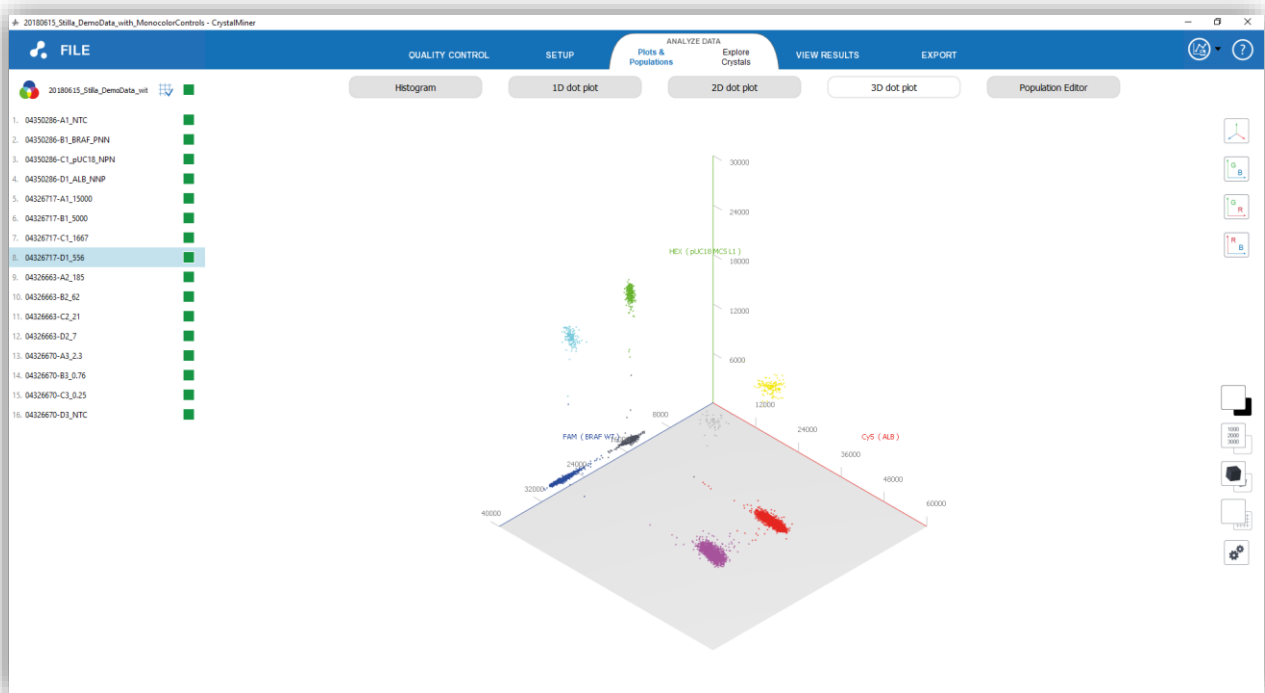




# Crystal Miner™ for the Naica™ System



## User Manual v2.0



## TECHNICAL SUPPORT

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## I. Introduction

### 1. Purpose of the document

This document is the specific “Crystal Miner” user manual. It provides all detailed information for the use of the “Crystal Miner” software application which is part of the Naica™ System.

Please refer to the general user manual named “Naica Crystal Digital PCR System” for complete information about the use of the materials and equipment composing the Naica™ System.

### 2. What is Crystal Miner?

The Crystal Miner™ software component of the Naica™ System is dedicated to the last step of the Crystal Digital™ PCR workflow: the intuitive visual inspection and analysis of the digital PCR experiment.

Once the image acquisition step has been performed using the Prism3™ instrument and the Crystal Reader™ software, you are ready to analyze the experiment and calculate your nucleic acid concentrations using the Crystal Miner software.

### 3. Intended use

The Naica system is a laboratory instrument for use by qualified personnel in a climate-controlled environment. The Naica system is intended for high-resolution genetic analysis based on digital PCR.

The Naica system is intended to be used for research use only, not for diagnostic procedures.

## II. Context & Prerequisite

### 1. Definition of an experiment

An experiment is a set of chambers which:

- Belong to unique Stilla chips (defined by a unique ID)
- Share the same experimental conditions, i.e.:
  - the same primers and the same probes (which implies the same fluorophores and the same targets)
  - the same Master Mix
- Have been imaged at 6.7 microns / pixel by the Prism3 instrument with the same scanning parameters, i.e.:
  - the same exposure times (in ms) for each of the 3 LEDs
  - the same focus value (in mm)

### 2. Prerequisite for using Crystal Miner software

This experiment analysis step can be performed either on the PC provided by Stilla or on any other PC (portable or fixed PC) which has the following specifications:

- **Operating System:** Windows 10 in 64 bits
- **RAM:** at least 16 Go
- **Processors:** Intel Core i5 or higher, at least 2 cores of 2 GHz or higher
- **Graphical Card:** recommended (equivalent to NVIDIA GeForce GT 1030 or higher)
- **Screen resolution:** at least 1920 x 1080; aspect ratio 16:9

To install the Crystal Miner software on another PC, plug the Stilla USB key then go to the “Crystal Miner Installer” directory. You can also download the installer from the website [www.stillatechnologies.com/technical-resources](http://www.stillatechnologies.com/technical-resources)

If you run the Crystal Miner application on a portable PC, the laptop should be plugged in to a power source for optimal use.

### III. Workflow of Experiment Analysis

- Double-click on the desktop icon to launch the Crystal Miner application.



Fig. 1 - Launch icon

The 5 main steps of the experiment analysis workflow using Crystal Miner software are as follows:

- (1) Load an experiment
- (2) Check the fluorescence spill-over compensation
- (3) Check the fluorescence thresholds
- (4) Get the concentration results
- (5) Export the data

#### 1. Step (1) - Load an experiment

- To load an experiment, click on the “FILE” menu, then click on “Open” and select the experiment file to load.

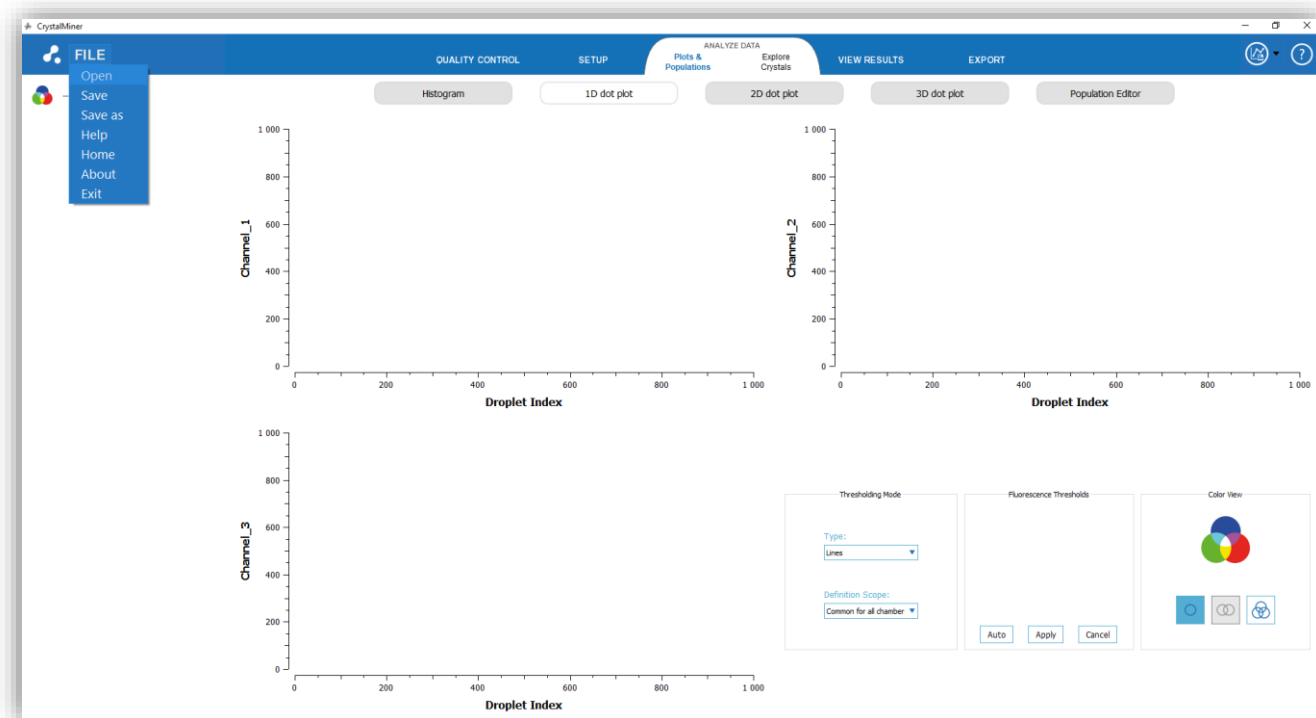


Fig. 2 - Step(1): Load an experiment

Once the experiment is loaded, the “ANALYZE DATA ” > “Plots and Populations” menu is displayed, showing by default the 1D dot plots concatenated for the all the chambers of the experiment:



Fig. 3 – Default view: 1D dot plots concatenated for all the chambers of the experiment (sequential view)

## a) Input file format

The input file format can be:

- **.ncx format**

The “Naica Crystal Experiment” (ncx) format is the format of the original file generated by the Crystal Reader software. The .ncx file contains the original high-quality images in 16 bits TIFF format required for the image analysis process.

The indicative size for the .ncx file is: 60 MB / chamber.

- **.ncr format**

The “Naica Crystal Result” (ncr) format is the format of the post-analysis file generated by the Crystal Miner software from the .ncx format file. The size of the .ncr file is much smaller (5 to 6 times smaller) than the size of the original .ncx file.

The indicative size for the .ncr file is: 10 MB / chamber.

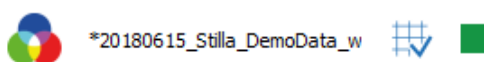
Note that it is also possible to drag & drop a .ncx file or a .ncr file in the application window in order to load it.

## b) Experiment status

The name of the loaded experiment is displayed in the top left side of the application window. It corresponds to name which has been previously entered in the Crystal Reader software.

There are 3 experiment status displayed next to the experiment name:

- **Modification status:** if the “\*” character is displayed just before the experiment name it means that the experiment has been modified without being saved, otherwise it means that it has not been modified or that the last modification has been saved
- **Compensation status:** if an icon showing a validated grid is displayed just after the experiment name it means that a fluorescence spillover compensation has been applied (in particular if a compensation file has been loaded in the Crystal Reader software), otherwise it means that the current experiment is not compensated for fluorescence spillover.
- **A global quality flag:** this quality flag value corresponds to the lowest of the chamber’s quality flags. Note that this flag becomes slashed with yellow lines if the user has excluded some droplets or regions from the crystal in one or more chambers composing the experiment.



*Fig. 4- From left to right: 3-color rosace logo;  
experiment name with the modification status (\* on left side if modified);  
compensation status (validated grid icon if compensated);  
global quality flag of the experiment.*

Note that the “\*” character before the experiment name indicates that the experiment has been modified but not saved. To save the experiment, click on the “FILE” menu, then click on “Save” or “Save as”.

## c) Chamber list

All the chambers of the experiment are listed in the left side panel.

Each chamber is named using the following convention:

*< Chip ID >\_< Chamber Position >\_< Sample Name >\_< Chamber Context >*

where:

- *< Chip ID >* is the unique ID of the chip which the chamber belongs to. This information should have been previously entered in the Crystal Reader software.
- *< Chamber Position >* is the position of the chamber in the chip (A, B, C, D from top to bottom) followed by the index of the slot receiving this chip on the chip holder (1, 2, 3 from left to right on the chip holder). This information is automatically defined by the software (e.g. “A1” or “D3”).



- [< Sample Name >](#) is the name of the sample inside the chamber. This information should have been previously entered in the Crystal Reader software.
- [< Chamber Context >](#) is an optional additional information allowing to make the scan data unique (useful if scanning the chamber multiple times). This contextual information is set in Crystal Reader and cannot be edited in Crystal Miner.

**CAUTION:**




*All the chambers of an experiment should share the same conditions otherwise the experiment should be divided into smaller experiments including subsets of chambers (to this purpose you may use the “Save as” button in the “FILE” menu and the chamber removal icon “-” above the chamber list in the “EDIT EXPERIMENT” menu).*

#### d) Quality flags

A [chamber quality flag](#) is displayed next to each chamber name in the top left side of the application window. This chamber quality flag provides a visual feedback for quality control at the chamber level.

An [experiment quality flag](#) (global quality flag) is displayed next to the experiment name in the left panel. This experiment quality flag provides a visual feedback for quality control at the experiment level and corresponds to the worst chamber quality flag among the chambers belonging to the experiment.

There are 3 types of quality flags:

-  Green flag: [high quality](#)
-  Green & Yellow flag: [moderate quality](#)
-  Yellow flag: [low quality](#)

For more details about Quality Control, see the section “*How to perform quality control?*” in the Advanced Functionalities

#### e) Data visualization and interpretation

- To visualize the graphs generated by Crystal Miner, click on the “ANALYZE DATA” > “Plots and Populations” menu, then click on one of these sub-menus: “1D dot plot”, “2D dot plot”, or “3D dot plot”.

The unit of the fluorescence axis in the dot plots is RFU (Relative Fluorescence Unit).

Each point in the dot plot graph represent one particular droplet in the selected crystal(s). If the fluorescence of the droplet in a given channel is higher than the fluorescence threshold defined for this channel, then the droplet is said as “positive”, otherwise it is said as “negative”.

As each droplet may be negative or positive in each of the 3 channels (“Blue”, “Green” and “Red” channels), there are  $2^3 = 8$  possible classes for a crystal droplet.

The Crystal Miner application uses the following 8 color-codes for each droplet class:



*Fig. 5 - The 8-color diagram used for 3D data visualization in Crystal Miner (the background of this diagram corresponds to the black / dark gray color)*

- (1) Black / Dark gray: negative in “Blue”, negative in “Green”, negative in “Red”
- (2) Blue: positive in “Blue”, negative in “Green”, negative in “Red”
- (3) Green: negative in “Blue”, positive in “Green”, negative in “Red”
- (4) Red: negative in “Blue”, negative in “Green”, positive in “Red”
- (5) Cyan: positive in “Blue”, Positive in “Green”, negative in “Red”
- (6) Yellow: negative in “Blue”, positive in “Green”, positive in “Red”
- (7) Magenta: positive in “Blue”, negative in “Green”, positive in “Red”
- (8) White / Light gray: positive in “Blue”, positive in “Green”, Positive in “Red”

- To visualize in 8 color-mode, click on the icon representing 3 overlapping circles in the “Color mode” widget



*Fig. 6 - Icon for visualization mode in 8 colors*

- To visualize in simplified color-mode, go to the in the “Color mode” widget and click on:
  - The icon representing 1 circle in the “1D dot plot” menu



Fig. 7 - Icon for visualization mode in 2 colors



Fig. 8 - The 2-color diagrams used for 1D data visualization in Crystal Miner (the background of these diagrams corresponds to the black / dark gray color)

- The icon representing 2 overlapping circles in the “2D dot plot” menu



Fig. 9 - Icon for visualization mode in 6 colors



Fig. 10 - The 4-color diagrams used for 2D data visualization in Crystal Miner (the background of these diagrams corresponds to the black / dark gray color)

- In a given 1D graph, the vertical axis shows the fluorescence value of the droplets in the detection channel of interest (“Blue”, “Green” or “Red” channel) and the horizontal axis shows the droplet index in the crystal if only one chamber is selected or the chamber names if multiple chambers are selected.

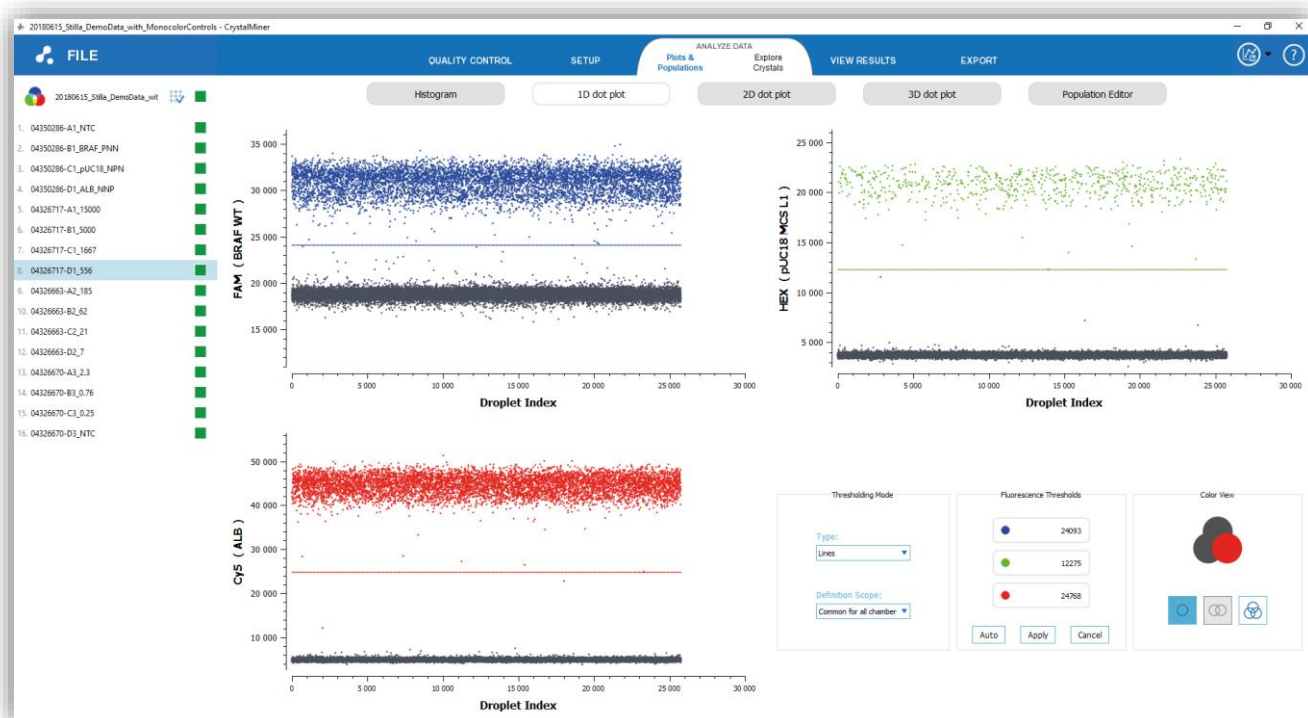


Fig. 11 - 1D dot plots in simplified colors

- In a given 2D graph, the vertical axis shows the fluorescence value of the droplets in one channel of interest and the horizontal axis shows the fluorescence value of the droplets in another channel of interest.

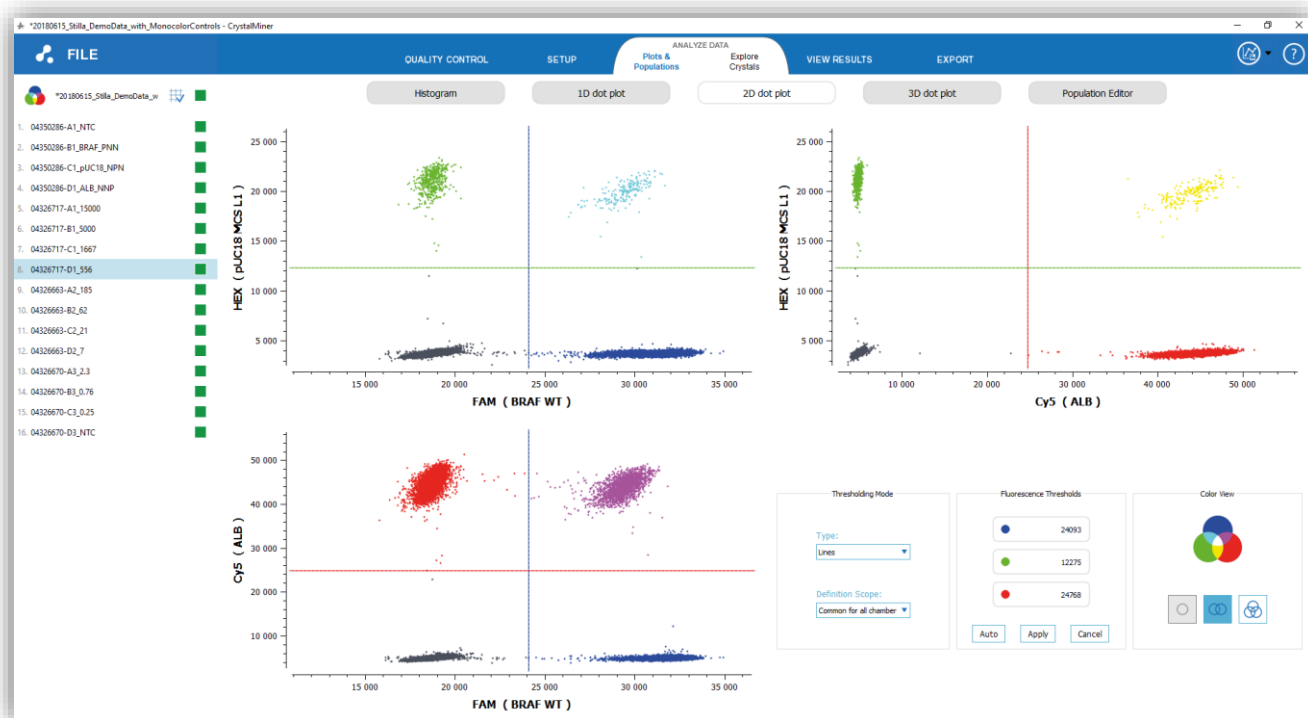


Fig. 12 - 2D dot plots in simplified colors

- To navigate in a graph:
  - Use mouse scroll to zoom in or out in the graph
  - Use left mouse click to translate the graph
  - Double-click inside the graph with left mouse button to reset the view
  - When applicable, double-click on any axis to edit the graph's settings (for example: min and max limits)

## 2. Step (2) - Check the fluorescence spill-over compensation

- To compensate the fluorescence spill-over, click on the menu “SETUP” > “Compensate Spillover”.

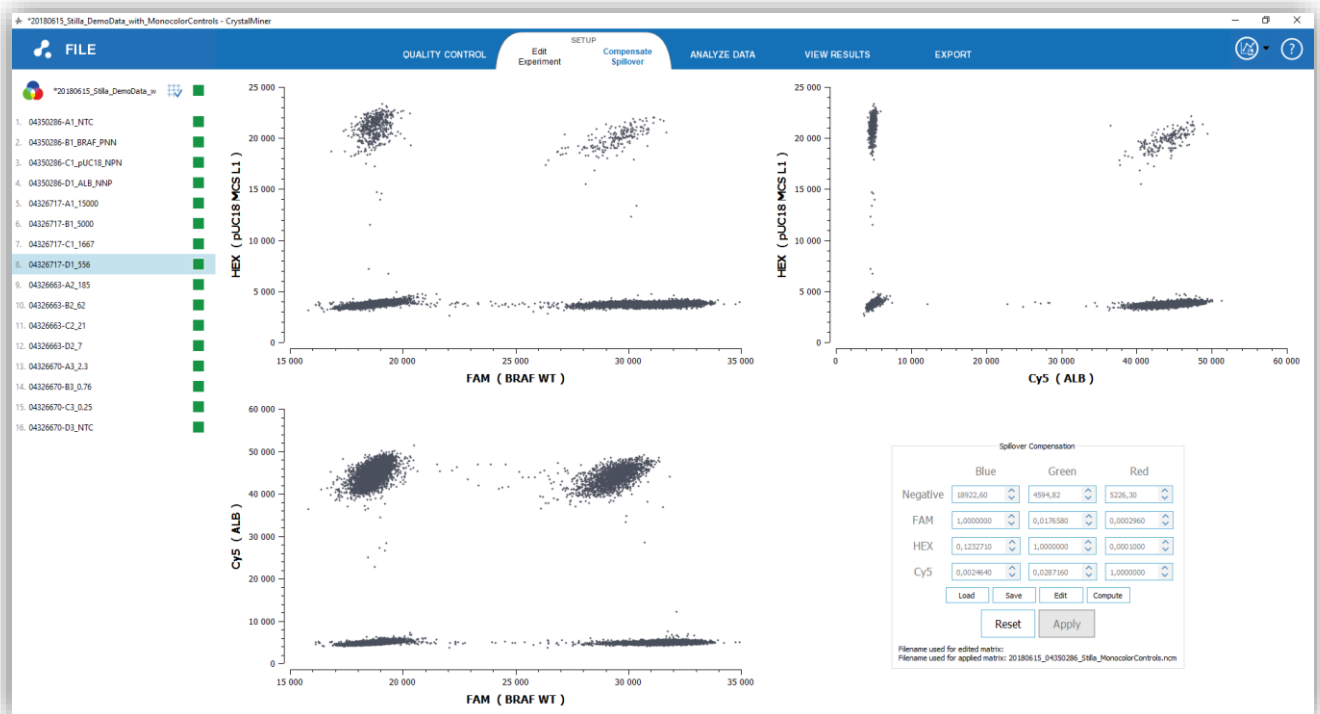


Fig. 13 - Step (2): Compensate fluorescence spill-over

The fluorescence spill-over compensation coefficients are divided into 2 categories:

- The 9 coefficients of the LED-fluorophore excitation matrix
- The 3 coefficients of the fluorescence background vector

For more details on the computation of these coefficients, see the section “Computation of the fluorescence spill-over compensation coefficients” in the Annexes.

	Blue	Green	Red
Negative	18922,60	4594,82	5226,30
FAM	1,0000000	0,0176580	0,0002960
HEX	0,1232710	1,0000000	0,0001000
Cy5	0,0024640	0,0287160	1,0000000

Fig. 14 - Example of a compensation matrix, with the translation vector in line 1 (“Negative” line), and the excitation matrix in lines 2, 3 and 4, where the fluorophore FAM is associated with the “Blue” LED, the fluorophore HEX with the “Green” LED and the fluorophore “Cy5” with the “Red” LED.

