, purity and high purity.

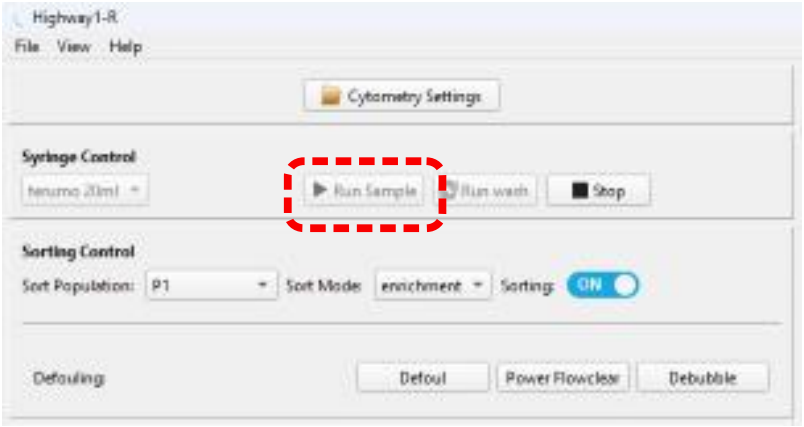
Table 1 below lists the maximum cell concentrations and total cell numbers for the purity and enrichment modes.

Table 1 Summary of Purity and Enrichment modes

	Purity mode	Enrichment mode
Max Throughput (cells/s)	10,000	37,000
Full cartridge capacity (22 mL runs in 83 min)	50e6 cells (2.3e6/mL)	185e6 cells (8.4e6/mL)

7.5. Running a sort

1. Load new cartridge with sample and place on instrument; close door.
2. Wait until status bar states *Ready to run sample*.
3. Run sample. Wait until status bar states *Acquiring. Ready to sort.*
4. Switch Sorting to *On*.
5. When the status bar changes to *Sorting*, you can walk away from the machine.
6. The status bar will say *Sorting complete* once sorting has finished.



Note: when sorting is completed (or if the stop button is pressed), the machine vision panel of HighwayR may still show stray particles passing through the chip. This can be ignored as it is a small residual flow that does not reach the Sort1 and Sort 0 outputs.

8. Data storage

Data from each sort operation (and all Daily QC data) is automatically saved on the Host PC.

Data is automatically organised in the Cellular Highways folder in the Documents. There will be two folders: Experiments and Daily QC.

The data in the Experiments folder is structured as follows:

1. All the samples within one experiment are saved in a folder named with the experiment name and accompanied by a .hwe file of the same name. The .hwe file is used to reopen the experiment in HighwayR.
2. Each time a new acquisition or sort is initiated, a new sample will start recording in HighwayR's native format (.hwr).
3. Once the run is complete the sample will automatically be exported as a standard FCS file (.fcs). The acquired data is subsampled automatically to reduce the file size (500,000 events default).

The Daily QC folder contains one experiment folder (DailyQC), a sample file (DailyQC.hwe), and a Daily QC Reports folder.

Highway1 data files:

1. There are several files saved in each run. The following are primarily intended for the User:
    - <Experiment name>.hwe – the file used to re-open an experiment on HighwayR software.
    - <Sample name>.hwr – HighwayR's native data file containing the full cytometry data.
    - <Sample name>.fcs file – the data file required to load a sample in a range of analysis software, for example, FlowJo.
    - Sort Report <DATE> <TIME>.pdf – a sort report summary including sort counts, and flow cytometry plots.
    - config.ini – configuration of the Highway1 instrument saved at the time that the run was completed.
    - log.txt – a human-readable log of the run, including errors and warnings.
    - Other files are primarily intended for Cellular Highways Support and troubleshooting. These include instrument_log.csv, which contains all internal sensing and process analytical data of the instrument during a sample run. A subsample of event data is also recorded as raw images and channel traces.

9. Shutdown

1. Close HighwayR software.
2. Switch off the Highway1 by pressing the power button once; the lights on the Highway1 will then flash blue.
3. Press the power button a second time; the lights on the Highway1 will flash yellow and the Highway1 will shut down within 10 seconds.
4. Shutdown the PC.

10. Troubleshoot guide

If an issue occurs, first try the following quick solutions. Otherwise, contact Cellular Highways Support (contact details below).