By default, the original experiment is not compensated, which means that:

- the translation coefficients (line 1 in the table) are equals to “0”
- the excitation matrix (lines 2, 3 and 4 in the table) is equal to the identity matrix (i.e. values “1” in the diagonal and values “0” outside the diagonal)

	Blue	Green	Red
Negative	<input type="text" value="0,00"/>	<input type="text" value="0,00"/>	<input type="text" value="0,00"/>
FAM	<input type="text" value="1,0000000"/>	<input type="text" value="0,0000000"/>	<input type="text" value="0,0000000"/>
HEX	<input type="text" value="0,0000000"/>	<input type="text" value="1,0000000"/>	<input type="text" value="0,0000000"/>
Cy5	<input type="text" value="0,0000000"/>	<input type="text" value="0,0000000"/>	<input type="text" value="1,0000000"/>

*Fig. 15 - Default compensation matrix coefficients  
(fluorescence spill-over is not compensated by default)*

To compensate the spill-over for the loaded experiment, there are 3 compensation methods:

- the template-based method
- the automated method
- the manual method

#### a) Template-based method

This method can be applied if the fluorescence spill-over compensation has already been performed on a previous experiment which has the same conditions as the current experiment, i.e. the same fluorophores and targets, and the same focus value.

- To use the template-based spill-over compensation method, click on the “Load” button, then select the file in .ncm format which represents the compensation template.

The “Naica Compensation Matrix” (.ncm) format is the format used in the Crystal Miner application to store the spill-over compensation coefficients (i.e. the excitation coefficients and the background translation coefficients).

- To generate a new compensation template from the current experiment, click on the “Save” button, then choose a name for the .ncm file.

Note that the spill-over compensation file can also be exported in the EXPORT menu (see the step “Export the data” for more details).

## b) Automated method

This method can be applied if the current experiment contains at least one mono-fluorophore positive control sample for each of the used fluorescence channels

- To use the automated spill-over compensation method, click on the “Compute” button, then select in the pop-up window the appropriate control chambers (note that control chambers are automatically selected if sample types have been previously defined by the user in Crystal Reader software):
  - **Optional: one or several chamber(s) for the “Negative Control” (NNN)**, i.e. the chamber(s) containing only negative droplets for all fluorophores (which means that they contain no target at all)
  - **Required: at least one chamber for the “Fluorophore 1 Positive Control” (PNN)**, i.e. the chamber(s) containing positive droplets for the fluorophore “Fluorophore 1” and only negative droplets for all the other fluorophores (which means that they contain only the target of the “Fluorophore 1”)
  - **Required: at least one chamber for the “Fluorophore 2 Positive Control” (NPN)**, i.e. the chamber(s) containing positive droplets for the fluorophore “Fluorophore 2” and only negative droplets for all the other fluorophores (which means that they contain only the target of the “Fluorophore 2”)
  - **Required: at least one chamber for the “Fluorophore 3 Positive Control” (NNP)**, i.e. the chamber(s) containing positive droplets for the fluorophore “Fluorophore 3” and only negative droplets for all the other fluorophores (which means that they contain only the target of the “Fluorophore 3”)



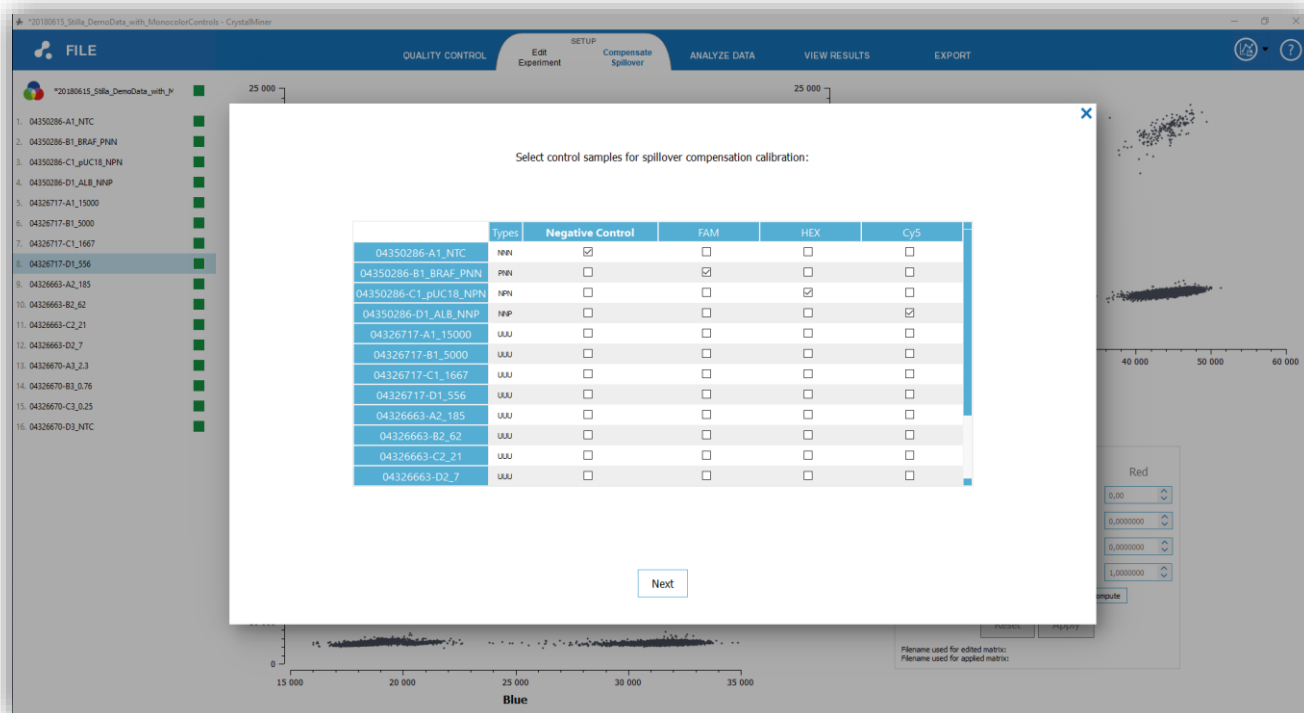


Fig. 16 - First step of the automated method for fluorescence spill-over compensation

- Then click on “Next” and check the control fluorescence thresholds in each graph (you can adjust them by editing their value or by right-clicking in the dot plots to drag & drop the threshold lines), then click on “Compute compensation”.

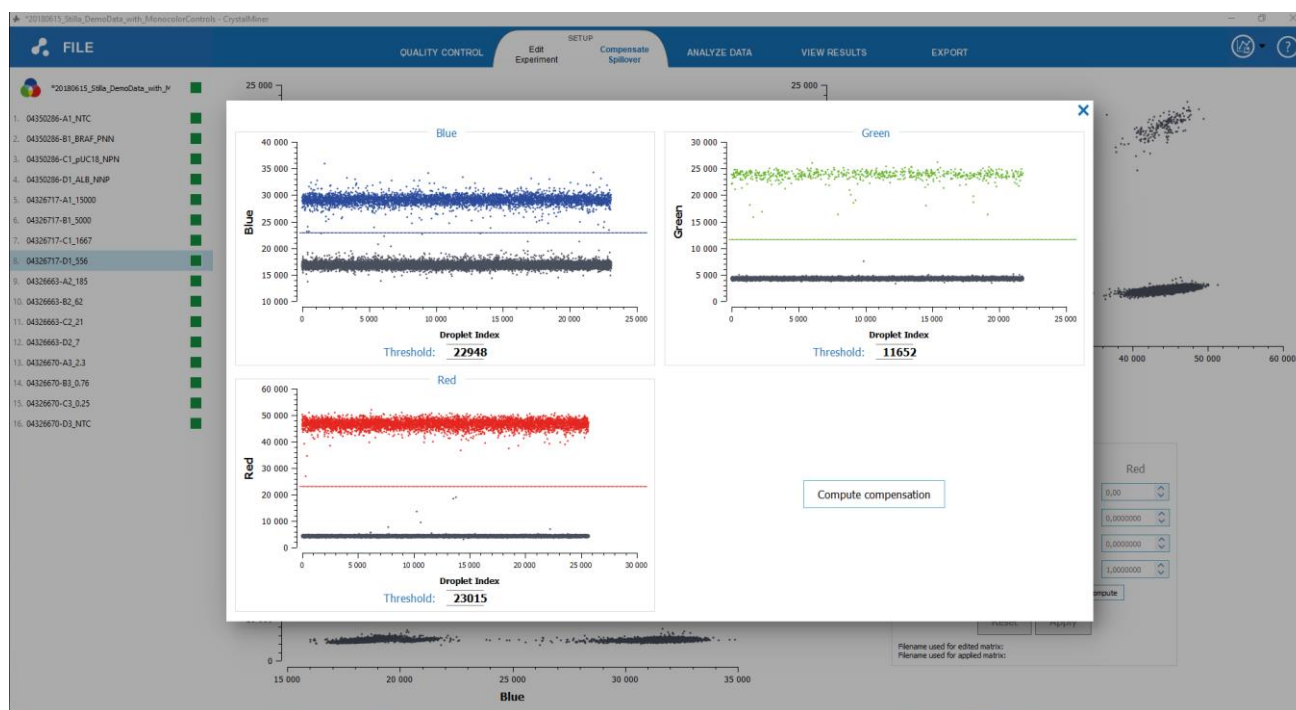


Fig. 17 - Second step of the automated method for fluorescence spill-over compensation

## c) Manual method

### CAUTION:

*The manual spill-over compensation method assumes that there is no non-specificity issue, otherwise it is not recommended.*

This method must be applied if neither the template-based method nor the automated method can be applied.

- To use the manual spill-over compensation method, click on the “Edit” button, then edit the values of the coefficients in the compensation matrix.

For example, in the “Green vs. Blue” graph (top left graph):

- If the droplet population which is positive for the fluorophore “HEX” but negative the fluorophore “FAM” is not vertically aligned with the double-negative droplet population, it means that the “HEX” fluorophore is partially excited by the “Blue” LED, so the matrix coefficient (“HEX”, “Blue”) should be increased to correct this spill-over phenomenon.
- If the droplet population which is positive for the fluorophore “FAM” but negative the fluorophore “HEX” is not horizontally aligned with the double-negative droplet population, it means that the “FAM” fluorophore is partially excited by the “Green” LED, so the matrix coefficient (“FAM”, “Green”) should be increased to correct this spill-over phenomenon.

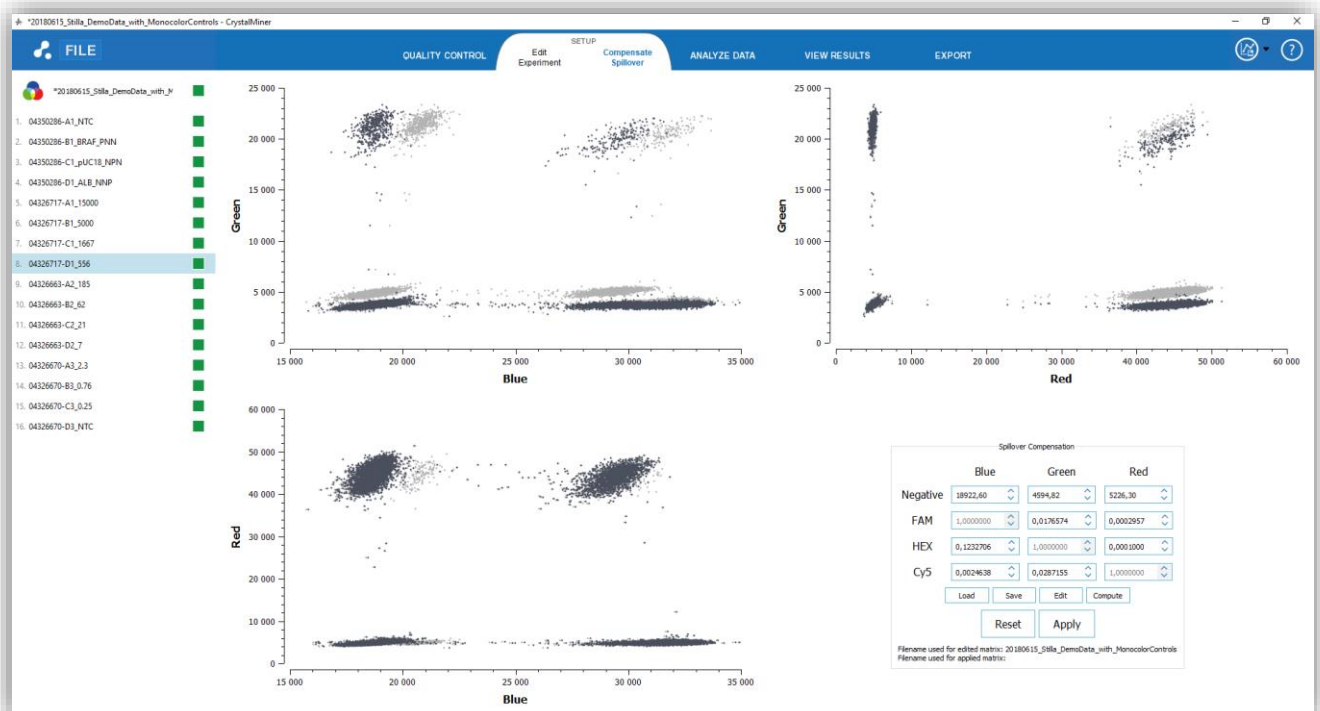


Fig. 18 - Modification of the fluorescence spill-over coefficients, with the non-compensated populations in light gray and the compensated populations in dark gray.

Once the compensation matrix has been modified, the compensated droplet populations are overlapped in the graphs where they appear darker than the non-compensated droplet populations.

- To apply the spill-over compensation to the whole experiment, click on the “Apply” button, then click on “Yes” if you want to update the automated fluorescence thresholds (recommended).

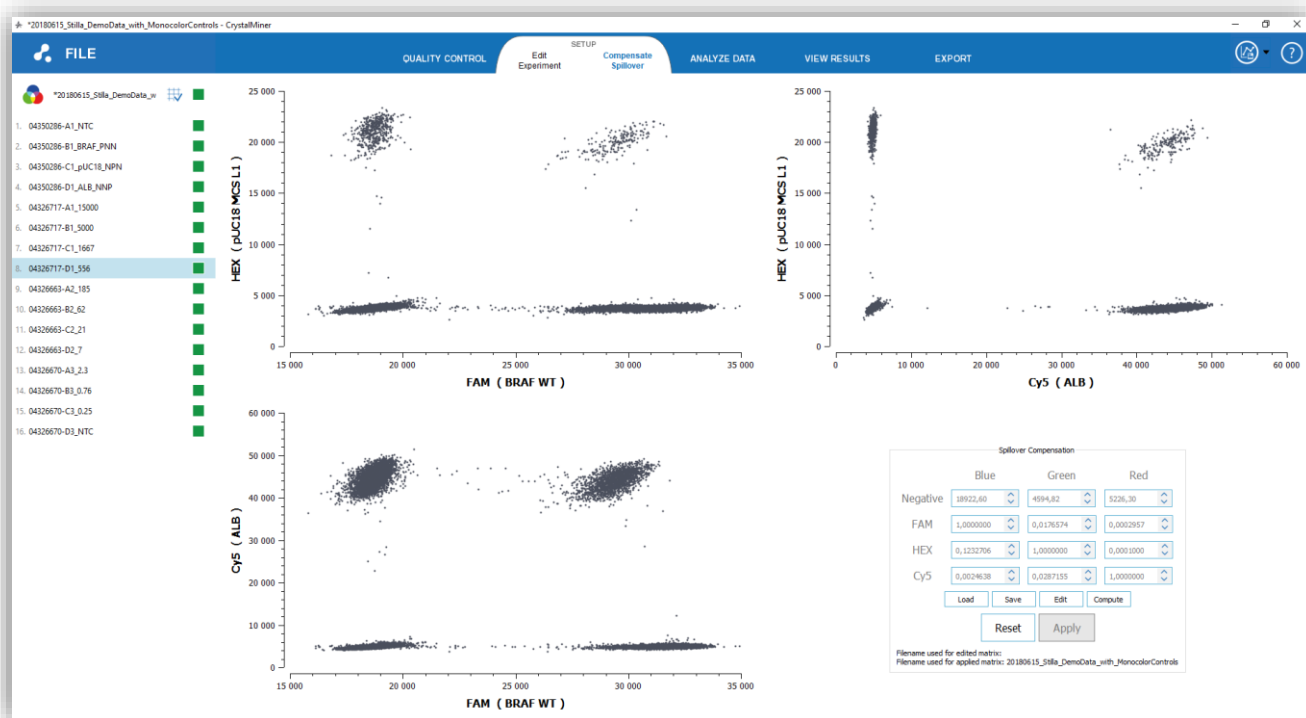


Fig. 19 - Fluorescence compensation has been applied to the whole experiment

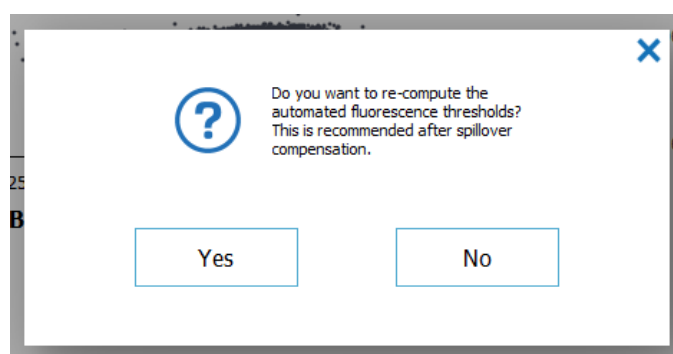


Fig. 20 - Automated fluorescence thresholds should be updated after spillover compensation

Note that once the spillover compensation is applied, the fluorescence values are not anymore displayed in the LED domain but in the fluorophore domain. For this reason, the axes of all fluorescence graphs are not named with the name of the LEDs (“Blue”, “Green”, “Red”) anymore but with the name of the used fluorophores.

- To reset the spill-over compensation and come back to a non-compensated experiment, click on the “Reset” button.

Note that the 8-colors mode in the 1D and 2D dot plot graphs may help to identify the misaligned droplet populations.

Below are examples of 1D fluorescence graphs in 8-colors mode, before and after spill-over compensation:

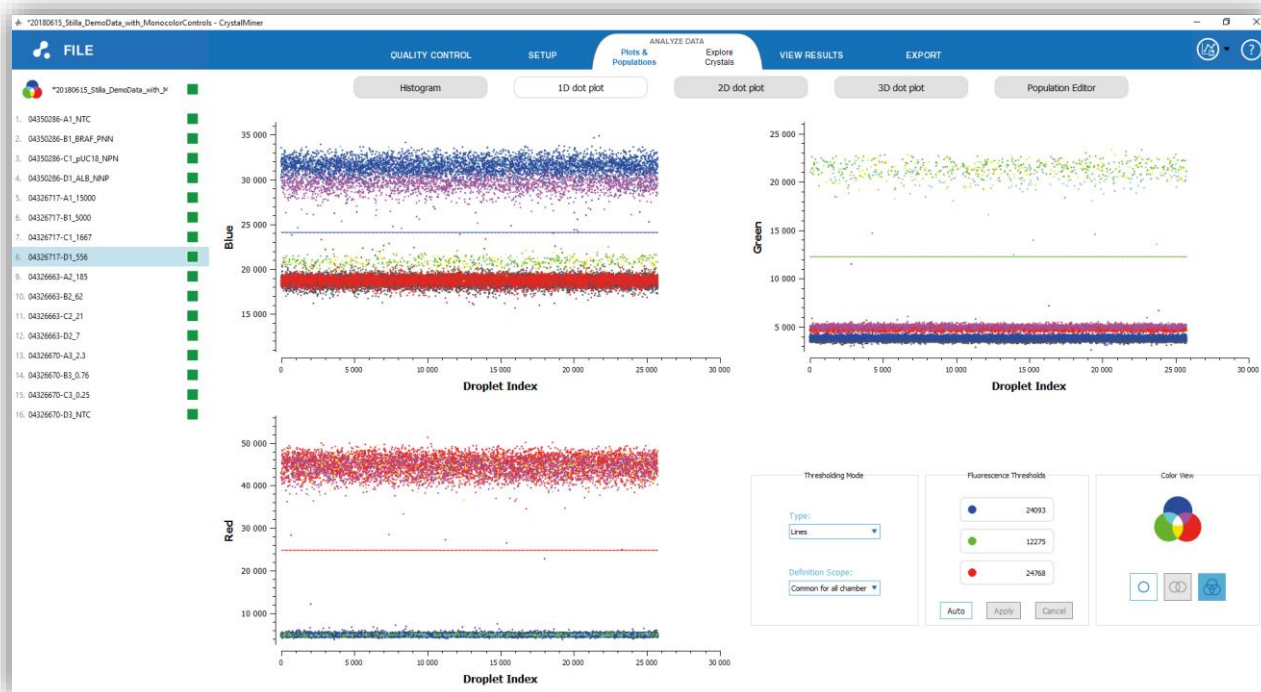


Fig. 21 - Example of non-compensated 1D dot plots in 8-colors mode

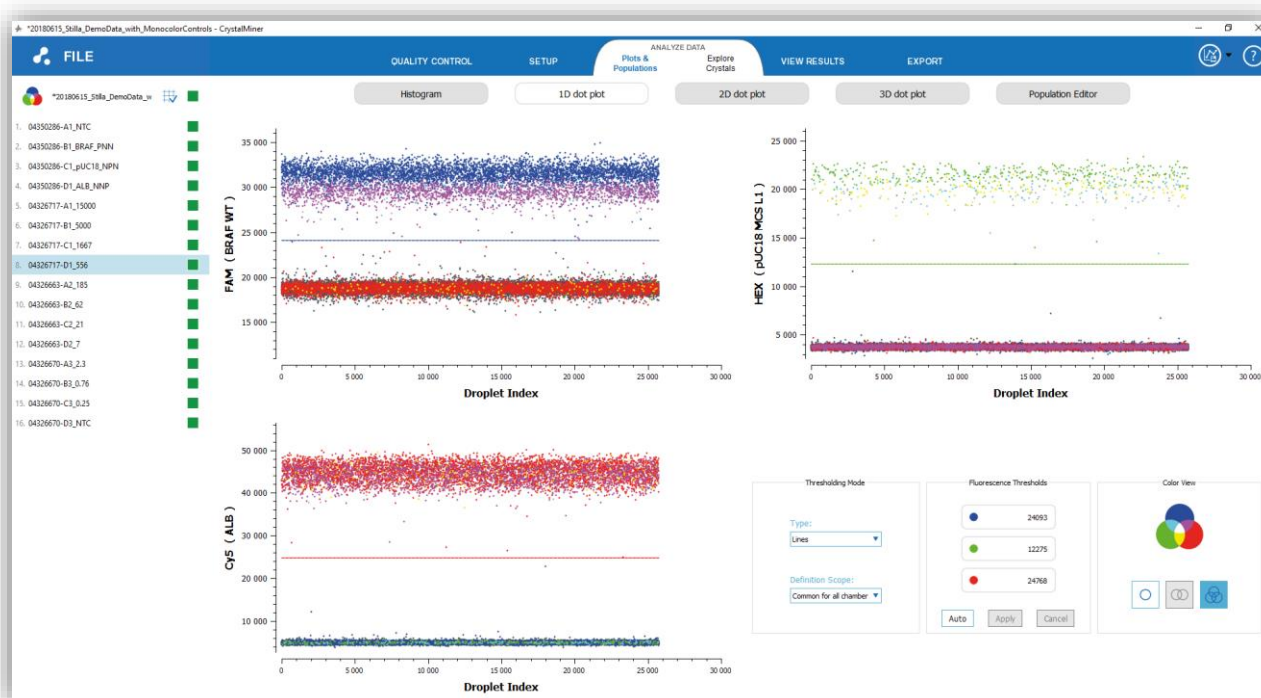


Fig. 22 - Example of compensated 1D dot plots in 8-colors mode

Below are examples of 2D fluorescence graphs in 8-colors mode, before and after spill-over compensation:

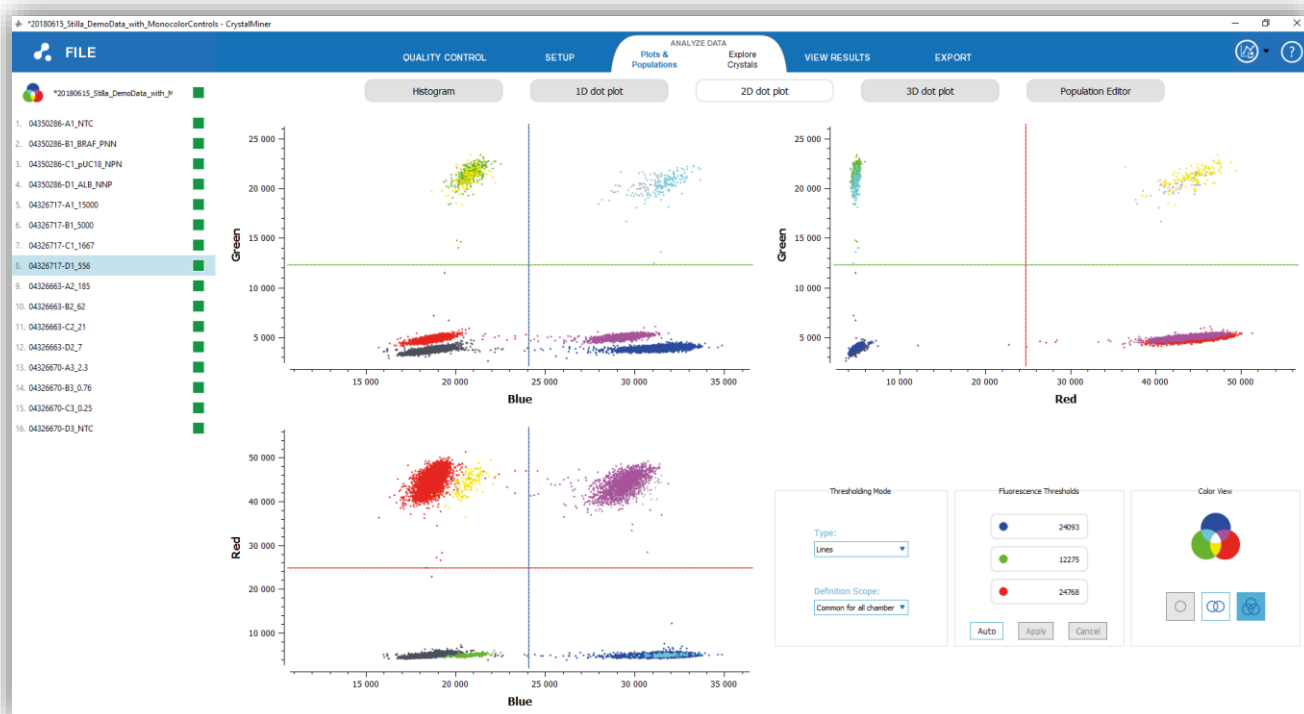


Fig. 23 - Example of non-compensated 2D dot plots in 8-colors mode

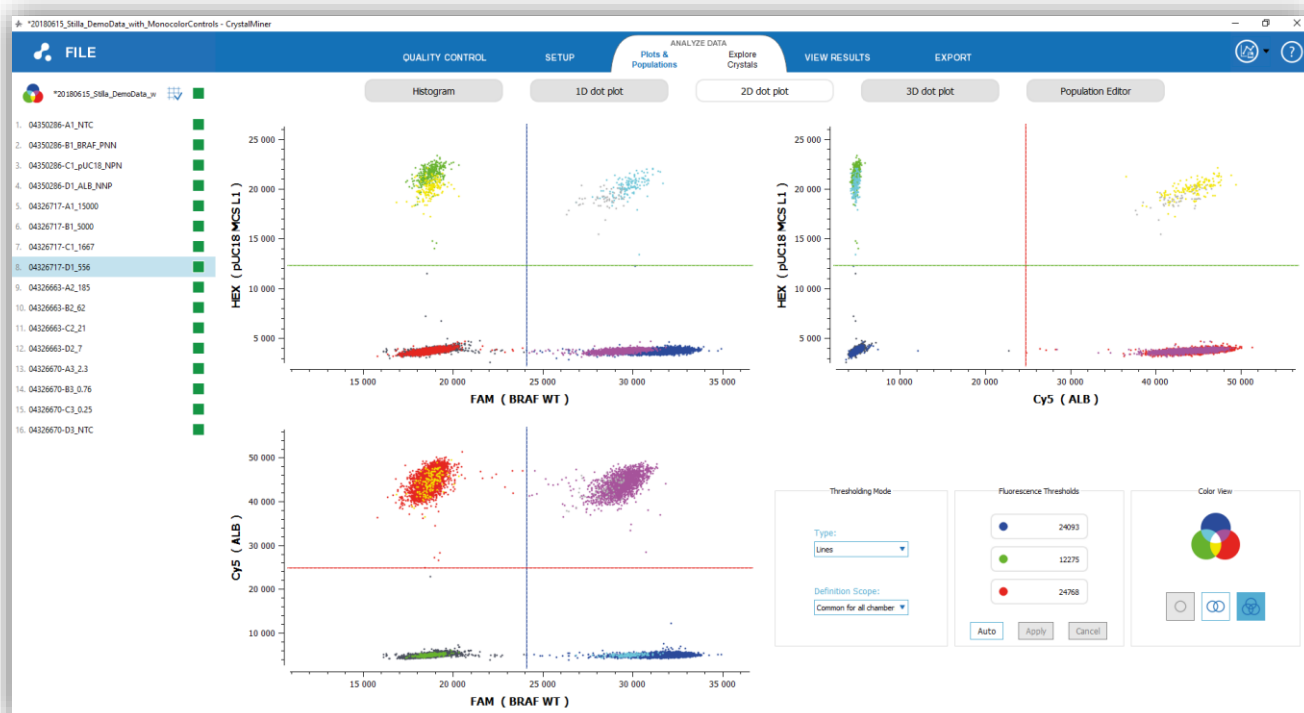


Fig. 24 - Example of compensated 2D dot plots in 8-colors mode



### 3. Step (3) - Check the fluorescence thresholds

By default, the fluorescence threshold values are automatically estimated by the software given all the samples of the experiment and the current spill-over compensation values.

The automated estimation is performed such that the fluorescence thresholds best discriminate between the positive and negative droplet populations in each channel and for the whole experiment. More precisely, the automated threshold maximizes the inter-class variance and minimizes the intra-class variance by considering the fluorescence points aggregated from all chambers.

The default automated threshold estimation is more accurate if both negative and positive droplet populations are represented in the experiment and in each channel, which is for example the case if the experiment includes at least one negative control chamber and one positive control chamber for each of the 3 channels.

- For an efficient adjustment of the fluorescence thresholds, it is recommended to inspect the sequential view of the 1D fluorescence graphs including all the chambers, by clicking on the “1D dot plot” and selecting all the experiment chambers listed in the left panel (Shift+Click or Ctrl+A).

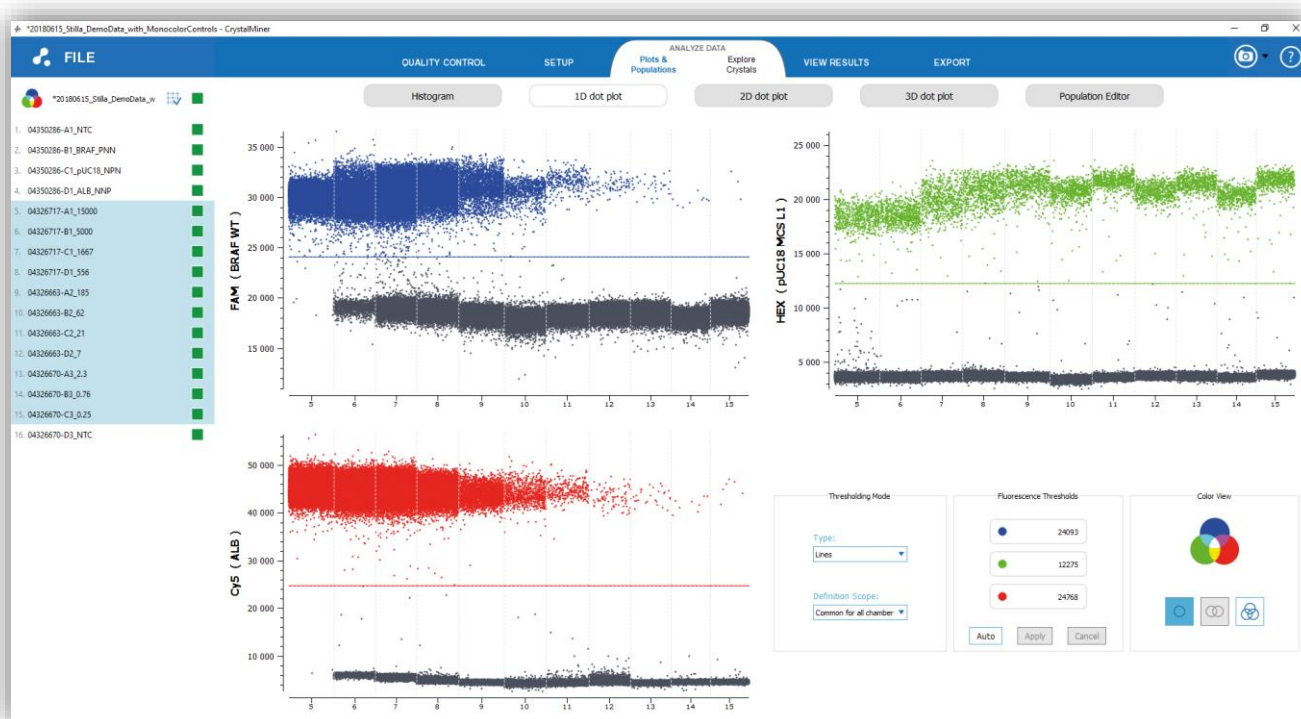
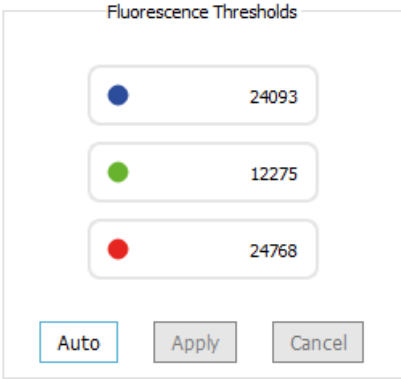


Fig. 25 - Step (3): Sequential view of all the experiment test chambers in the 1D dot plots for a more efficient verification of the 3 fluorescence thresholds

- To get the automated estimation of the fluorescence thresholds, click on the “ANALYZE DATA” menu, then click on any of the dot plot sub-menu (“1D dot plot”, “2D dot plot”, “3D dot plot”) and click on the “Auto” button in the “Fluorescence Thresholding” widget (note that automated thresholding is done by default at experiment load)



The dialog box titled "Fluorescence Thresholds" contains three input fields, each with a colored circle (blue, green, red) to its left. The values in the fields are 24093, 12275, and 24768 respectively. At the bottom are three buttons: "Auto", "Apply", and "Cancel".

Color	Value
Blue	24093
Green	12275
Red	24768

Fig. 26 - Step (3): Check the fluorescence thresholds

- To manually adjust the fluorescence thresholds, you can right-click in the dot plots to drag & drop the threshold lines, or you can edit the value of the fluorescence thresholds in the "Fluorescence Thresholding" widget (unit: RFU standing for Relative Fluorescence Unit).

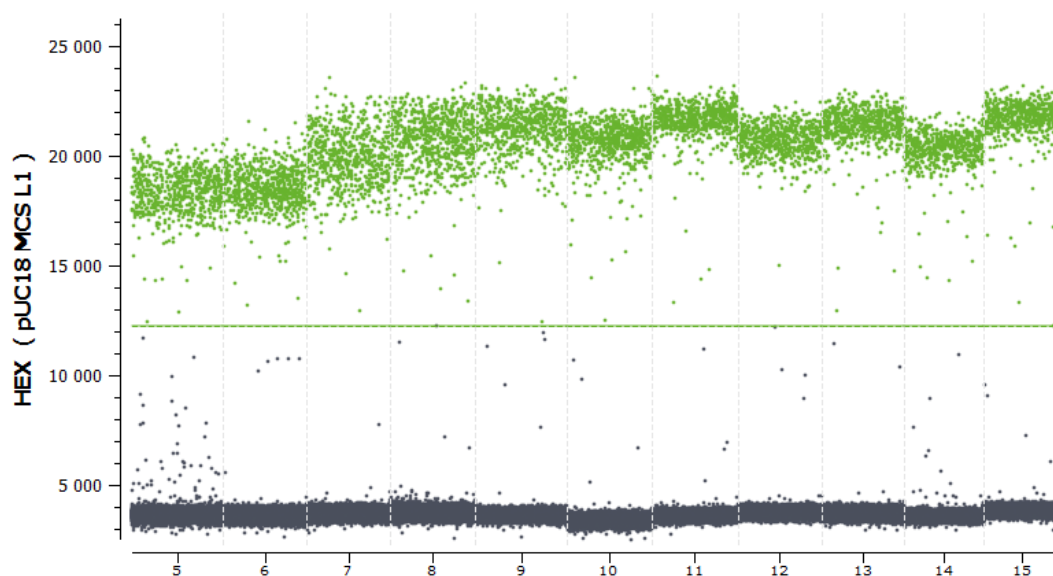


Fig. 27 - Manual adjustment of the fluorescence threshold using Right-Click to drag & drop

- To apply the modified values to the whole experiment, click on "Apply"
- To recover the thresholds values set before you started editing (i.e. the last time "Apply" has been clicked), click on "Cancel"
- To set the values back to their automated estimation for the whole experiment, click on the "Auto" button

#### 4. Step (4) - Get the concentration results

- To get the concentration results, click on the “VIEW RESULTS ” > “Result Table” menu.

The estimated concentrations are the concentrations of the target molecule in the initial stock in copies /  $\mu\text{L}$ .

The absolute nucleic acid concentrations are computed based on the standard Poisson statistics that are commonly used in digital PCR (see the section “*Digital PCR statistics*” in the Annexes for more details).

Note that the total number of chambers belonging to the experiment is indicated above the chamber list in the left panel.

- Click on the “Result Table” sub-tab to see the table of results.

The colors of the column titles indicate the color of the corresponding channel:

- In blue color: the results for the fluorophore-target couple associated with the “Blue” LED
- In green color: the results for the fluorophore-target couple associated with the “Green” LED
- In red color: the results for the fluorophore-target couple associated with the “Red” LED

Note that if the sample has been diluted 10-fold, then the value “10” should have been entered for the dilution factor, and the stock concentration is equal to the chamber concentration multiplied by 10.



Crystal Miner Software Interface - Step (4): Get the concentration results in table representation

Experimental Details:

- Fluorophore names: FAM, HEX, Cy5
- Target names: BRAP WT, pUC18 MCS.L1, ALB


Scanning Parameters:

- Exposure times: 50 ms, 150 ms, 50 ms
- Focus: 0.84 mm

Comments: Demo data by Sabine Müller

Chamber	Context	Nb Droplets	Dilution	Blue Channel		Green Channel		Red Channel	
				C (cp/mL)	Nb Pos	C (cp/mL)	Nb Pos	C (cp/mL)	Nb Pos
1. 04350286-A1_NTC		25666	1	0	0	0	0	0	0
2. 04350286-B1_BRAP_PNN		23072	1	371.9	4517	0	0	0	0
3. 04350286-C1_pUC18_NPN		21785	1	0	0	49.2	619	0	0
4. 04350286-D1_ALB_NNP		25674	1	0	0	0	0	369.7	5000
5. 04326717-A1_15000		27175	1	14367	27169	41.8	657	16242	27173
6. 04326717-B1_5000		25582	1	5257	24407	47.2	698	5303	24438
7. 04326717-C1_1667		25123	1	1687	15777	45.6	663	1684	15762
8. 04326717-D1_556		25746	1	561.8	7221	48.5	721	554.1	7137
9. 04326663-A2_185		27499	1	173.6	2660	41.9	667	174.7	2675
10. 04326663-B2_62		25748	1	58.3	864	46.2	688	55.7	827
11. 04326663-C2_21		25890	1	19.5	294	46	688	20.4	308
12. 04326663-D2_7		25457	1	5.84	87	46.6	686	6.11	91
13. 04326670-A3_2,3		24903	1	2.47	36	43.7	630	1.99	29
14. 04326670-B3_0,76		23976	1	0.64	9	44.6	618	0.57	8
15. 04326670-C3_0,25		23434	1	0.22	3	44.5	603	0.44	6
16. 04326670-D3_NTC		24733	1	0	0	0	0	0	0

Fig. 28 - Step (4): Get the concentration results in table representation

Click on the “expand” icon  located above the chamber list in the left panel to see more detailed results, including the number of negative droplets, the confidence intervals and the relative uncertainties at 95% confidence level (see the “Digital PCR statistics” chapter in the Annexes for more information).

FILE

QUALITY CONTROL

SETUP

ANALYZE DATA

VIEW RESULTS

Result Table

Advanced Graphs

EXPORT

20180615\_Skiba\_DemoData\_v1

16 Chambers

Fluorophore names: FAM, HEX, Cy5

Target names: BRAP WT, pUC18 MCS.L1, ALB

Scanning Parameters

Exposure times: 50 ms, 150 ms, 50 ms

Focus: 0.84 mm

Comments

Demo data by Sabine Muller

	Chamber Context	Nb Droplets	Dilution	Blue Channel		Nb Neg	C_min (cp/uL)	C_max (cp/uL)	Relative Uncertainty (CI 95%)	Dilution	Green Channel		Nb Neg	C_max
				C (cp/uL)	Nb Pos						C (cp/uL)	Nb Pos		
1. 04350286-A1_NTC		25666	1	0	0	25666	0	0	inf %	1	0	0	25666	
2. 04350286-B1_BRAP_PNN		23072	1	371.9	4517	18555	361	382.8	2.92 %	1	0	0	23072	
3. 04350286-C1_pUC18_NPN		21785	1	0	0	21785	0	0	inf %	1	49.2	619	21166	
4. 04350286-D1_ALB_NNP		25674	1	0	0	25674	0	0	inf %	1	0	0	25674	
5. 04326717-A1_15000		27175	1	14367	27169	6	13364	17115	13.1 %	1	41.8	657	26518	
6. 04326717-B1_5000		25582	1	5257	24407	1175	5164	5355	1.81 %	1	47.2	696	24604	
7. 04326717-C1_1667		25123	1	1687	15777	9346	1690	1715	1.62 %	1	45.6	683	24460	
8. 04326717-D1_556		25746	1	561.8	7221	18525	548.8	574.8	2.32 %	1	48.5	721	25025	
9. 04326663-A2_185		27499	1	173.6	2660	24839	167	180.2	3.8 %	1	41.9	667	26832	
10. 04326663-B2_62		25748	1	58.3	864	24884	54.4	62.1	6.67 %	1	46.2	688	25060	
11. 04326663-C2_21		25890	1	19.5	294	25596	17.3	21.7	11.4 %	1	46	688	25202	
12. 04326663-D2_7		25457	1	5.84	87	25370	4.62	7.07	21 %	1	46.6	686	24771	
13. 04326670-A3_2,3		24903	1	2.47	36	24867	1.66	3.28	32.7 %	1	43.7	630	24273	
14. 04326670-B3_0,76		23976	1	0.64	9	23967	0.22	1.06	65.3 %	1	44.6	618	23358	
15. 04326670-C3_0,25		23434	1	0.22	3	23431	-0.03	0.47	113.2 %	1	44.5	603	22831	
16. 04326670-D3_NTC		24733	1	0	0	24733	0	0	inf %	1	0	0	24733	

Fig. 29 - Expanded view of the result table

- Click on the “Advanced Graphs” tab, and on the “Concentration Graphs” sub-menu to see the graphs of concentrations (use the radio-buttons to switch between “Linear” and Log” modes)

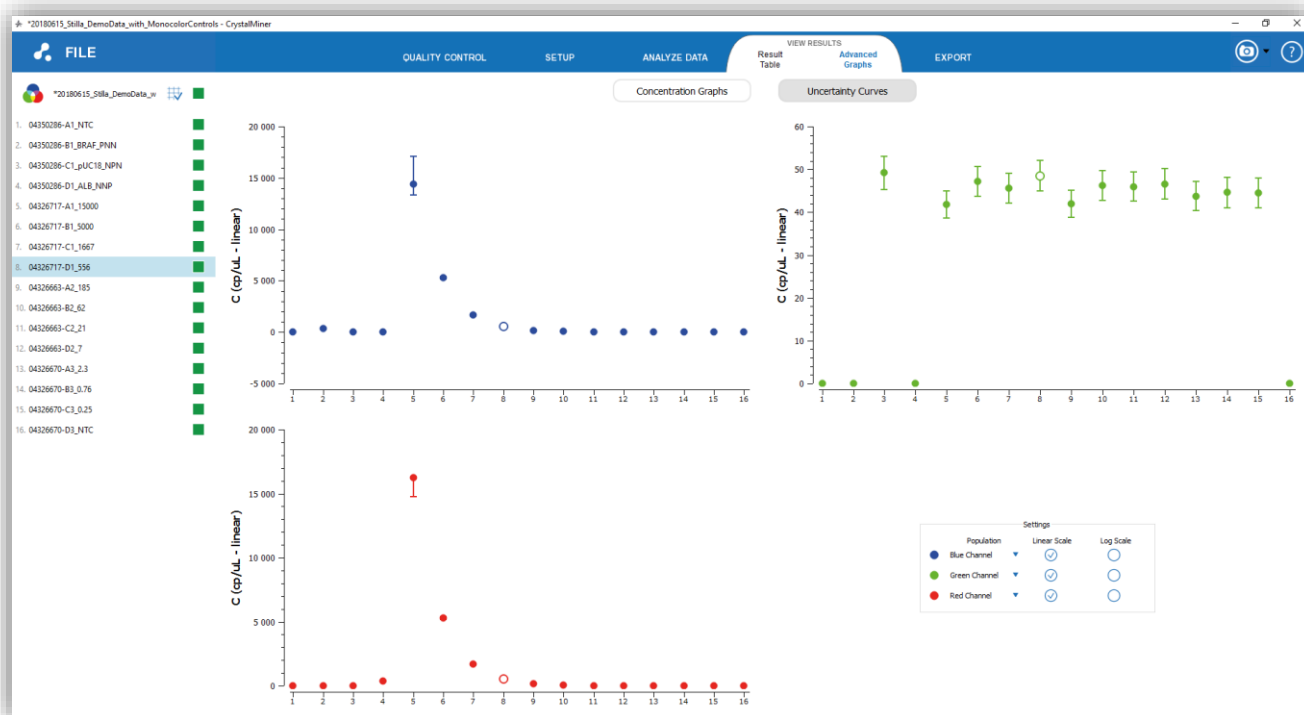


Fig. 30 - Get the concentration results in graph representation (linear view)

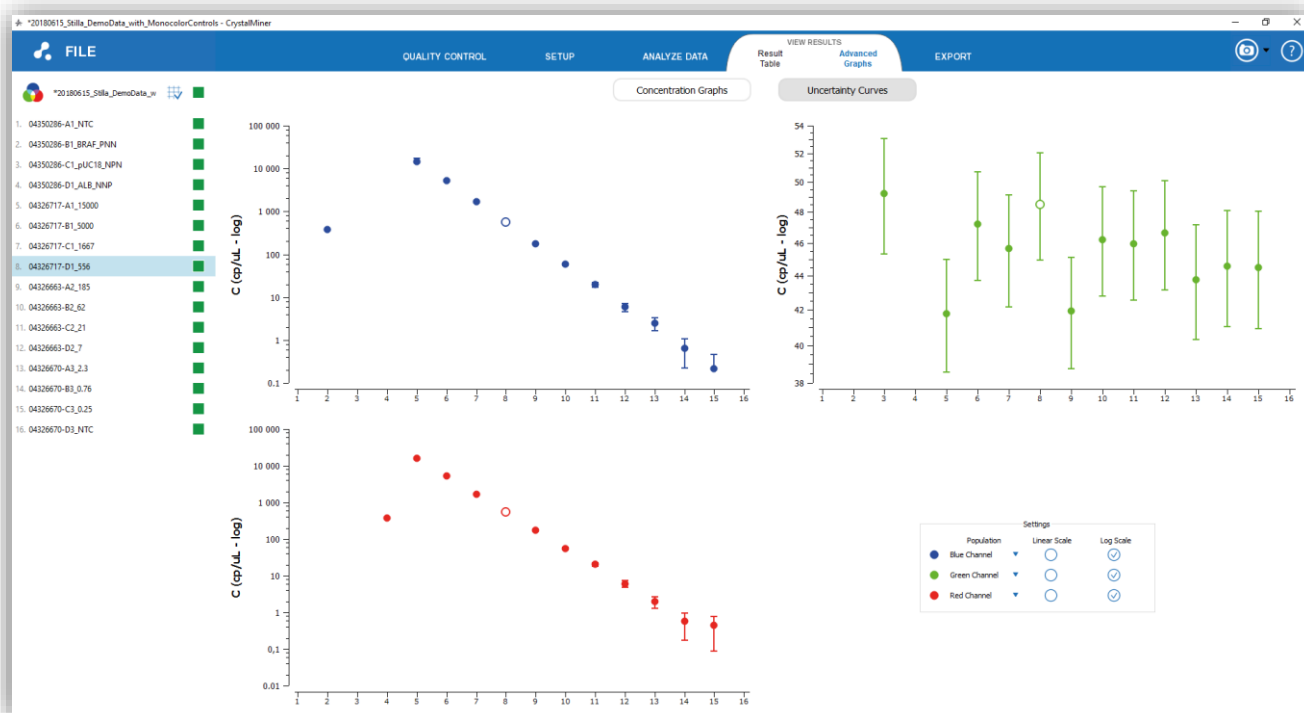


Fig. 31 - Get the concentration results in graph representation (log view)

Note: the perceived absolute data variability depend on the min & max values of the vertical axis, which should be taken into account to infer relative data variability (e.g. coefficient of variation).

## 5. Step (5) - Export the data

- To export the data, click on the “EXPORT” menu, then select the data you wish to include, choose the output directory, and click on the “Export” button.