Startup	
Machine or computer not turning on	1. Check all cables are plugged in and switched on at the mains
Machine not connecting to computer	1. Check all cables are connected 2. Close the HighwayR software and open it again 3. Restart instrument and host PC 4. Shut down instrument and host PC, turn off both at mains switch, turn on and try again
Lasers not turning on/ Laser error reported	1. Close HighwayR 2. Shut down instrument 3. Restart both
High chip resistance error reported	1. Change to a new cartridge
Alignment error reported	1. Make sure door is closed properly until it clicks 2. Reseat cartridge and close door 3. Try a new cartridge
Flow rate outside range error reported	1. Check correct syringe and adapter are selected 2. Remove cartridge and check for bubbles in syringe. Purge any bubbles present 3. Replace cartridge
Pressure instability or blockage error reported	1. Check for clumps in sample 2. Run again and manually activate *Power Flow Clear*. See whether gunk is shifted and whether run proceeds without issues 3. Run wash

	4. Change cartridge
Leak error reported	1. Check correct syringe and adapter are selected. (Incorrect syringe or adapter may be falsely reported as a leak.) 2. Check whether there really is a leak – is the cartridge wet or has any liquid dropped from the bottom of the cartridge? 3. Change cartridge

Acquisition	
Cytometry unstable error reported: • **Splice error** • **Laser delay instability** • **Stream correction error**	1. Run Daily QC. If unsuccessful, replace cartridge 2. Increase user threshold so that it is just below where cells are located on the FSC plot 3. Try to reduce sample debris and clumping by: ▪ Filtering reagents through <0.2µm filter ▪ Filtering cells/beads prior to running ▪ Adding 0.1% P188 to bead/ cell solutions to reduce sticking ▪ Adding DNase or EDTA to cell samples
No events seen	1. Are bubbles trapped in the laser pockets on the live machine vision panel? Press de-bubble manually if any bubbles are seen 2. Run QC beads through Highway1 at recommended concentration
Outgassing of bubbles in laser pockets	1. Check for large bubbles in sample syringe and purge 2. Warm sample up to room temperature or if sample is cooled, attach cooling blocks

	3. Resuspend cells in pre-warmed buffer if applicable
Event rate too low	1. If population of interest is clipped, decrease user threshold until it is just below where cells are situated on FSC plot 2. Resuspend sample at higher concentration
Event rate too high	1. Increase user threshold to just below where cells are located on the FSC plot Note: user threshold sets the FSC-A level of debris that can be ignored from coincidence rejection in purity mode sorting. Debris will therefore still be sorted if in same envelope as target 2. Dilute sample
High gunk level	1. Try to reduce sample debris and clumping by: ▪ Running samples with cooling blocks attached ▪ Filtering reagents through 0.02µm filter ▪ Filtering cells/beads prior to running ▪ Adding 0.1% P188 to bead/ cell solutions to reduce sticking ▪ Adding DNase or EDTA to cell samples Please note: if none of these options work and there is still too much gunk, we recommend improving sample preparation before sorting on the Highway1. Otherwise less than optimal results will be achieved.

Sorting	
Low sort fidelity error reported	1. Replace cartridge 2. If cartridge has been re-used, run wash solution through cartridge

Low efficiency	1. Ensure most appropriate sort mode selected. 2. Increase user threshold to ignore debris from sort decisions 3. Reduce cell concentration to reduce number of coincidence-based aborts Note: purity mode rejects coincidence events. At the recommended maximum purity mode event rate of 10,000 cells/s, efficiency should be around 70-80% due to about 20-30% coincidence events. In enrichment mode however, efficiency should be close to 100% provided that the maximum sustained sort rate is not exceeded, i.e. target rate below 4000/s.
High focussing error rate	1. Try to reduce sample debris and clumping by: ▪ Filtering reagents through 0.02µm filter ▪ Filtering cells/beads prior to running ▪ Adding 0.1% P188 to bead/ cell solutions to reduce sticking ▪ Adding DNase or EDTA to cell samples 4. Try setting sort mode to High Purity instead of Purity 5. Dilute sample to reduce sort rate
Target rate greater than 4000/s	1. Dilute sample to improve recovery
Low yield caused by high rate of actuator fouling	1. Use sort media with lower protein concentration 2. Add P188 to sample 3. Dilute sample to reduce sort rate 4. Wash cartridge between runs with protein-free media 5. Change cartridge

Failing Daily QC	
Fluorescent or scatter parameters are failing	1. Prepare fresh sample of QC and rerun 2. Wait 10 minutes and rerun 3. Try a new cartridge
Fluorescent or scatter parameters passes but sorting fails	1. Try a new cartridge

11. Contact CHW Support

support@cellularhighways.com, include screenshot, log file or Daily QC report showing the issue, if applicable

Call +44 1763 262626, ask for Cellular Highways Support, Mon-Fri 09:00 – 17:30 UK time

WhatsApp Messenger send text to +44 7356 115489, Mon-Fri 09:00 – 17:30 UK time