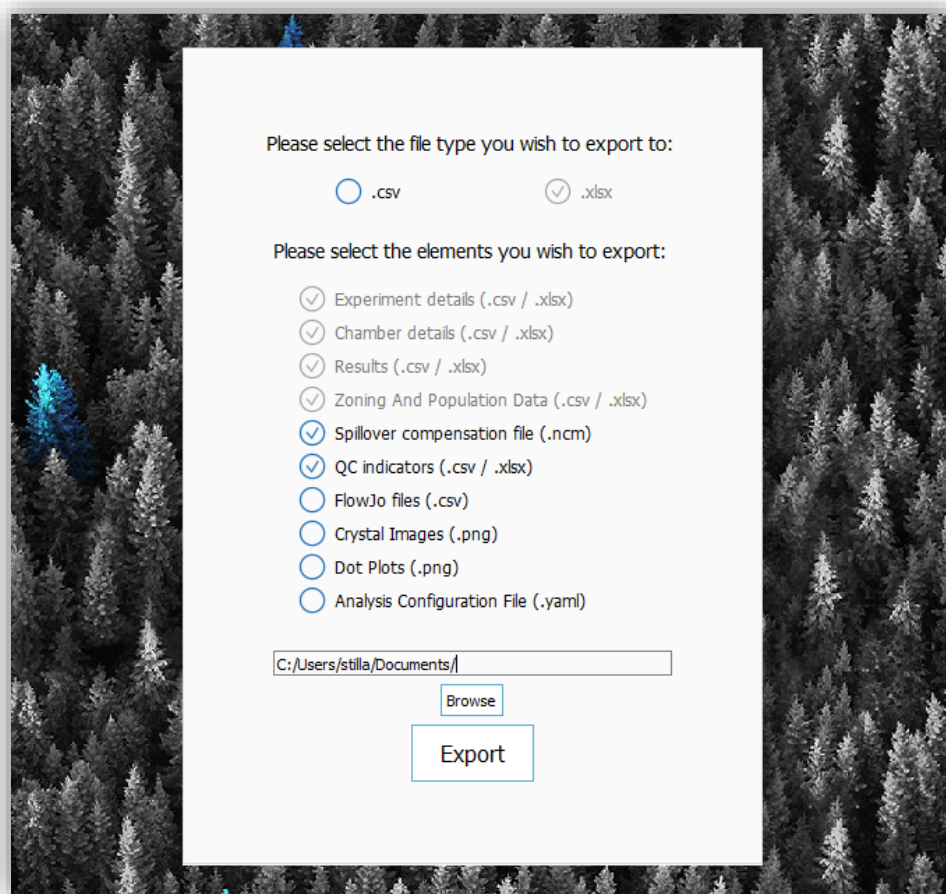


Fig. 32 - Step (5): Export the data

The exported data includes:

- **Experiment details** (.csv / .xlsx file), including:
  - Experiment name
  - Fluorophore and target names
  - User comments
  - Focus value (in mm) and scanning parameters: exposure times (in ms) in each channel
  - Spill-over compensation status (compensated or not)
  - Software version of Crystal Miner version used to export.
  - Timestamp of data export.

- Thresholding type (“Line” vs. “Polygon” zoning) and definition scope (“common for all chambers” vs. “individual per chamber”) for the experiment (see the section “*Custom population definition*” in the Advanced Functionalities)
- **Chamber details** (.csv / .xlsx file), including for each chamber:
  - Chip ID; Chamber position (“A”, “B”, “C”, “D”) Sample name; Chamber context  
For each detection channel: sample type (“U”, “N”, “P”, or “S”); reference concentration (in copies /  $\mu$ L, applicable only if sample type = “S”)
  - Original experiment name (might differ in case of a merging of various .ncx files)
  - Software version of Crystal Reader used to scan (might differ in case of a merging of various ncx files)
  - Scanning timestamp (might differ in case of a merging of various ncx files)
  - Serial number of the Prism3 instrument used to scan (might differ in case of a merging of various ncx files)
  - Scanner resolution ( $\mu$ m / pixel) of the Prism3 instrument used to scan (might differ in case of a merging of various ncx files)
  - Profile name in Crystal Reader used to scan (might differ in case of a merging of various ncx files)
  - Index of chip position (slot 1, 2, 3) and index of chip holder at the time of the scan (might differ in case of a merging of various ncx files)
  - Number of manually removed droplets (see the sections “*How to explore the crystal populations?*” and “*How to perform quality control?*” in the Advanced Functionalities)
- **Results** (.csv / .xlsx file), including for each chamber:
  - Chip ID and chamber position, sample name and chamber context
  - Total number of analyzed droplets
  - For each population:
    - sample type (only in “Line” thresholding mode)
    - dilution factor (N/A if not applicable)
    - estimated stock concentration (in copies /  $\mu$ L)
    - number of positive droplets (i.e. belonging to the population)
    - number of negative droplets (i.e. not belonging to the population, if not specifically defined as “negatives” with respect to the population)
    - minimum and maximum limits of the 95% confidence interval for the estimated stock concentration (in copies /  $\mu$ L)
    - 95% relative uncertainty for the estimated stock concentration (equal to the length of 95% confidence interval divided by 2 times the estimated concentration)

**Zone Details** (see the section “*Custom population definition*” in the Advanced Functionalities), including for each fluorescence zone:

S

- Chip ID and chamber position, sample name and chamber context
- Zone ID (note that in “Line” thresholding mode there are always 8 fluorescence zones, while in “Polygon” thresholding mode there is one zone per user-defined polygon)
- Zone type (quadrant in “Line” mode, polygon in “Polygon” mode)
- The x and y channels used to define the zone, and the z channel (where “Chan1” stands for “Blue”, “Chan 2” for Green and “Chan 3” for “Red”).

- List of x-y coordinates of the 2D points defining the zone (note that in “Line” thresholding mode there are always 5 points defining a square)
  - Coordinates of the z boundaries of the zone (note that in “Polygon” thresholding mode the z-values for the boundaries are always 0-65535 RFU)
- [Population Details](#) (see the section “*Custom population definition*” in the Advanced Functionalities), including for each population:
    - Population name
    - Population color (RGB values)
    - List of the ID(s) of the zone(s) from which the population have been built  
*Optional:* list of the ID(s) of the zone(s) specifically defined as “negatives” for the population
  - *Optional:*  
[QC Indicators](#) (.csv / .xlsx file), including for each chamber:
    - Chip ID and chamber position, sample name and chamber context
    - Value of the global quality flag for the experiment
    - Value of the quality flag “Image Sharpness”, and its exact score value
    - Value of the quality flag “Number of Analyzable Droplets”, and its exact number value
    - Value of the quality flag “Number of Saturated Objects”, and its exact number value
  - *Optional:*  
[Advanced QC Indicators](#) (.csv / .xlsx file), including for each chamber:
    - Chip ID and chamber position, sample name and chamber context
    - Automated threshold values for each channel
    - In “Line” thresholding mode only:
      - manual threshold values for each channel
      - separability,  $\mu$ pos,  $\mu$  neg, std-dev pos, std-dev neg values for each channel
  - *Optional:*  
[FlowJo files](#) (.csv files compatible with FlowJo v10), for each chamber and each compensation status (compensated and not compensated):
 




In these files, for each analyzable droplet is defined:

    - the x and y coordinates of the droplet center in the chamber
    - the 3 fluorescence values (in RFU) of the droplet in each channel
    - the numerical index of the droplet
  - *Optional:*  
[Spillover compensation file](#) (.ncm format), including:
    - the translation vector for the fluorescence background in each channel
    - the excitation matrix for the partial excitation of each fluorophore by each LED
  - *Optional:*  
[Crystal images](#) (.png format in 8 bits), including the images of each chamber in each channel

- *Optional:*  
**Dot Plots** (.png format), including:
  - the 1D dot plots of each chamber, in each channel
  - the 2D dot plots of each chamber, in each channel pairs
  - the concatenated 1D graphs with all the chambers in sequential view, in each channel
  - the concentration graphs, in each channel
  - the uncertainty curves, in each channel
  - the droplet fluorescence histograms, in each channel
- *Optional:*  
**Analysis configuration** file (exported in .yaml format), including all the image analysis parameters

Note that:

- Exporting all the chamber images or graphs will take more time (they will be exported in 8 bits PNG format)
- The indicative size for the exported chamber images in PNG format is 8 MB / chamber
- From each menu of the application, it is also possible to save a given image view or graph view by activating one of the following states

- |        |   |                         |
|--------|---|-------------------------|
| - Save |  | as an image file (.png) |
| - Save |  | as a vector file (.svg) |
| - Copy |  | to clipboard            |

And then clicking on the image or graph of interest.

Finally, to save the experiment, simply click on the “FILE” menu and click on “Save” or “Save as” depending on what is needed.

## IV. Advanced Functionalities

### 1. How to edit the experiment?

- To edit the experiment, click on the “SETUP” > “Edit Experiment” menu.

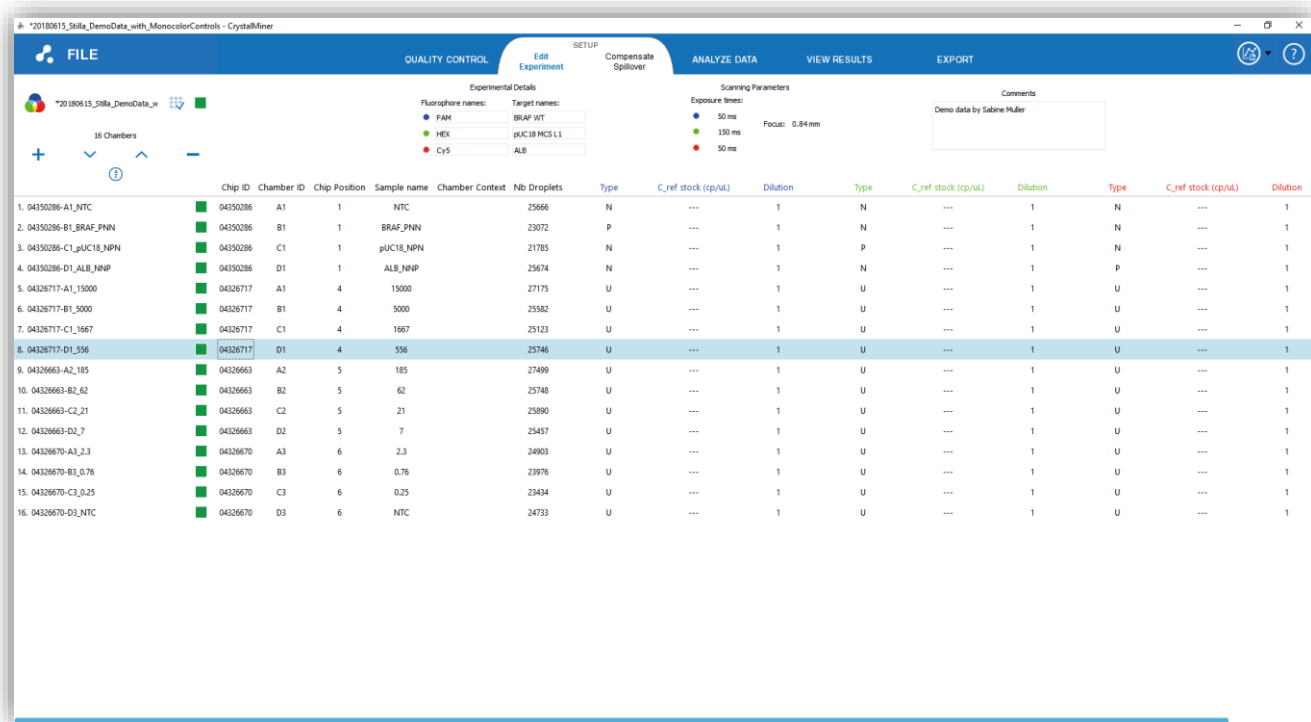


Fig. 33 - Edit the experiment properties

The editable information regarding the context of the experiment is:

- The fluorophore names (for each fluorescence channel)
- The target names (for each fluorescence channel)
- The user comments

The editable information regarding the context of each chamber is:

- The sample name
- The sample type (U: unknown; N: negative; P: positive; S: standard)
- The dilution factor, when 10 means that the mix has been diluted 10 times from the stock
- The standard concentration, which is the known stock concentration in the case when the sample type is “S”

Note that if you modify the dilution factor from “1” to “10”, then the estimated stock concentration, will be multiplied by 10.

Some fields are read-only because their content is used to identify the chambers..

- To remove one or several chamber(s) from the current experiment, select the chamber(s) in the left panel list, then click on the “-” icon above the list
- To add the chambers of another experiment into the current experiment, you can click on the “+” icon above the chamber list, then select the experiment to be imported (in .ncx or .ncr format) or you can drag & drop the file in the application window.

Note that the imported experiment should have exactly the same experiment parameters as the current experiment, otherwise the results may be compromised.

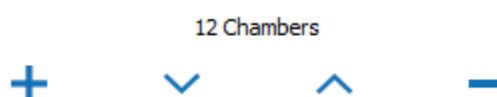


Fig. 34 - Icons to add, sort or remove experiment chambers in the EDIT EXPERIMENT menu

### CAUTION:

**The experiment to be added should have exactly the same experiment parameters as the current experiment, i.e.:**

- **Fluorophore names and target names should be the same, otherwise they will be erased by that of the current experiment**
  - **Scanning parameters (exposure times and focus value) should be the same, otherwise they will be erased by those of the current experiment**
  - **Image analysis parameters should be the same, otherwise a warning message will be displayed, and it is recommended to perform image re-analysis after chamber addition**
- To modify the order of the chambers in the list, click on the “up” and “down” icons above the chamber list to sort them by name, or click on the title of any column to sort them according to the column value in increasing or decreasing order.

For example: the chambers can be sorted according to increasing stock concentration values in the “Blue” channel, in order to visualize increasing values in the concentration graph of the “Blue” channel.



## 2. How to navigate in the 3D graph?

- To visualize the 3D graph, click on the “ANALYZE DATA ” > “Plots and Populations” menu, then click on the “3D dot plot” sub-menu. The 3D graph is displayed, where each axis represents the fluorescence value of the droplets in a given channel.

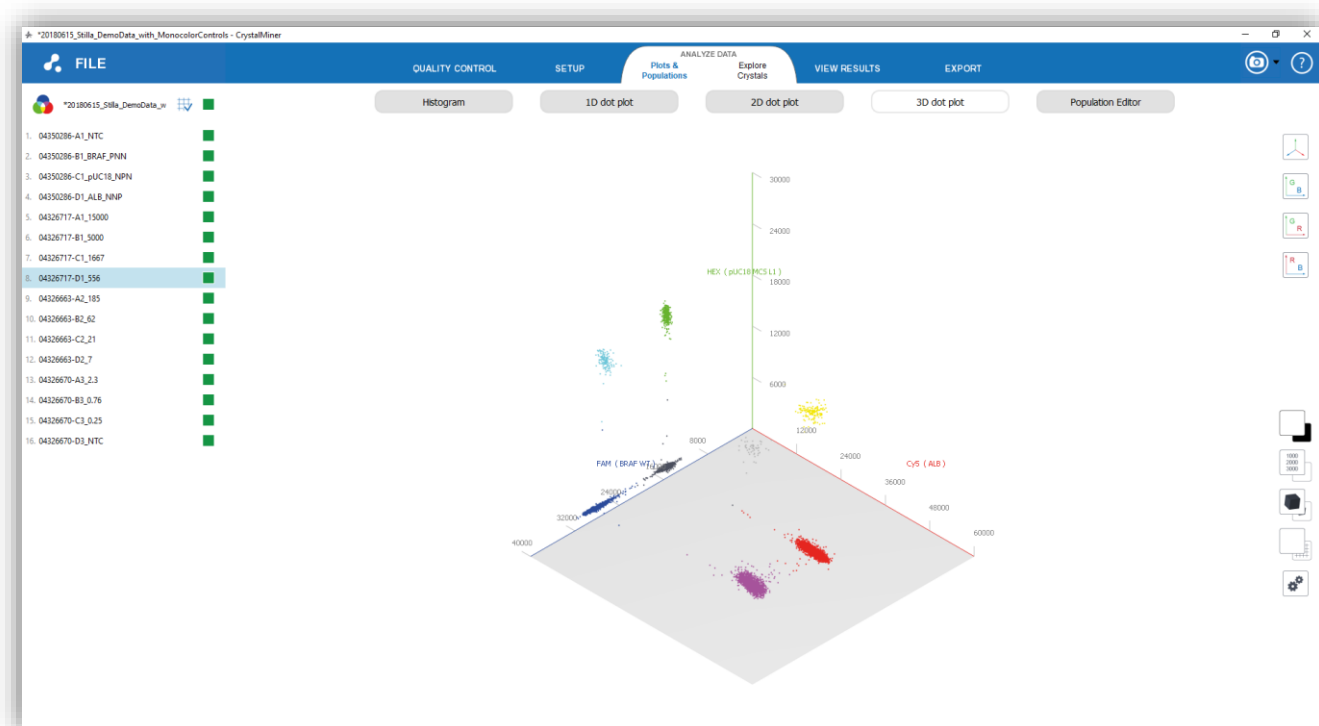


Fig. 35 - Navigation in the 3D dot plot

- To navigate in the 3D graph:
  - Use mouse scroll to zoom in in the graph
  - Use right mouse click to rotate the graph
  - Use left mouse click to translate the graph (and the rotation center)
  - Double-click with left mouse button to reset the view

- Icon description:



Reset the view



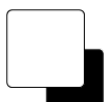
Project the view on the Green and Blue axis



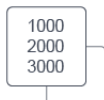
Project the view on the Green and Red axis



Project the view on the Red and Blue axis



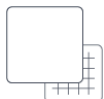
Switch between black and white backgrounds



Show or hide the fluorescence values



Switch between the perspective and isometric views



Hide or show the grid



Modify the point size, the line width, or the grid step

### 3. How to read the droplet fluorescence histograms?

- To read the histograms of the droplet fluorescence values, click on the “ANALYZE DATA ” > “Plots and Populations” menu, then click on the “Histogram” sub-menu.

The histograms of the currently selected chamber(s) are displayed for each channel, where the x-axis shows the fluorescence value (in RFU) and the y-axis shows the number of associated droplets:



Fig. 36 - Histograms of fluorescence

This histogram view is useful to appreciate the separability between the negative population (on the left side of the histogram) and the positive droplet population (on the right side of the histogram), and thus to check or adjust the fluorescence thresholds.

If multiple chambers are selected, then the number of droplets in each fluorescence bin are simply added together.

#### 4. How to interpret the uncertainty curves?

- To visualize the curve of relative uncertainty at 95% confidence level, click on the “VIEW RESULTS ” > “Advanced Graphs” menu, then click on the “Uncertainty Curves” sub-menu.

The 95% relative uncertainty curves are displayed for each channel, where:

- The x-axis shows all possible chamber concentrations (in copies /  $\mu\text{L}$ )
- The y-axis shows the 95% relative uncertainty (in %) of the chamber concentration (which is based on the total number of droplets analyzed in the currently selected chamber)
- The colored curve corresponds to the relative uncertainty curve of the currently selected chamber (the higher the total number of droplets in the chamber, the lower the curve)
- The upper gray curve corresponds to the relative uncertainty curve of the chamber having the minimum total number of droplets in the experiment
- The lower gray curve corresponds to the relative uncertainty curve of the chamber having the maximum total number of droplets in the experiment
- The filled point(s) located on the curve corresponds to the predicted concentrations of the currently selected chamber(s) on the left panel (be careful to distinguish between the chamber concentration and the stock concentration which is equal to the chamber concentration times the dilution factor)
- The white point located on the curve represents the chamber concentration which minimizes the relative uncertainty (this indicative value may help to estimate to optimal dilution for the experiment; note that whatever the number of droplets and the confidence level, the white point is reached where ~79.7% of the droplets are positive).

See the “*Digital PCR statistics*” chapter in the Annexes for more information about the computation of the 95% relative uncertainty.

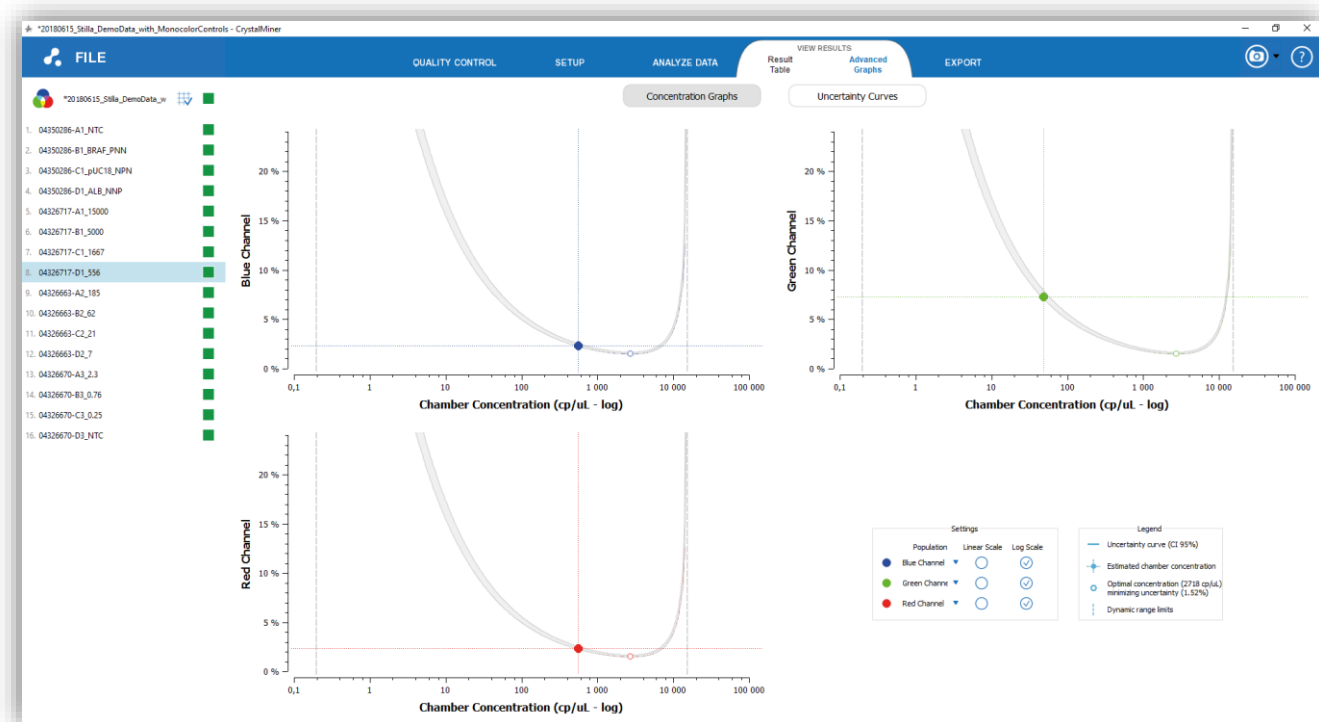


Fig. 37- Relative Uncertainty Curve

## 5. How to concatenate or aggregate graphs?

- To concatenate 1D graphs, select multiple chambers in the left panel (using “Ctrl+Left-Click”). The 1D graphs of the selected chambers are then concatenated in a sequential view (according to the order of the chamber list):

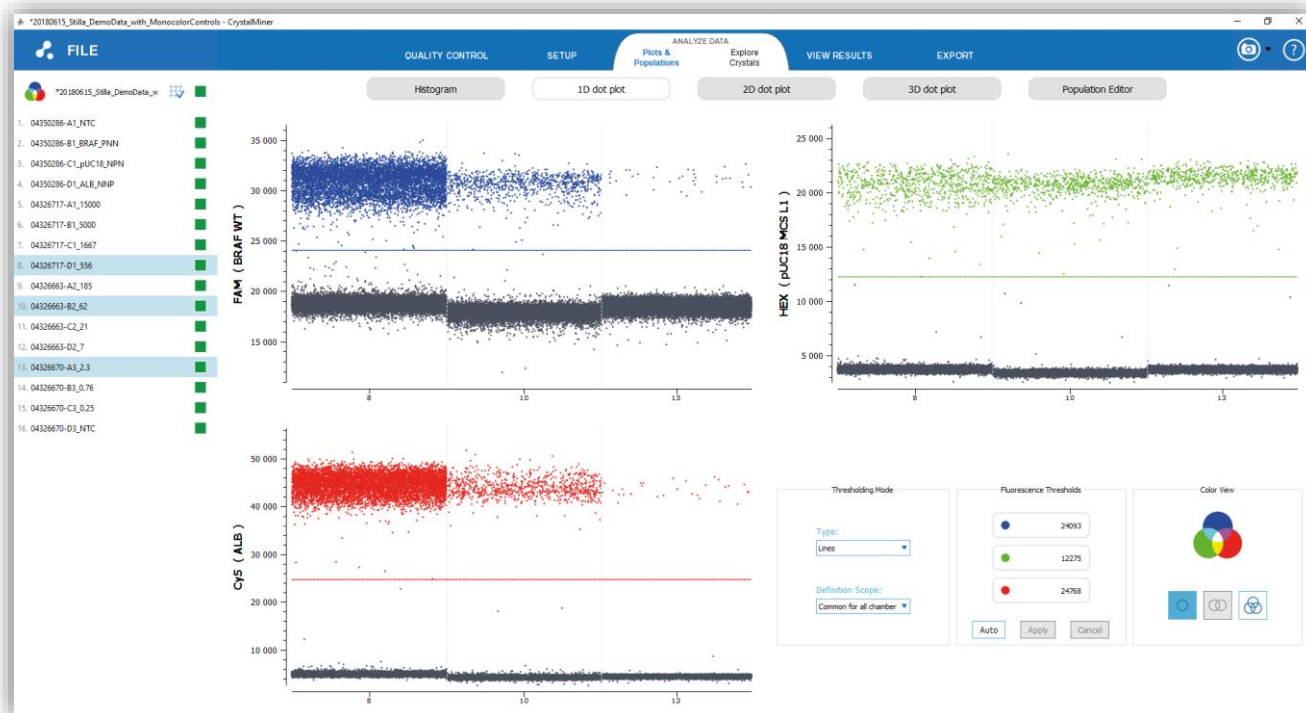


Fig. 38 - Concatenated view of 1D dot plots

- To aggregate 2D graphs, select multiple chambers in the left panel (using “Ctrl+Left-Click”). The 2D graphs of the selected chambers are then aggregated:

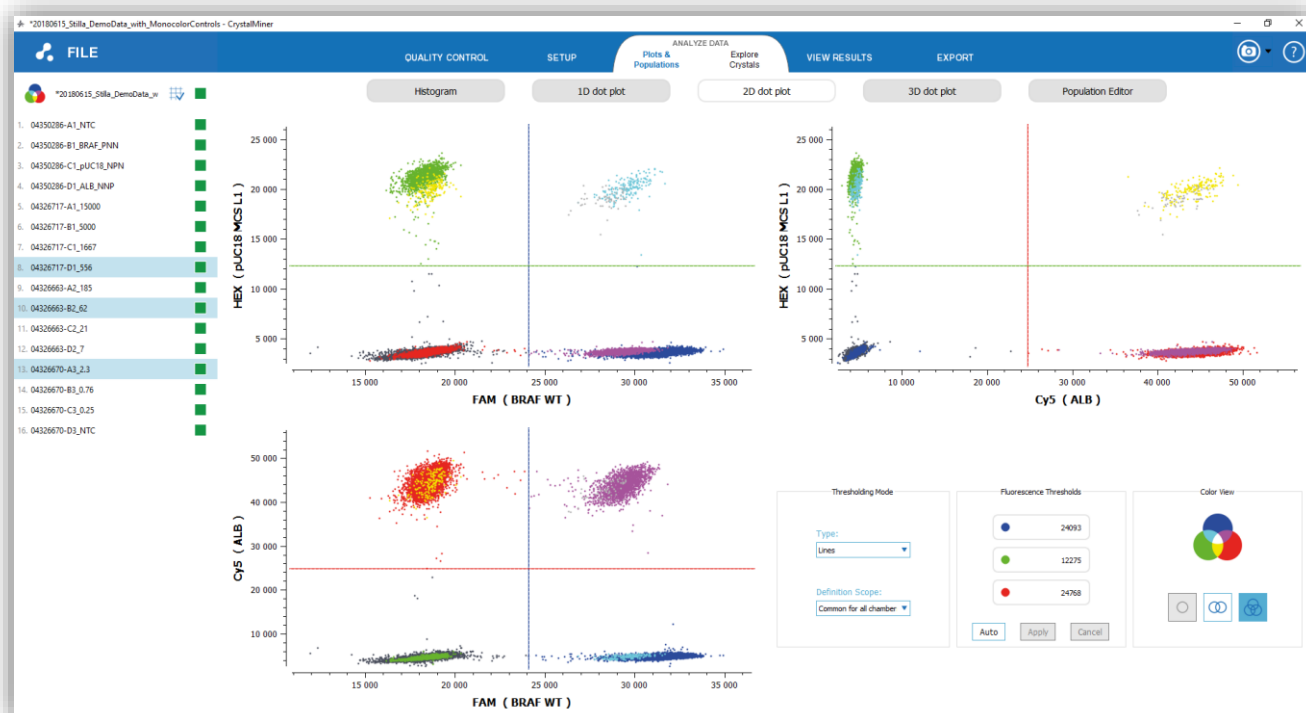


Fig. 39- Concatenated view of 2D dot plots

- To aggregate 3D graphs, select multiple chambers in the left panel (using “Ctrl+Left-Click”). The 3D graphs of the selected chambers are then aggregated:

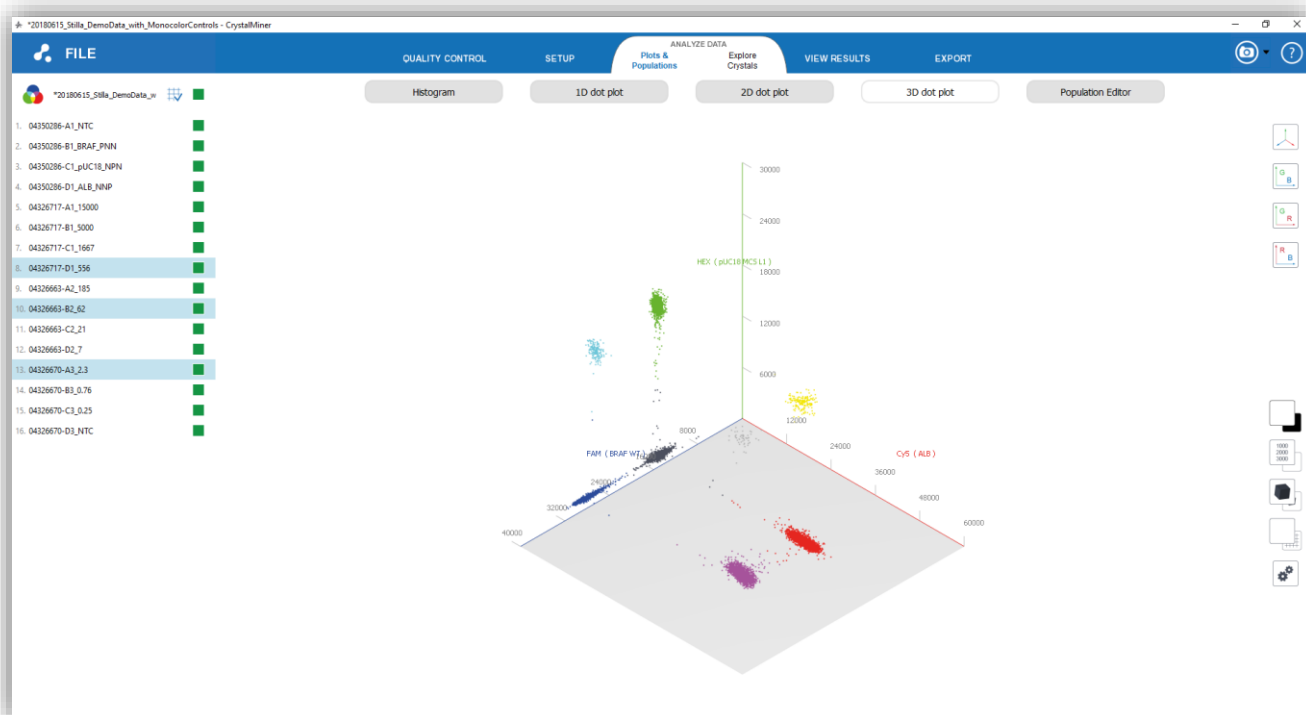


Fig. 40 - Concatenated view of 3D dot plots

- You can perform the similar operations on the histogram graphs and on the uncertainty curves:



Fig. 41 - Concatenated view of Histograms

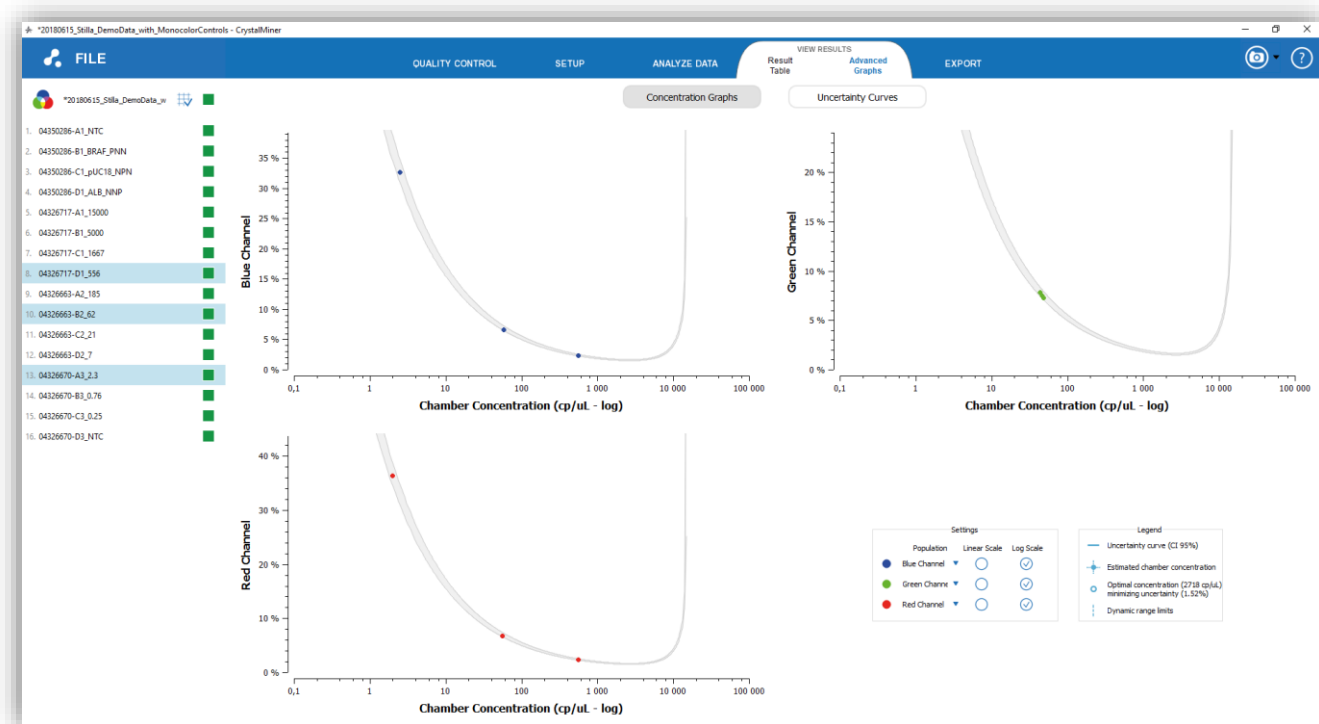


Fig. 42 - Concatenated view of Uncertainty Curves

## 6. How to explore the crystal populations?

- To explore the crystal populations, click on the “ANALYZE DATA” - “Explore Crystals” menu, then select in the left panel the chamber of interest.
- To navigate in the crystal image of a chamber:
  - Use mouse scroll to zoom in and out in the image
  - Use left mouse click to translate the image
  - Double-click with left mouse button to reset the view
  - Click on the LED icons in the “Channel selection” widget to change the detection channel (“Blue”, “Green” or “Red” LED)
  - Click on the “Auto” button (resp. “Reset” button) in the “Contrast Adjustment” widget to automatically adjust image contrast (resp. reset image contrast)



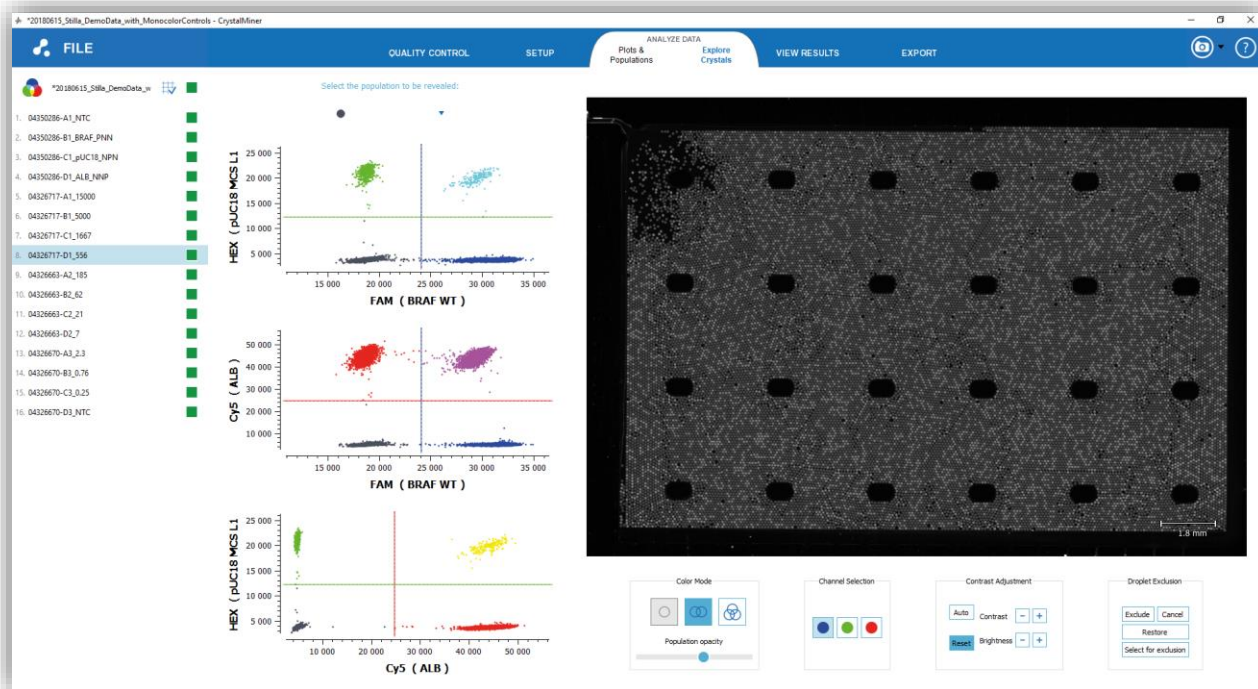


Fig. 43 - Explore crystals in 8 colors mode

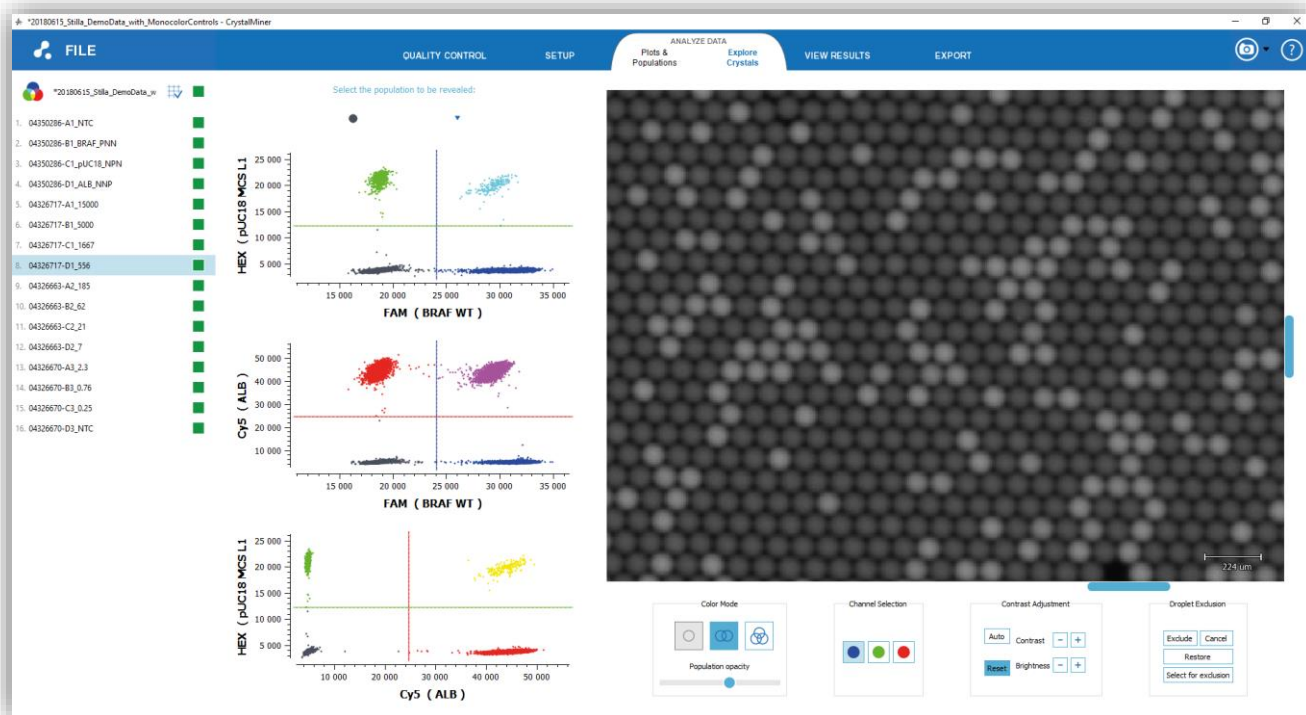


Fig. 44 - Zoom in the crystal

- To highlight specific droplet in the crystal image:
- To activate / deactivate the highlight of droplets included in one quadrant of a 2D graph, left-click on the quadrant of interest.

The activated quadrant will be filled in blue in the graph and the droplets included in this quadrant will be highlighted in their current color-codes in the image. You may use the “Population opacity” cursor to adjust the alpha value.

Use “Shift + Left-click” to select multiple quadrants in a given 2D graph and highlight multiple droplet populations.

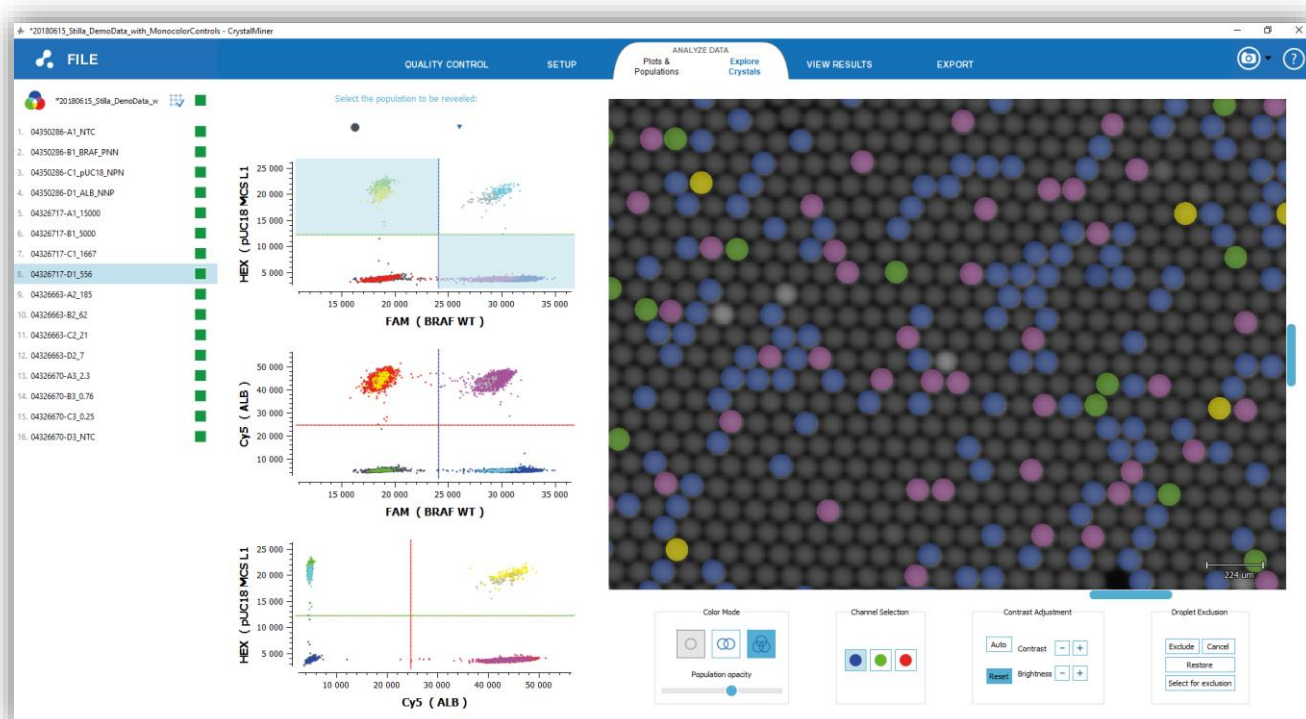


Fig. 45 - Highlighted droplet populations using graph quadrants

- To highlight the droplets belonging to a given population, select the population name in the drop-down menu above the 2D dot plots.

The associated zones (quadrants or polygons) will be filled in blue in the graph and the droplets included in these zones will be highlighted with the population color in the image. You may use the “Population opacity” cursor to adjust the alpha value.

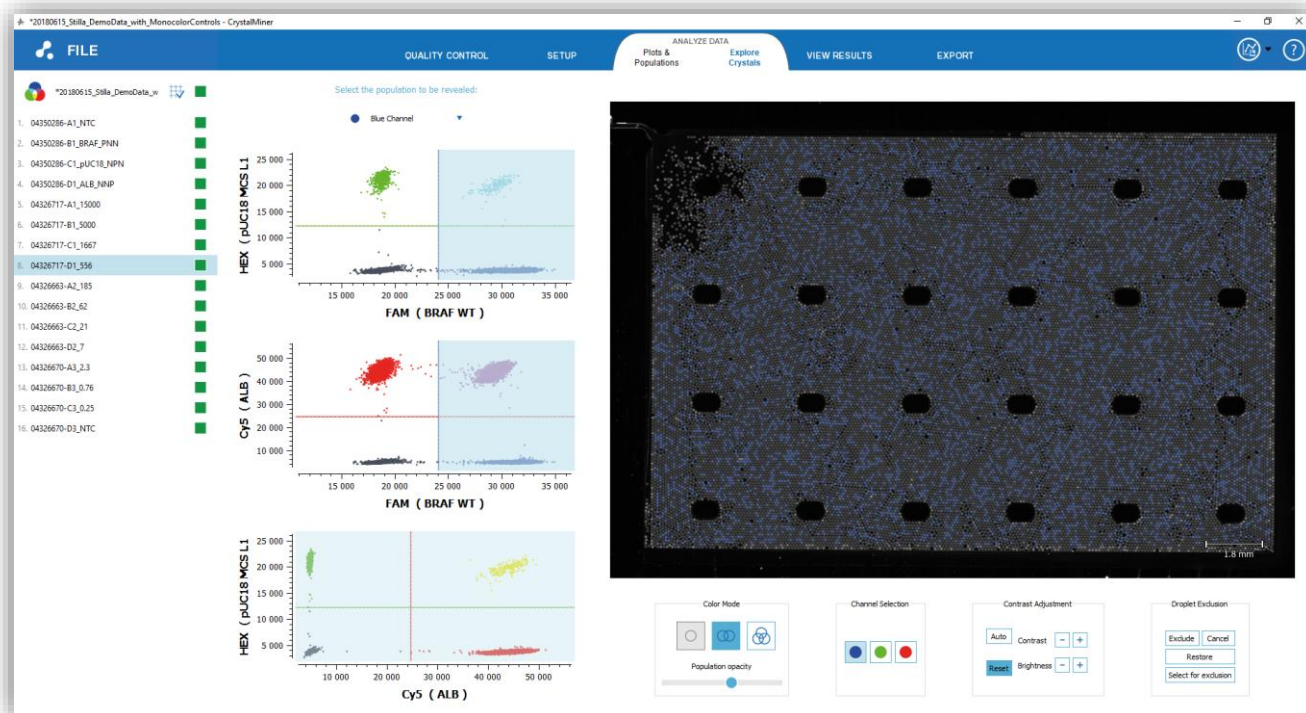


Fig. 46 - Highlighted droplet populations using graph quadrants

- To highlight droplets included in one user-defined polygonal region of a 2D graph, press the “Ctrl” key, then right-click several times to define the polygonal region to be drawn (one click = one vertex), and leave the “Ctrl” to define the last polygon vertex.

The polygon will then be drawn with orange edges the 2D graph and the droplets included in this polygon will be highlighted in orange color in the image. You may use the “Population opacity” cursor to adjust the alpha value.

You may then edit the polygon:

- Move one of its vertices by left-clicking and dragging it where you want
- Add a vertex by right clicking in on the edge to be split
- Remove a vertex by right clicking on it

You can also define a basic diamond polygon in one click by holding “Shift” key while right-clicking. Then edit it normally.



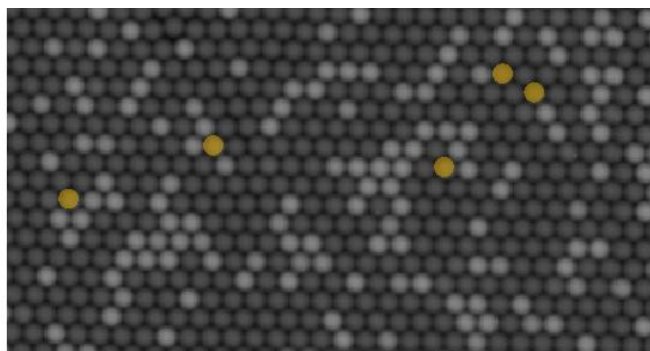
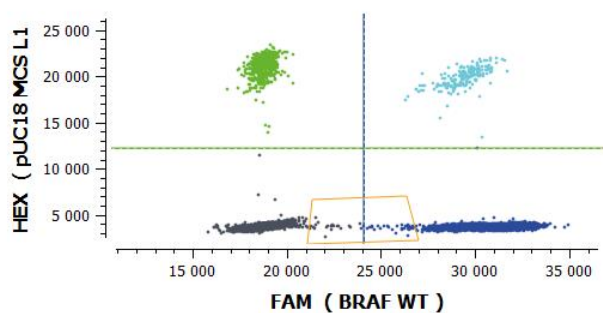


Fig. 47 - Highlighted droplet population using graph-based polygonal region (left: polygon drawn in the 2D graph; right: included droplets are highlighted in orange color)

- To exclude highlighted droplets from the analysis, click on the “Select for exclusion” button in the “Droplet Exclusion” widget, then you may manually deactivate / activate some of them by right-clicking on them in the image, and finally click on the “Exclude” button in the “Droplet Exclusion” widget to remove them from the analysis.

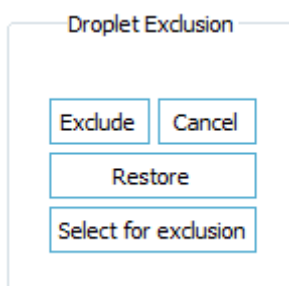


Fig. 48 - Widget for manual droplet exclusion in EXPLORE CRYSTAL menu

Manually excluded droplets will be marked with yellow hexagons in the crystal images and will be removed from any dot plot:

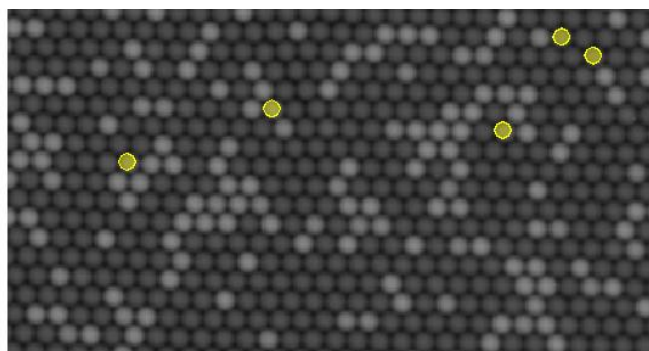
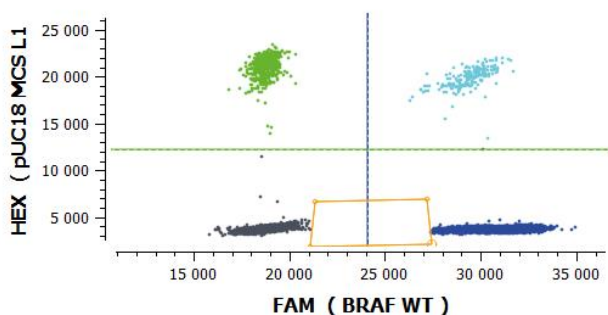





Fig. 49 - Excluded droplets are removed from the 2D graph and marked with yellow hexagons in the image

- To restore all the manually excluded droplets, click on the “Restore” button in the “Droplet Exclusion” widget.

## 7. How to perform quality control?

- To perform quality control, click on the “Quality Control” menu.

For quality control, 3 types of quality flags are proposed:

-  Green flag: high quality
-  Green & Yellow flag: moderate quality
-  Yellow flag: low quality

These quality flags are automatically computed and provided at 3 levels:

- **Experiment level:** the experiment flag corresponds to the worst quality flag at the chamber level, among the chambers belonging to the experiment
- **Chamber level:** the chamber flag corresponds to the worst quality flag at the criterion level, among the criteria computed for the chamber
- **Criterion level:** 3 specific flags are used
  - **“Image Sharpness”:** the higher the score the better the sharpness in chamber images.  
Note that a yellow flag may be caused by an out-of-focus issue (this can be corrected when using Crystal Reader by checking that chip holder is clean and chamber is well placed)
  - **“Number of analyzable droplets”:** the higher the number the better the confidence in predicted concentration results.  
Note that saturated droplets and droplets covers by artefacts are not analyzable (this can be corrected when using Crystal Reader by checking that exposure times are not too high, and chamber is cleaned up).
  - **“Number of saturated objects”:** the lower the number the better the chamber quality.  
Note that saturated objects are either saturated droplets caused by too high exposure time or saturated artefacts (this can be corrected when using Crystal Reader by checking that exposure times are not too high and chamber is cleaned up).

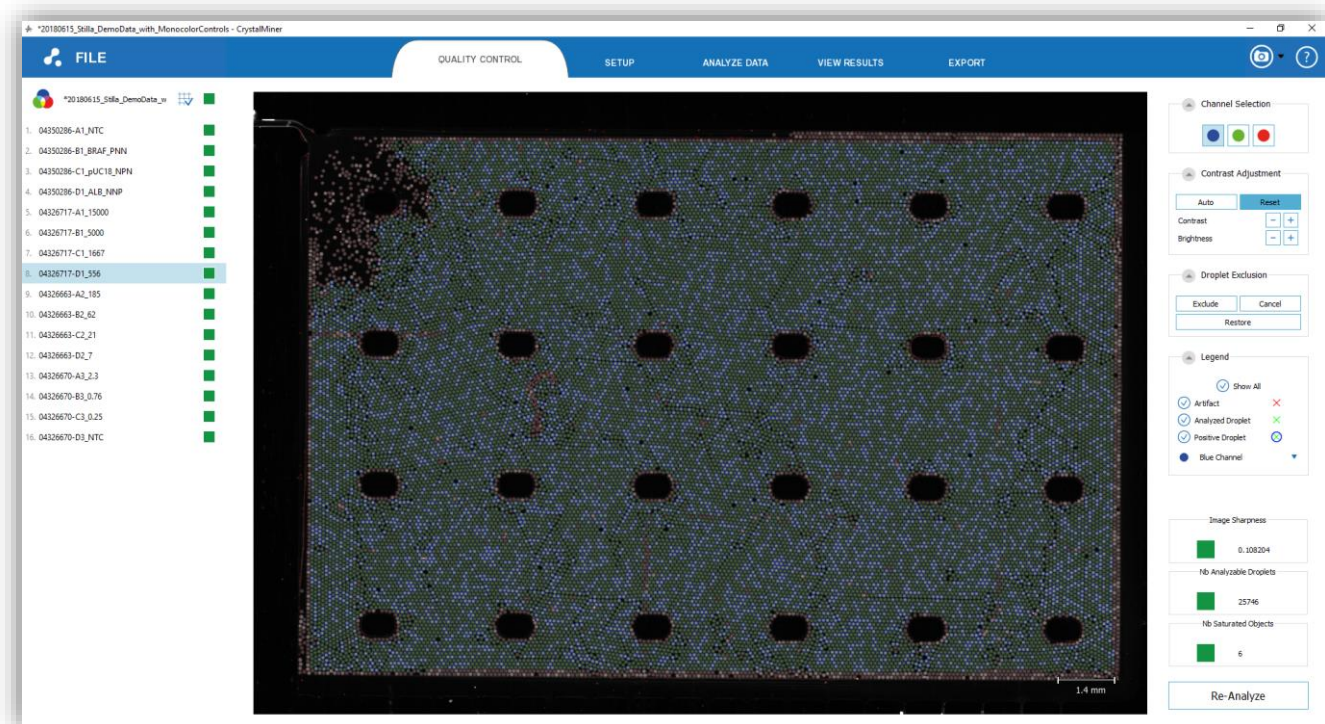


Fig. 50 - Annotated crystal for quality control

- To inspect the crystal of a given chamber, click on the chamber in the chamber list. The chamber crystal is then displayed with the following annotations:

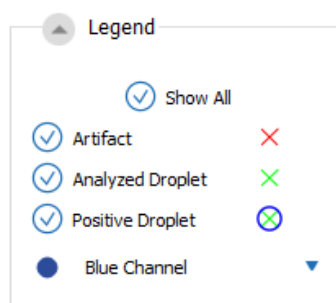





Fig. 51 - Legend for image annotation in QC menu (checkboxes allow to show / hide specific annotations)

-  Red Cross: artefact (e.g. dust, too small droplet, too large droplet, too irregular droplet, boundary droplet, etc.) not counted as an analyzable droplet
-  Green Cross only: negative droplet in the currently selected channel (counted as an analyzable droplet). **Note that all negative droplets should be visible in the “Blue” channel image thanks to the addition of the reference fluorophore (e.g. Fluorescein or FITC fluorophore).**
-  Blue Circle around Green Cross: positive droplet in the currently selected population in the drop-down menu just below (counted as an analyzable droplet)

- To navigate in a chamber crystal:
  - Use mouse scroll to zoom in and out in the image
  - Use left mouse click to translate the image
  - Double-click with left mouse button to reset the view
  - Click on the LED icons in the “Channel selection” widget to change the detection channel (“Blue”, “Green” or “Red” LED)
  - Click on the “Auto” button (resp. “Reset” button) in the “Contrast Adjustment” widget to automatically adjust image contrast (resp. reset image contrast)

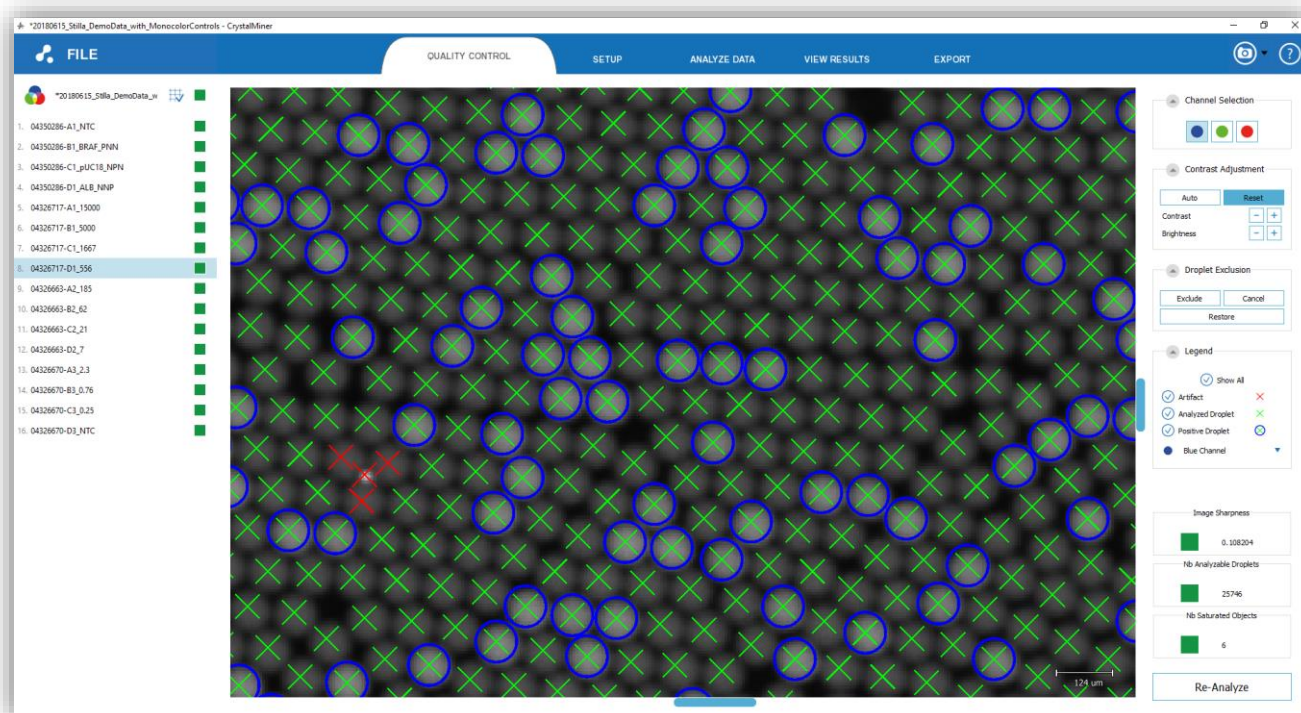


Fig. 52 - Zoom in the annotated crystal for quality control

- To exclude one or several droplet(s) considered as false positive(s) (i.e. artefacts not to be counted as analyzable droplets):
  - Right-click on each droplet to be excluded
  - Click on the “Exclude” button in the “Droplet Exclusion” widget

Note that excluded droplets are marked with yellow hexagons.



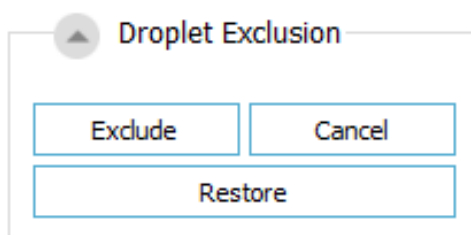


Fig. 53 - Widget for manual droplet exclusion in QC menu

- To exclude all the droplets belonging to an image region:
  - Press the “Ctrl” key, then right-click several times to define the polygonal image region to be excluded (one click = one vertex), then leave the “Ctrl” to define the last polygon vertex
  - Click on the “Exclude” button in the “Droplet Exclusion” widget

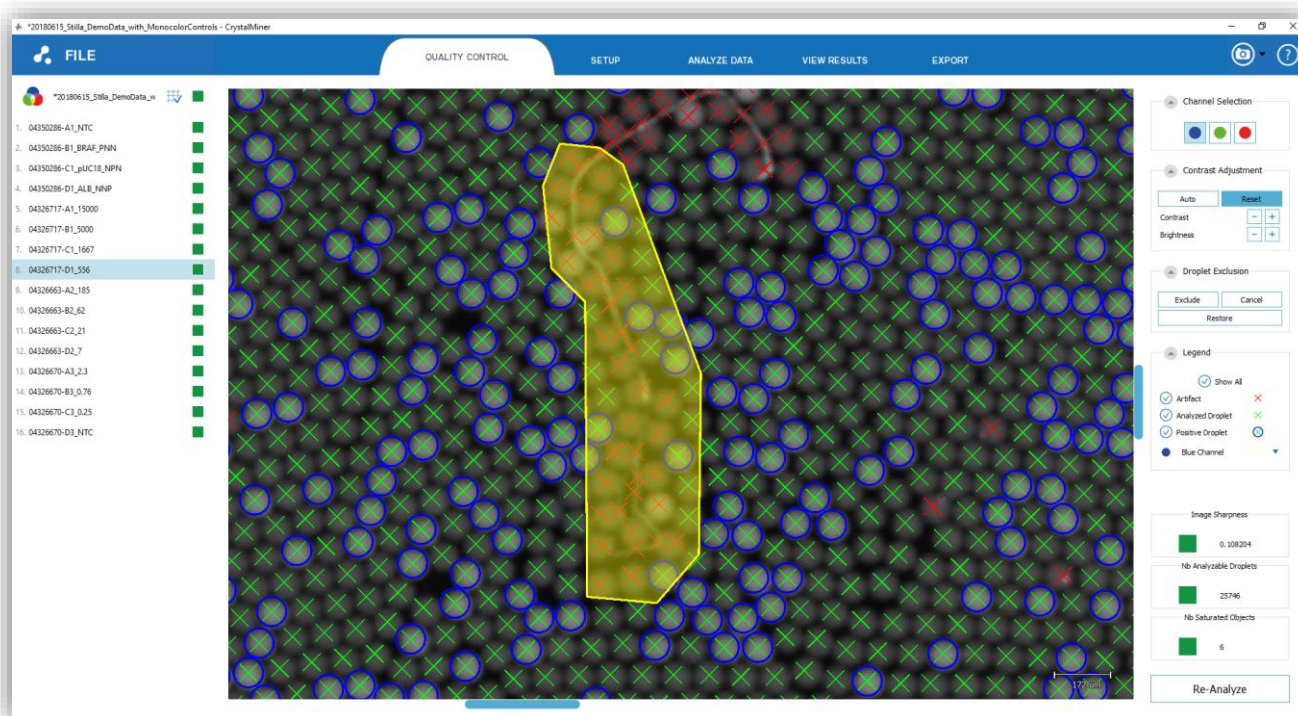
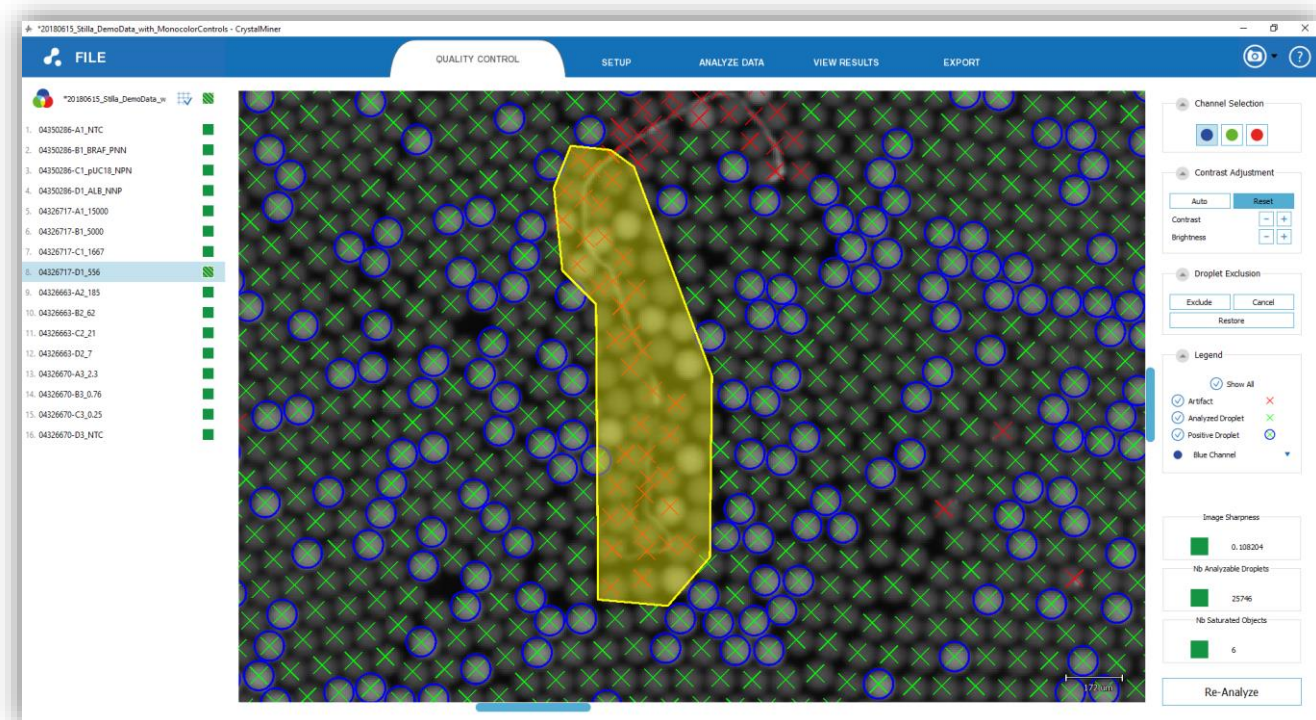


Fig. 54 - Selection of droplet or polygonal image regions to be excluded (note that droplet annotations are still visible in the yellow color before clicking on “Exclude”)





*Fig. 55 - All selected droplet or image regions are removed  
(note that droplet annotations are not visible anymore in the yellow color after clicking on “Exclude”)*

Note that all droplets belonging to the polygonal region will be removed from the analysis and marked with yellow color.

- To cancel the current selection of droplets to be removed, click on the “Cancel” button in the “Droplet Exclusion” widget.
- To restore all the manually excluded droplets, click on the “Restore” button in the “Droplet Exclusion” widget.

## 8. How to perform image re-analysis?

- To perform image re-analysis, click on the “Quality Control” menu (Quality Control menu), then select the control chamber for image re-analysis in the left panel, and click on the “Re-Analyze” button on the bottom right.
- In the first pop-up window:
  - Click on the “browse” icon and select the analysis configuration file in .yaml format to be applied for image re-analysis (note that this .yaml file should have been duplicated from the template analysis configuration file presented in the Annexes part of this document, before being modified)
  - Select the runtime strategy: "Sequential analysis" (default, longer, but compatible with other tasks) or "Parallel analysis" (shorter, but may slow down other tasks)
  - Click on the “Analyze” button to launch the image re-analysis on the control chamber

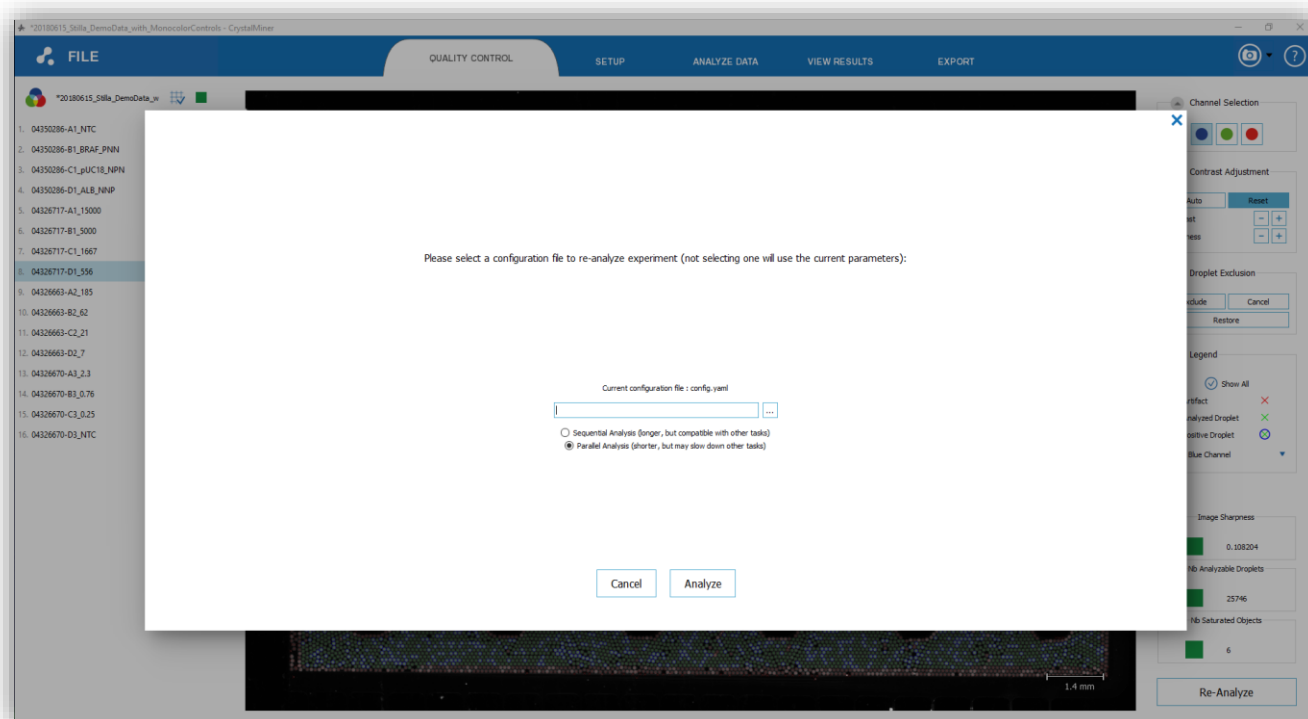


Fig. 56 - Select the input analysis configuration file (.yaml file) for experiment re-analysis

- When image re-analysis is done on the control chamber, a second pop-up window is displayed, showing the new results:
  - You can navigate in the new annotated chamber image
  - If you are satisfied, click on “Apply” to apply the re-analysis to the whole experiment with the new analysis parameters (this will take around 30 sec. per chamber with 16 Go of RAM), else click on “Cancel”



Fig. 57 - Apply or Cancel experiment re-analysis

Note: At the end of image re-analysis, the fluorescence spill-over is not compensated anymore so spill-over compensation should be performed again.

## 9. How to adjust thresholds at chamber level?

By default, the fluorescence thresholds automatically computed by Crystal Miner for each detection channel are 1D “Line” thresholds that are common to all the chambers of the experiment.

Any manual adjustment of these thresholds is by default also commonly applied to all chambers. This allows to ensure both objectivity and repeatability in the quantification results, assuming that all the chambers of the experiment share the same conditions.

If common thresholds are not relevant given the observed chamber-to-chamber variability, it is possible to adjust the fluorescence thresholds at the chamber level.

- To this purpose, go to “ANALYZE DATA” > “Plots & Populations” > “2D dot plot”, then go to the “Thresholding Mode” widget and select “Individual per chamber” (instead of “Common for all chambers”) in the “Definition Scope” combo box.
- You will then be able to manually adjust the thresholds at the level of each chamber (click on “Apply” to validate any adjustment).

Note that the easiest way to view the “Line” thresholds of all the chambers is to use the concatenated view in the 1D dot plots by selecting all the chambers in the left panel:

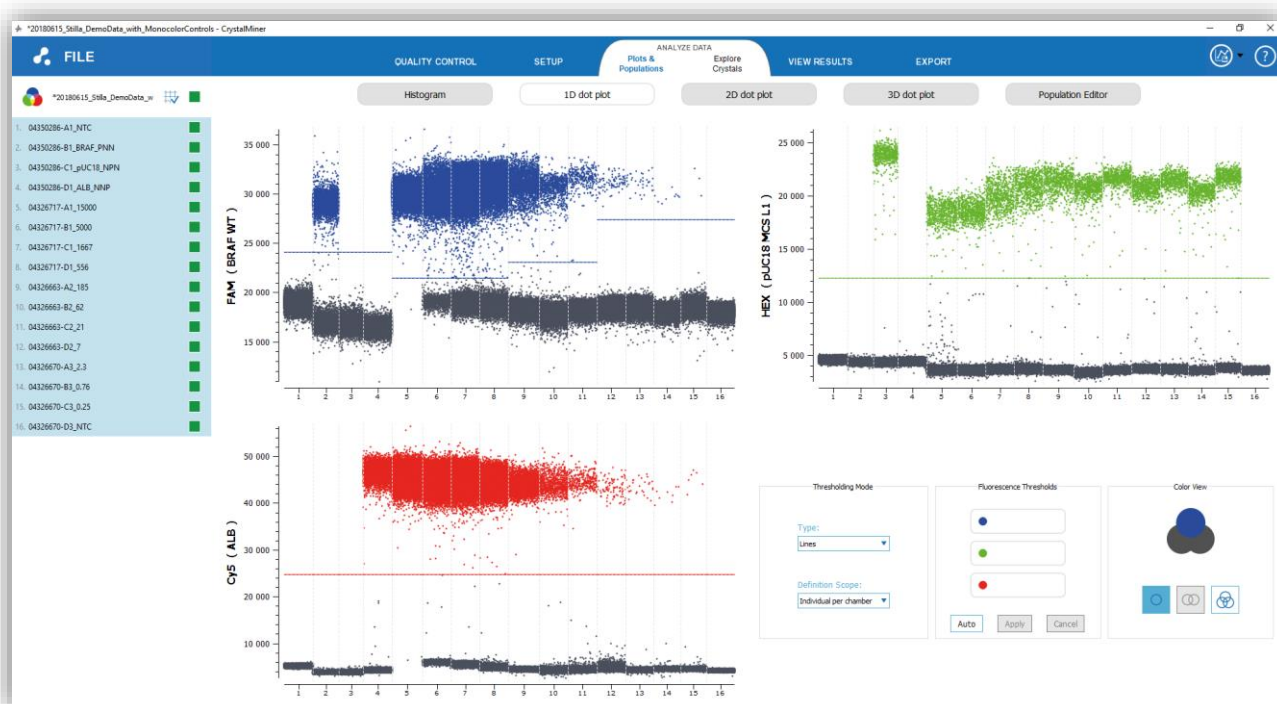


Fig. 58 – Visualization of all the individual thresholds in the 1d dot plots

## 10. How to define custom populations?

The process of defining more refined populations than the 3 default ones proposed for each experiment (i.e. Blue, Green, Red populations) is split into two distinct parts: zoning definition and population definitions.

The zoning definition consist of describing the way you want to partition the 3D fluorescence space into zones.

Once this is done, you can define populations based on the zones you created previously, and Crystal Miner will automatically compute all the usual results (number of positive and negative droplets, concentration, confidence interval) for all these populations.

### ○ Zoning definition:

There are two ways to partition the 3D fluorescence space with Crystal Miner: 1D “Line” thresholds and 2D “Polygon” thresholds.

To select the appropriate thresholding type to be applied to the whole experiment, go to “ANALYZE DATA” > “Plots & Populations” > “2D dot plot”, then go to the “Thresholding Mode” widget and use the “Type” combo box.

- The 1D “Line” thresholds are the default zoning for any freshly scanned experiment.

Each 1D line splits each channel in 2 parts, resulting in a total of 8 zones (see the section “*Data visualization and interpretation*”). See the section “*Check the fluorescence thresholds*” for more details on how to manipulate Line thresholds.

- The 2D “Polygon” thresholds provide a more flexible way to define zones in the 3D fluorescence space by drawing 2D polygons and projecting them along the third axis.

By default, when you switch to the “Polygon” thresholding type, the experiment does not contain anymore the populations and zones created by default.

You have to manually create Polygon zones to suit your needs. To this purpose, go to “ANALYZE DATA” > “Plots & Populations” > “2D dot plot” and select “Polygons” in the “Type” drop-down button of the “Thresholding Mode” widget:



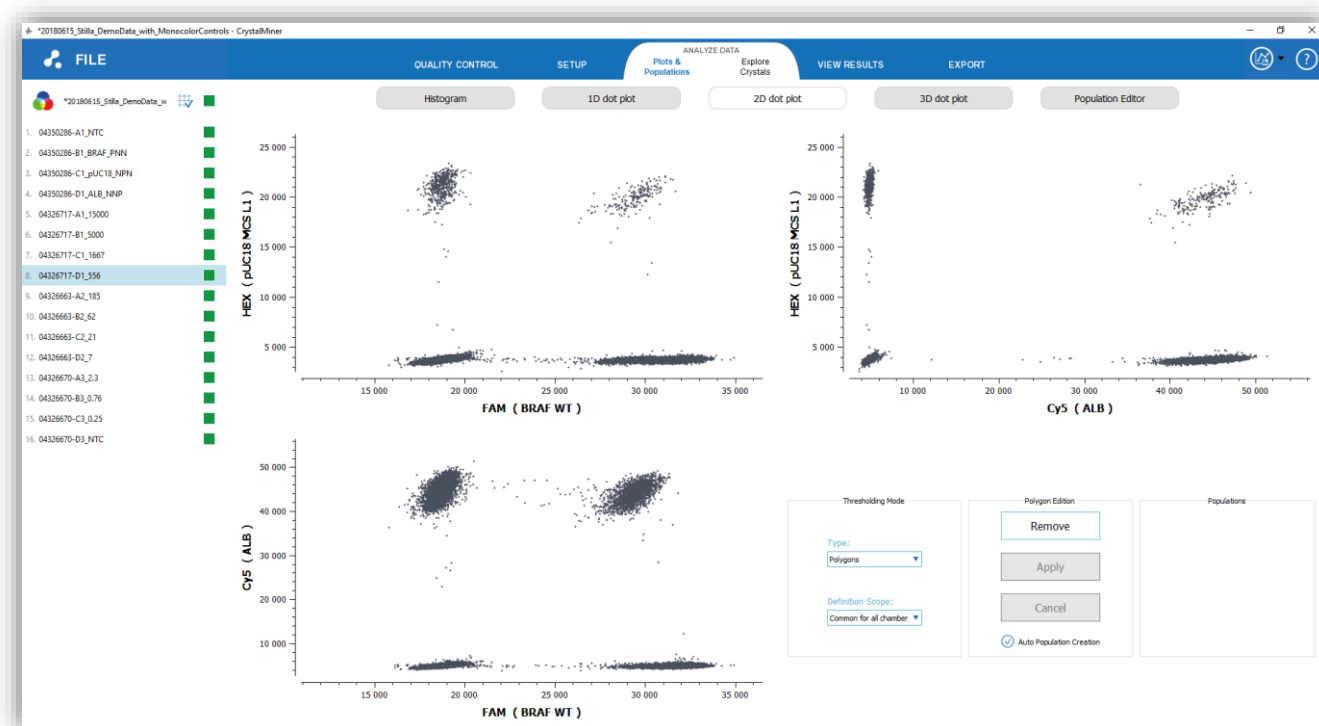


Fig. 59 – An experiment freshly converted to “Polygon” thresholding mode

You can then create and edit polygons in the same way than in the Explore Crystal plots (see the section “How to explore the crystal populations?”)

By default, the checkbox “Auto Population Creation” is checked, prompting you to create a new population each time you create a polygon.

You can uncheck it if you’d rather create all your polygons first and then create populations from one or multiple polygons.

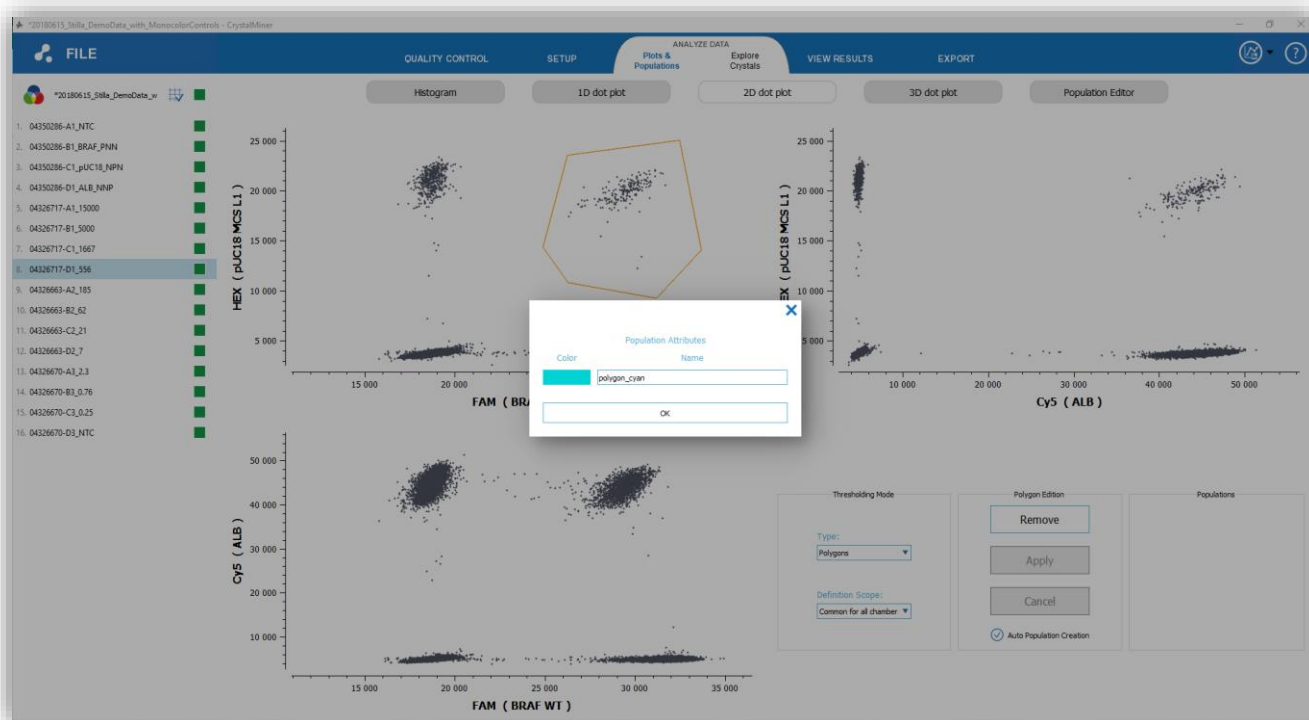


Fig. 60 – Creating a new population upon Polygon creation

If a polygon is associated with only one population, it will be displayed with the color of the population, otherwise it will be orange.

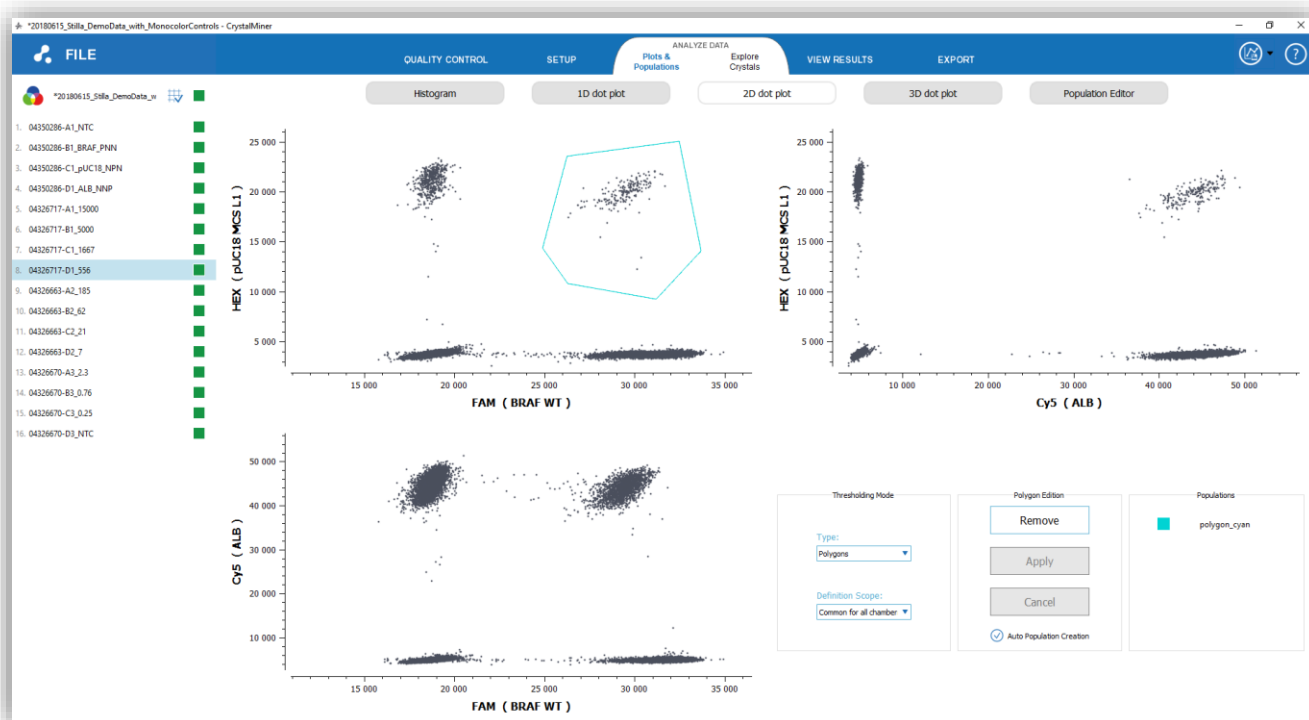


Fig. 61 – A polygon is colored with the population color if it is associated with only one population

You can edit Polygon thresholds (i.e. drag & drop, add or remove summits and edges), and apply or cancel the changes as needed:

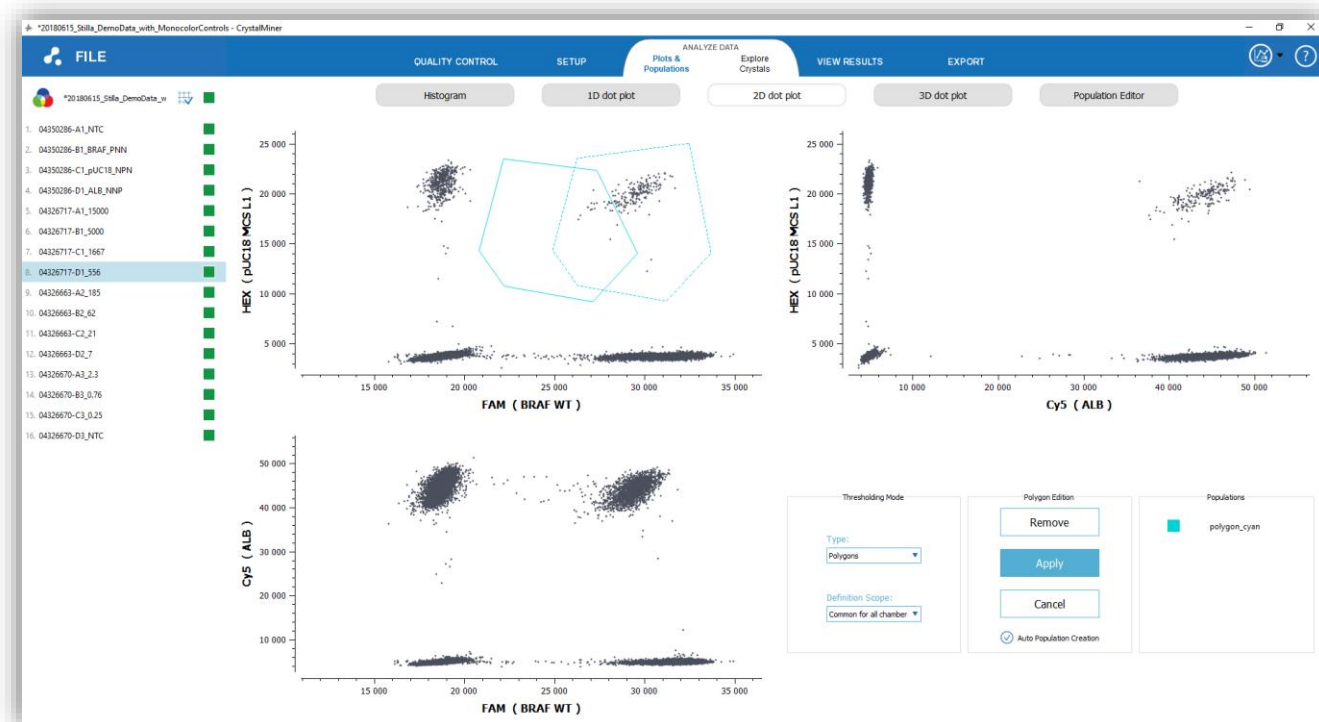


Fig. 62 – If one polygon has been moved or edited, the change can be applied or cancelled

As for the Line thresholds, you can choose to either set the Polygon thresholds uniformly across the whole experiment, or adjust each Polygon zone chamber by chamber as far as you have selected the mode “Individual per chamber” in the “Definition Scope” combo box of the “Thresholding Mode” widget.

Note that in the “Individual per chamber” mode, that you can only view the Polygon zones adjusted for one chamber in the same 2D dot plots (no polygon zones will be displayed if you select more than one chamber in the left panel).



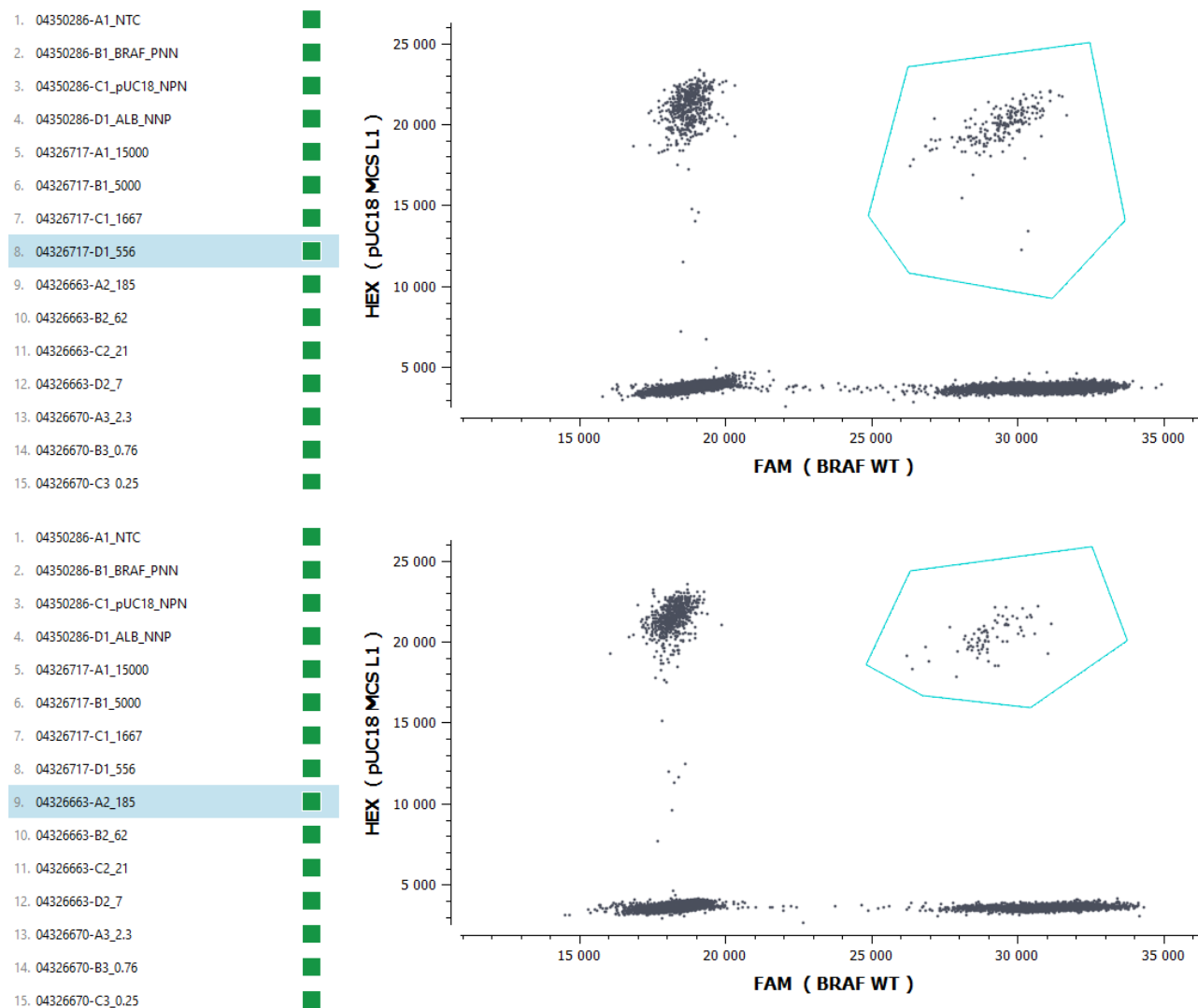


Fig. 63 – Example of Polygon zone that is adjusted differently in 2 different chambers

### ○ Population definition:

Once your fluorescence zones are defined, you can define the populations you need to quantify using a building block approach.

Go to the “ANALYZE DATA ” > “Plots and Populations” and in the “Population Editor” sub-menu.

You can see in the right panel the current list of the populations for the experiment (by default the “Blue channel”, “Green channel” and “Red channel” populations), and in the center the list of the zones already defined along with dot plots to help visualize them.

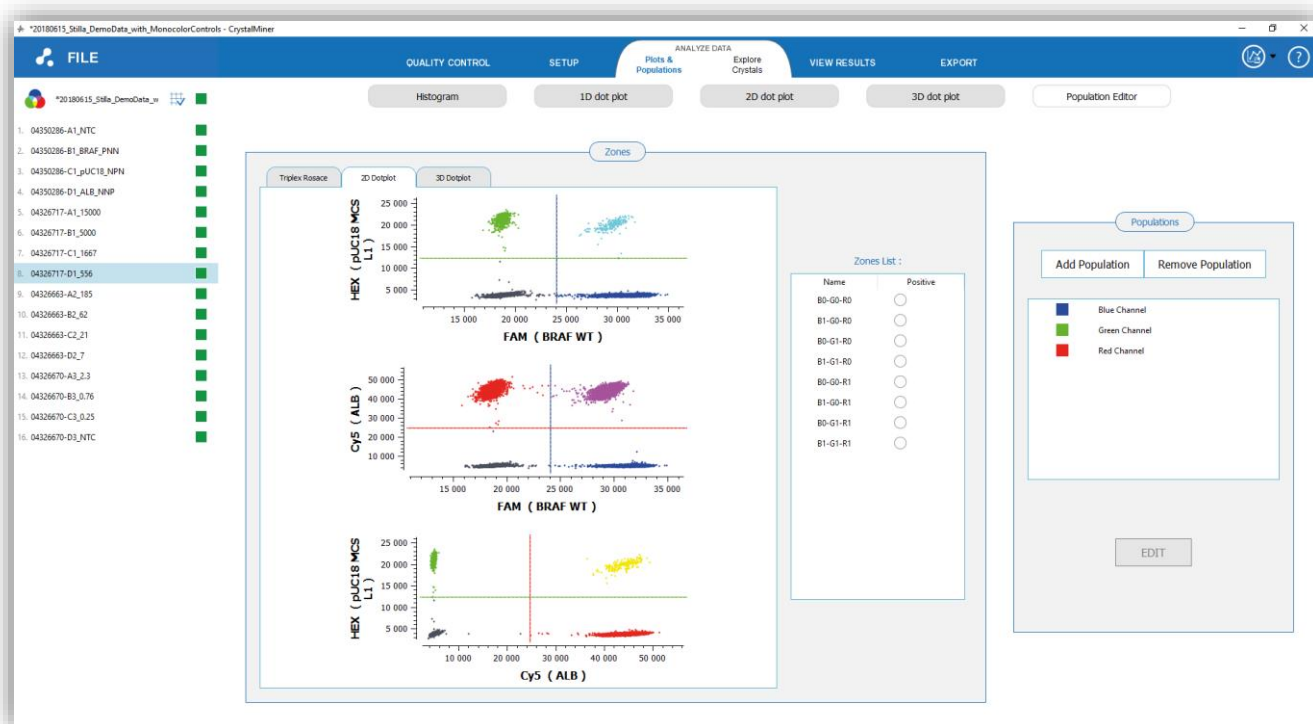


Fig. 64 - Population Editor for a default triplex experiment

For each population, you can display its associated zones (both in the list and in the plots) by simply clicking on the population name:

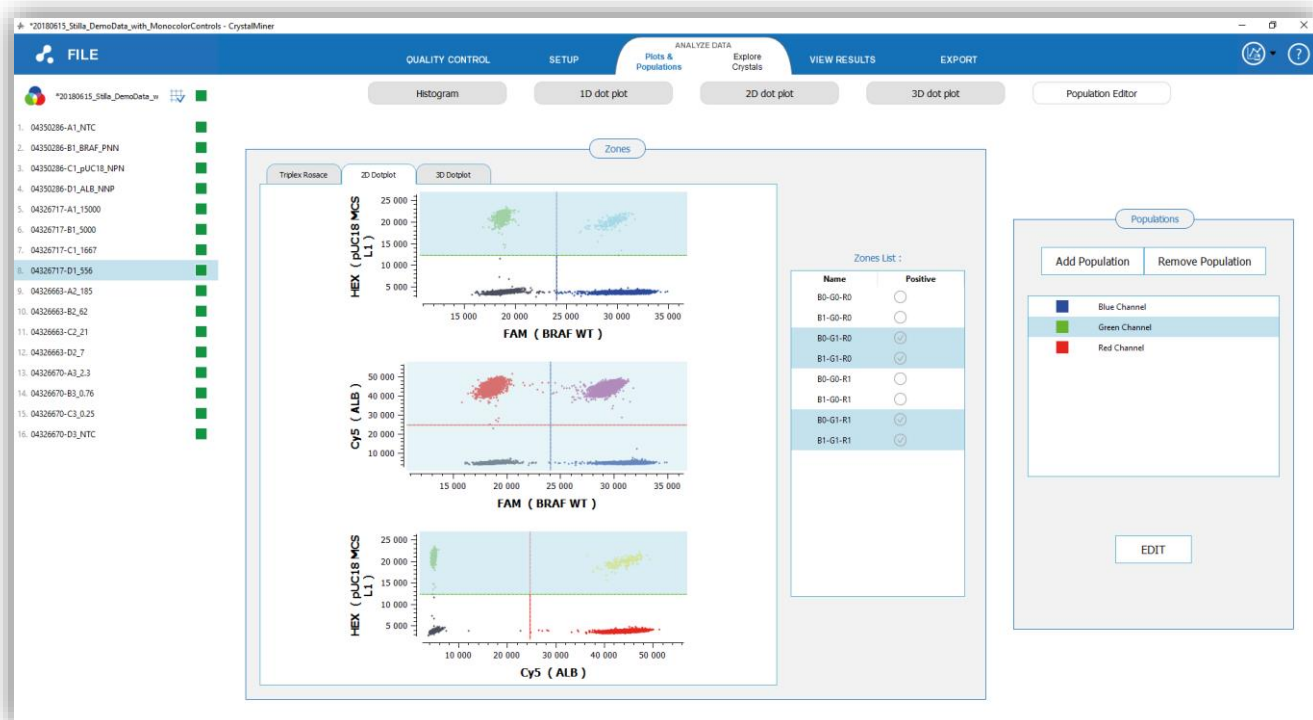


Fig. 65 - Population Editor for a default triplex experiment

By clicking on “Add Population” (resp. “Remove Population”) you can create (resp. delete) a population:

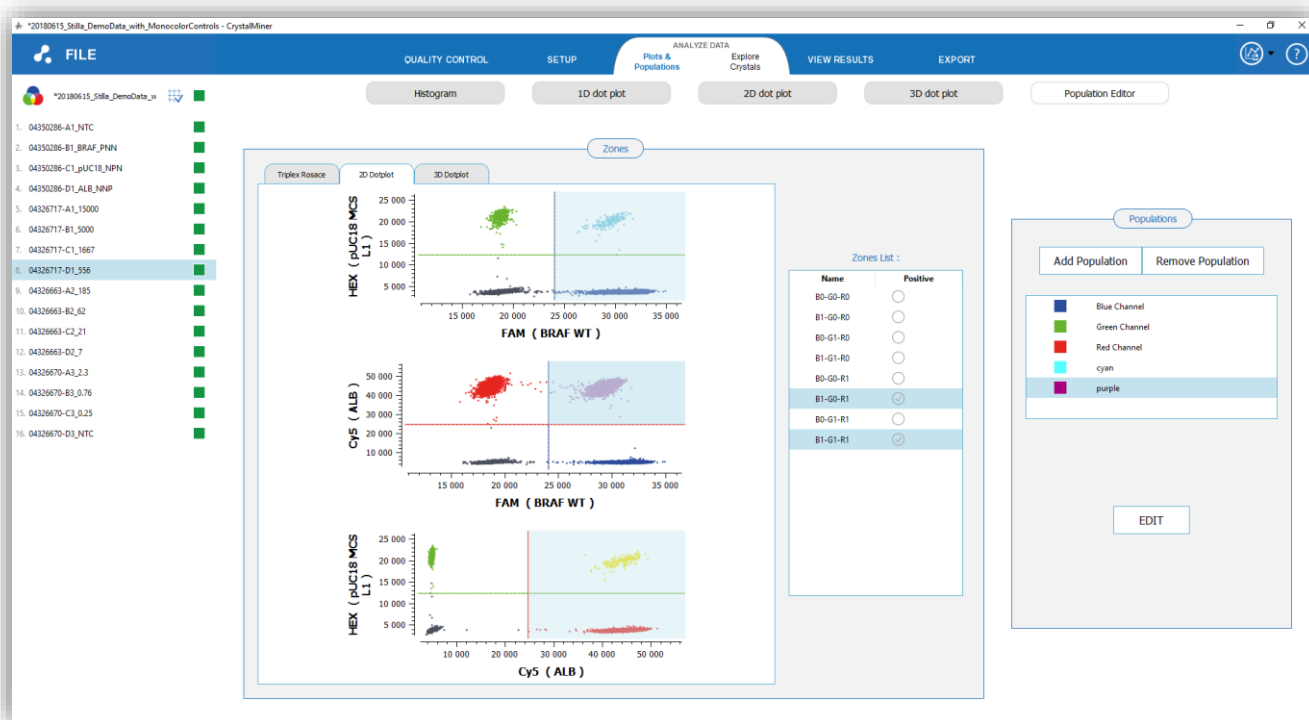


Fig. 66 – Examples of additional populations for some double positive droplets

By clicking on the “Edit” button, you can change the color and the name of a population, as well as the zones to be included in this population (click on “APPLY” to validate your changes):

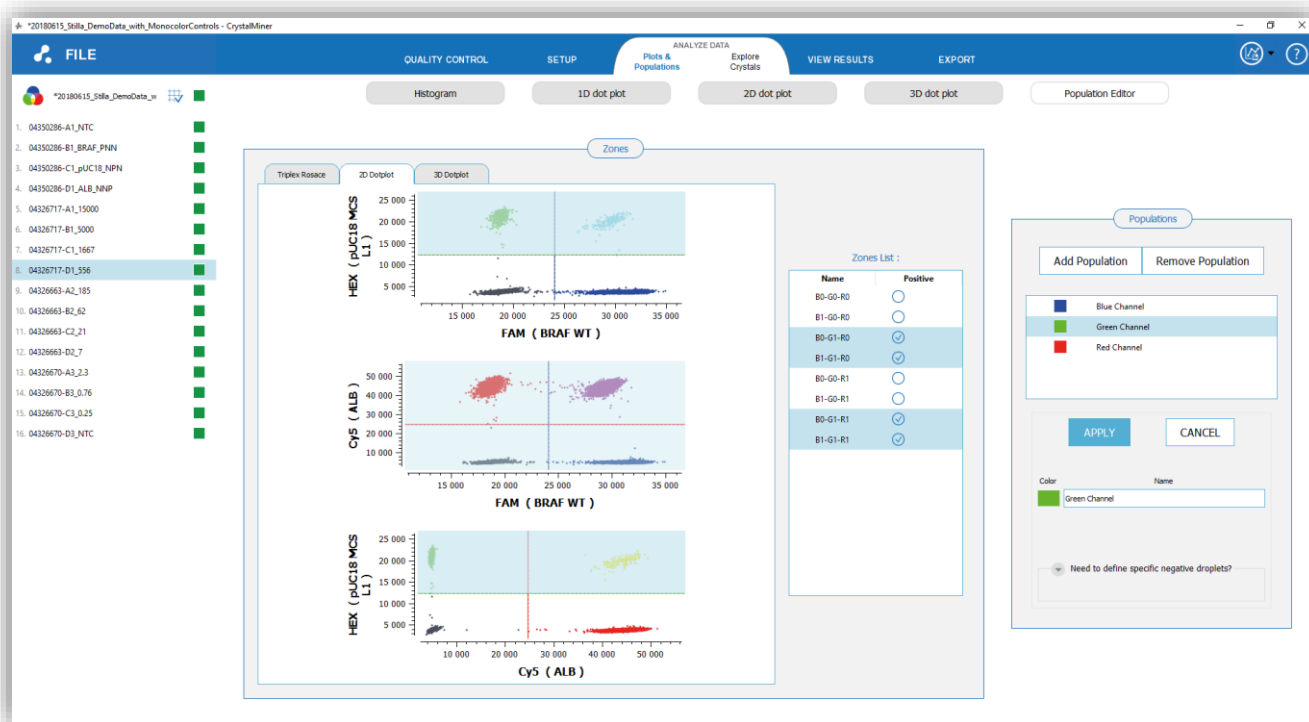


Fig. 67 – Edition of a population

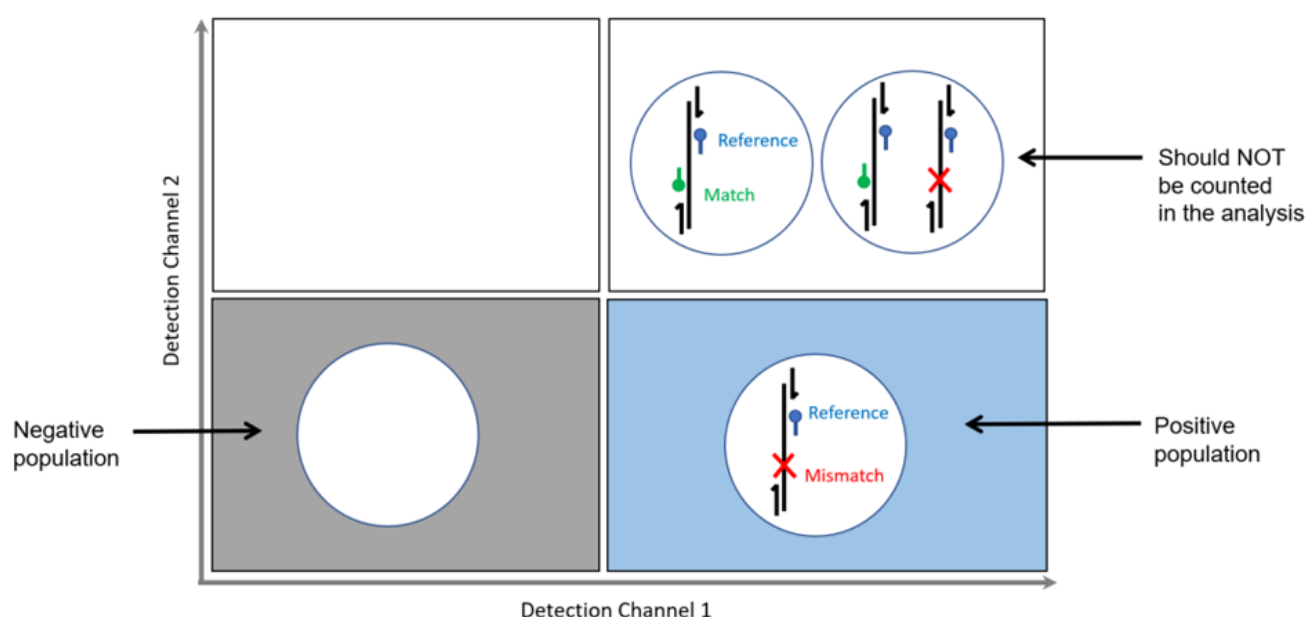
By definition, the droplets included in the population are counted as positive droplets.

By default, all the droplets that are not included in the population are counted as negative droplets for this population.

- However, for some applications such as drop-off applications, it might be necessary to restrict the negative droplet population by specifying the “negative droplets” to be considered for a give population:

### Why defining a specific negative droplet population with Crystal Miner™ ?

For all assays where a target is differentiated using reference & mismatch probes (e.g. drop-off use cases), the target can be coencapsulated with the background population in the same droplet partition:



For this type of assay, **the negative population should be re-defined** to the double-negative droplets instead of “all droplets except positive ones”, otherwise the target concentration will be underestimated.

If you expand the panel “Need to define specific negative droplets?” and click on the button “Edit Negatives”, a new column appears in the Zone list, allowing you to define the negative zones you need:



Fig. 68 – Edition of the negative zones of a population

Once all your populations are defined, you can see their results in the “VIEW RESULTS” panel:

FILE

QUALITY CONTROL

SETUP

ANALYZE DATA

Result Table

VIEW RESULTS

Advanced Graphs

EXPORT

\*20180615\_S18a\_DemoData\_m

16 Chambers

Experimental Details

Fluorophore names:  
● FAM  
● HEX  
● Cy5

Target names:  
BRAP WT  
pUC18 MCS L1  
ALB

Scanning Parameters

Exposure times:  
● 30 ms  
● 150 ms  
● 50 ms

Focus: 0.94 mm

Comments

Demo data by Sabine Müller

Green Channel

Red Channel

Cyan

Purple

	Nb Pos	Dilution	C (cp/mL)	Nb Pos	Dilution	C (cp/mL)	Nb Pos	Dilution	C (cp/mL)	Nb Pos	Dilution	C (cp/mL)	Nb Pos
1. 04350286-A1_NTC	0	1	0	0	1	0	0	1	0	0	1	0	0
2. 04350286-B1_BRAF_PNN	4517	1	0	0	1	0	0	1	0	0	1	0	0
3. 04350286-C1_pUC18_NPN	0	1	49.2	619	1	0	0	1	0	0	1	0	0
4. 04350286-D1_ALB_NNP	0	1	0	0	1	369.7	5000	1	0	0	1	0	0
5. 04326717-A1_15000	27169	1	41.8	657	1	16242	27173	1	41.8	657	1	13876	27167
6. 04326717-B1_5000	24407	1	47.2	698	1	5303	24438	1	45.9	679	1	4137	23317
7. 04326717-C1_1667	15777	1	45.6	663	1	1684	15762	1	27.7	404	1	847.6	9834
8. 04326717-D1_556	7221	1	48.5	721	1	554.1	7137	1	13.1	197	1	134.4	1949
9. 04326663-A2_185	2660	1	41.9	667	1	174.7	2675	1	4.6	74	1	15.3	246
10. 04326663-B2_62	864	1	46.2	688	1	55.7	827	1	2.52	38	1	2.19	33
11. 04326663-C2_21	294	1	46	688	1	20.4	308	1	0.59	9	1	0.4	6
12. 04326663-D2_7	87	1	46.6	686	1	6.11	91	1	0.07	1	1	0	0
13. 04326670-A3_2.3	36	1	43.7	630	1	1.99	29	1	0	0	1	0	0
14. 04326670-B3_0.76	9	1	44.6	618	1	0.57	8	1	0.07	1	1	0	0
15. 04326670-C3_0.25	3	1	44.5	603	1	0.44	6	1	0	0	1	0	0
16. 04326670-D3_NTC	0	1	0	0	1	0	0	1	0	0	1	0	0

Fig. 69 – Results for the additional populations can be seen as usual in “View Results”

Note that populations for which specific negative droplets have been defined are indicated with the symbol “\*” added just after their name (meaning that the sum of positive and negative droplets for such populations may not be equal to the total number of droplets).



## V. Maintenance and Technical Support

For technical questions or dysfunction, refer to [www.stillatechnologies.com](http://www.stillatechnologies.com) or contact us directly at [support@stilla.fr](mailto:support@stilla.fr).

Tel: (+33) 9 82 27 47 47

Opening hours: 9h30-18h30 (GMT+2) on working days

***We will make our best to answer as promptly as possible.***

To enable remote software maintenance on the PC provided by Stilla, check that the PC is turned on and connected to the Internet, then double-click on the “Team Viewer” desktop icon and send both your ID & password information by email to [support@stilla.fr](mailto:support@stilla.fr).



*Fig. 71 - Desktop icon of the “Team Viewer” application for remote software maintenance*

Please prepare the following information for remote maintenance:

- The software version, which is available in “About” in the Home page
- The log files which have been generated in the directory:

“C:/Users/<UserName>/Stilla/CrystalMiner”

## VI. Troubleshooting

Observations	Recommended
a) The Crystal Miner application does not respond anymore	Go to "Control Panel" > "Task Manager" and kill the Crystal Miner process, then start it again
b) Data cannot be exported	Check that no already exported file is already opened in your export directory
c) The Crystal Miner application is too slow	<p>If you run the Crystal Miner application on a PC which is not the Naica PC, check that this PC meets the minimum requirements specified in this manual.</p> <p>If you run the Crystal Miner application on a portable PC, the laptop should be plugged in to a power source for optimal use.</p>
d) After the last action the displayed waiting mouse does not disappear	Go to "FILE" > "Save" or "FILE" > "Save as" to save your current experiment. This will make the frozen waiting mouse disappear.

If these observations persists, please contact the technical support :

Email : [support@stilla.fr](mailto:support@stilla.fr)  
Phone: (+33)0982274747



## VII. License Information

### 1. Crystal Miner license

*©2015 Stilla Technologies. All rights reserved.  
For research use only. Not for use in diagnostic procedures.*

### 2. Third-party licenses

The Crystal Miner software uses the following third-party software components:

- Boost
- Catch
- CMake
- CNTK
- cuDNN
- Docopt.cpp
- Eigen
- ETL
- GLC-Lib
- GSL (Guidelines Support Library)
- ITK
- JSON for Modern C++
- OpenCV
- Python
- Qt
- Quazip
- Qwt
- stlab
- xInt
- Yaml-cpp
- ZLib

Feel free to visit their websites or check your Crystal Miner installation directory to consult their license information (by default in "C:\Program Files\Stilla\CrystalMiner\licenses").

Note that the license information of all the third-part software components is also accessible in the Crystal Miner application, by clicking in "About" in the "FILE" menu.

## VIII. Annexes

### 1. Computation of the fluorescence spill-over compensation coefficients

The Prism3 instrument is composed of 3 excitation LEDs that are successively activated and deactivated to image the crystal regions. When a given LED is activated, the light sent by the LED is exciting some fluorophores contained in the droplets of the crystal. Then, each excited fluorophore emit specific wavelength lights that are filtered by the band-pass reception filter of the Prism3 instrument, before being detected by a sensor. As a result, 3 fluorescence images of the droplet crystal are produced, one for each activated LED.

The spill-over phenomenon exists when a given fluorophore is excited by at least 2 LEDs of the Prism3 instrument and is emitting through the band-pass filter of the Prism3 instrument. In this case, this fluorophore will have a first contribution in the fluorescence image associated with the first LED, and a second contribution in the fluorescence image associated with the second LED.

We assume that the main excitation LED of the fluorophore  $k$  is the LED  $k$ . The partial excitation of the fluorophores by the LEDs can be represented by the 3x3 excitation matrix  $E$  where:

- $E(i, i) = 1$
- $E(i, j)$  with  $i \neq j$  is an excitation percentage representing the partial excitation of the fluorophore  $i$  by the LED  $j$

In order to recover the 3 fluorescence values  $X$  emitted by the 3 possible fluorophores contained in a given droplet, a compensation matrix should be applied to the 3 fluorescence values  $Y$  recorded in the 3 images of this droplet. This compensation matrix is an affine transformation which transfers the 3 droplet coordinates from the “LED domain” to the “Fluorophore domain”.

The spill-over formula is:

$$X = \text{inv}(E) (Y - T) + T$$

where:

- $\text{inv}(E)$  is the inverse of the excitation matrix  $E$
- $T$  is the translation vector corresponding to the background fluorescence in the images

## 2. Digital PCR statistics

The digital PCR statistics are computed by the Crystal Miner software for each chamber of the experiment and for each fluorescence channel, based on the following variables:

- $N$  : total number of analyzable droplets in the chamber
- $p$  : number of positive droplets in the chamber for the given fluorescence channel
- $v$  : volume of the droplet (in  $\mu\text{L}$ ), assumed to be constant
- $d$  : the dilution factor used to dilute the sample from the stock to the chamber ( $d = 10$  means that the sample has been diluted 10 times)

We introduce the following variables:

- $V = N v$  : total droplet volume injected in the chamber
- $C_0$  : concentration of target molecules in the chamber (in copies /  $\mu\text{L}$ )
- $C = d C_0$  : concentration of target molecules in the stock (in copies /  $\mu\text{L}$ )
- $C_{max}$  : maximal limit of the confidence interval for the concentration of target molecules in the stock (in copies /  $\mu\text{L}$ )
- $C_{min}$  : minimal limit of the confidence interval for the concentration of target molecules in the stock (in copies /  $\mu\text{L}$ )
- $UC_{quant}$  : relative uncertainty for the concentration of target molecules in the stock (or in the chamber)
- $\lambda = C_0 v$  : average number of target molecules per droplet in the chamber

The distribution of target molecules in the droplets of the chamber follows a Poisson distribution of parameter  $\lambda$  :

$$\text{Proba ( droplet contains } k \text{ target molecules )} = \frac{\lambda^k}{k!} e^{-\lambda}$$

A droplet is said as:

- positive droplet if it contains at least 1 target molecule
- negative droplet if it contains 0 target molecule

The distribution of positive (resp. negative) droplets in the chamber follows a binomial distribution of probability  $1 - e^{-\lambda}$  (resp.  $e^{-\lambda}$ ):

$$\text{Proba ( chamber contains } p \text{ positive droplets )} = C_N^p (1 - e^{-\lambda})^p (e^{-\lambda})^{N-p}$$

$$\text{Proba ( droplet is negative )} = e^{-\lambda}$$

$$\text{Proba ( droplet is positive )} = 1 - e^{-\lambda}$$

If  $N$  is large enough:

$$\text{Proba ( droplet is positive )} = \frac{p}{N}$$

So the formula for the estimated stock concentration is:

$$C = -\frac{d}{v} \ln\left(1 - \frac{p}{N}\right)$$

The central limit theorem provides an approximation for the binomial confidence interval:

$$\frac{\hat{p}}{N} = \frac{p}{N} \pm z_c \sqrt{\frac{\frac{p}{N} \left(1 - \frac{p}{N}\right)}{N}}$$

For a 95% confidence level:  $z_c = 1.96$

Hence the formula for the confidence interval and the relative uncertainty (or uncertainty coefficient UC) of the estimated stock concentration:

$$C = -\frac{d}{v} \ln \left[ 1 - \left[ \frac{p}{N} \pm z_c \sqrt{\frac{\frac{p}{N} \left(1 - \frac{p}{N}\right)}{N}} \right] \right]$$

$$C_{min} = -\frac{d}{v} \ln \left[ 1 - \left[ \frac{p}{N} - z_c \sqrt{\frac{\frac{p}{N} \left(1 - \frac{p}{N}\right)}{N}} \right] \right]$$

$$C_{max} = -\frac{d}{v} \ln \left[ 1 - \left[ \frac{p}{N} + z_c \sqrt{\frac{\frac{p}{N} \left(1 - \frac{p}{N}\right)}{N}} \right] \right]$$

$$UC_{quant} = \frac{C_{max} - C_{min}}{2C}$$

Whatever the total number of droplets  $N$  and the confidence level  $z_c$ , the relative uncertainty is always minimized at  $\frac{p}{N} \sim 79.7\%$ .

Below are additional statistical indicators:

- Relative uncertainty for the sampling error at 95%:

$$UC_{sampling} \sim \frac{1.96}{\sqrt{C_0 V}}$$

- Minimal limit of Detection at 95% (smallest concentration in analyzed volume  $V$  for which the probability of having at least 1 positive droplet is more than 95%):

$$LOD_{min} = \frac{3}{V}$$

- Maximal limit of Detection at 95% (highest concentration in analyzed volume  $V$  for which the probability of having at least 1 negative droplet is more than 95%) for  $N$  ranging from 10 000 to 1 000 000:

$$LOD_{max} \sim 10 \frac{N}{V}$$

- Dynamic range of Detection at 95% (number of decades from  $LOD_{min}$  to  $LOD_{max}$  at 95%):

$$DrD \sim \log_{10}(N) + 0.5$$

### 3. Template of the analysis configuration file

Please find below the content of the analysis configuration template file “[AnalysisConfiguration\\_GenericTemplate.yaml](#)” which is located in “[C:\Program Files\Stilla\CrystalMiner\config](#)”.

This file should not be modified but you can duplicate it and modify it for re-analysis purposes. The .yaml files can be edited using any text editor. We recommend for example the use of the free editor “Notepad++”.

Note that a secondary template file named “[AnalysisConfiguration\\_GenericTemplate\\_mix-XLT.yaml](#)” is also provided for RNA analysis experiment using the XLT mix (with this mix, the droplet diameter should be set to 101.53 microns instead of 103.82 microns).

The template for the .yaml analysis configuration file is divided in several categories, and includes one line per analysis parameter. The syntax for the configuration of each parameter is:

*variable: value # comment*

where the “#” character is used as a comment tag.

The explanation of each important parameter is explained in the comment part after the “#” character:

---

```
#-----
# IMAGE ANALYSIS CONFIGURATION FILE FOR CRYSTAL MINER SOFTWARE
#
# This is a template file for the re-analysis functionality of the Crystal Reader software by Stilla Technologies.
# Note that the syntax is "variable: value # comment" where the "#" character is used as a comment tag.
#
#-----

m_identification_neural_network: "StillaNeuralNet_2017_03_14"
m_identification_neural_network_threshold: 0.5

#-----
# A. Geometry criteria
#-----
m_drop_size_min: 4 # minimum droplet diameter (in pixels)
m_drop_size_max: 18 # maximum droplet diameter (in pixels)
m_min_circularity: 0.4 # minimum droplet circularity
m_max_normalized_eccentricity: 0.6 # maximum droplet eccentricity
#
#-----
# C. Neighbourhood criteria
#-----
m_chamber_margin: 50 # margin region to be ignored for droplet detection (in pixels)
#
m_min_drop_density: 0.5 # minimum droplet density
m_max_drop_density: 1.35 # maximum droplet density
m_min_post_analysis_density: 0.2 # minimum droplet density after artefact exclusion
#
m_remove_crystal_boundary: true # controls whether or not the outside boundary of the crystal should be removed
m_remove_pillars_boundary: true # controls whether or not the pillars surroundings of the crystal should be removed
m_boundary_erosion_drop_nb: 1 # width of the crystal boundary to be ignored for droplet detection (in number of droplet layers)
m_artefact_dilatation_drop_nb: 0 # radius of the artefact neighbourhood to be ignored for droplet detection (in number of droplets)
#
#-----
# E. Low-level analysis parameters for image pre-treatment
#-----
# m_background: 10000 # TO BE UNCOMMENTED IF A FIXED VALUE SHOULD BE USED FOR IMAGE BACKGROUND (in RFU)
m_sigma_for_background_thresholding: 0.2
m_number_of_kmeans_iterations_for_background_thresholding: 6
```

```

m_kmeans_down_sampling: 2
m_kmeans_use_smoothed_image: true
#
m_pretreatment_gaussian_sigma: 0.2
#
#-----
# F. Low-level analysis parameters for droplet segmentation
#-----
m_segmentation_sigma: 0.25
m_segmentation_radius: 1
m_segmentation_preprocessing_enabled: true
m_segmentation_preprocessing_sigma: 3
m_segmentation_preprocessing_amount: 1
m_segmentation_preprocessing_smoothing_enabled: true
m_segmentation_preprocessing_smoothing_sigma: 1.5
#
#-----
# G. Low-level analysis parameters for image post-treatment
#-----
m_chip_gaussian_sigma: 2
#
#-----
# H. Low-level configuration parameters
#-----
m_resolution: 6.75 # default resolution of the scanner (in microns per pixel)
#
m_chip_version: 3 # version of the chip
m_theoretical_number_of_droplets_in_full_chamber: 30000 # theoretical maximum number of droplets per chamber
m_drop_diameter: 103.82 # theoretical average droplet diameter (in microns)
#
m_reference_channel: 0 # channel of the image used as reference for droplet detection
m_signal_channel_inclusion: false # TO BE SET TO "true" IF USING ALL CHANNELS FOR DROPLET DETECTION
m_saturation_removal: true # TO BE SET TO "false" IF SATURATED DROPLETS ARE NOT REJECTED
#
m_fluo_percentile: 0.5 # percentile value determining droplet fluorescence (applied to the RFU values of pixels belonging to the droplet)
#
channels:
- number: 0 # index of first channel (e.g. the "blue" LED)
  name: FAM # default name of the fluorophore associated with the first channel
- number: 1 # index of second channel (e.g. the "green" LED)
  name: Cy3 # default name of the fluorophore associated with the second channel
- number: 2 # index of third channel (e.g. the "red" LED)
  name: Cy5 # default name of the fluorophore associated with the third channel
#
#-----
# I. Quality Control (QC) thresholds
#-----
m_normalized_focus: true # enables normalization of the focus computation
m_qc_focus_flag_green_min: 0.06 # minimum threshold for good QC flag on image sharpness
m_qc_focus_flag_red_max: 0.04 # maximum threshold for bad QC flag on image sharpness
#
m_number_of_droplets_flag_green_min: 0.66 # minimum threshold for bad QC flag on total number of analyzable droplets
m_number_of_droplets_flag_red_max: 0.5 # maximum threshold for good QC flag on total number of analyzable droplets
#
m_qc_saturated_flag_green_min: 35 # minimum threshold for good QC flag on total number of saturated objects
m_qc_saturated_flag_red_max: 100 # maximum threshold for bad QC flag on total number of saturated objects
#
m_qc_separation_flag_red_max: 0.9 # minimum threshold for good QC flag on positive vs. negative fluorescence separability
m_qc_separation_flag_green_min: 0.95 # maximum threshold for bad QC flag on positive vs. negative fluorescence separability
#
#-----

```

# **Crystal Miner™** for the **Naica™ System**

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