Chapter 10

Multi-Color FLUOROSPOT Counting Using ImmunoSpot® Fluoro-X™ Suite

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Abstract

Multi-color FLUOROSPOT assays for simultaneous detection of several T-cell cytokines and/or classes/sub-classes of immunoglobulins secreted by B cells have recently become a major new avenue of development of ELISPOT technology. Advances in assay techniques and the availability of commercial test kits stimulated development of multi-color FLUOROSPOT data analysis platforms. The ImmunoSpot® Fluoro-X™ Software Suite was developed by CTL as an integrated data acquisition, analysis, and management solution for automated high-throughput processing of multi-color T- and B-cell FLUOROSPOT assay plates. The Fluoro-X™ software counting module is based on SmartSpot™/AutoGate™ technologies and utilizes CTL's Center of Mass Distance algorithm for the detection of multi-color spots. The Fluoro-X™ software provides an objective, user error-free means for analyzing multi-color FLUOROSPOT data. An integrated quality control module, with optional GLP and CFR Part 11 compliant package and role-based security, enables data validation, review, and approval with complete audit trails. The extensive multi-format data output and presentation capabilities of the Fluoro-X™ software allow further analysis of FLUOROSPOT data using any commercial flow cytometry software and facilitate the generation of professional reports and presentation. In this article, we present a detailed step-by-step workflow for the analysis of a human four-color IFN-γ, IL-2, TNF-α, and GzB antigen-specific T-cell assay using the Fluoro-X Software Suite.

Key words T cell, B Cell, Cytokines, Immunoglobulins, Antibodies, ELISPOT, FLUOROSPOT, Multiplex, Multi-Color, Spot counting, Objective, Center of mass, AutoGate™, SmartSpot™, SmartCount™ Quality Control, Software, Fluoro-X™, Fluorescence, Label

1 Introduction

Over past decade, ELISPOT has become the gold standard for monitoring T- and B-cell immunity in clinical trials. This is primarily to its capacity for single cell resolution, high throughput, and ability to detect antigen-specific responses directly ex-vivo. Recent advances in multi-color fluorescent spot detection made FLUOROSPOT a fast-developing method for simultaneous measurements of up to seven analytes at a time [1]. Whereas the main criteria for objective, user independent single color ELISPOT counting are well established [2, 3], and commercial analysis

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software has been available for a number of years, a multi-color FLUOROSPOT analysis software is new to the majority of the ELISPOT users. Here we present the main principles as well as a step-by-step workflow for four-color T-cell FLUOROSPOT data analysis. CTL's Fluoro-X™ multi-color assay platform utilizes individual monochromatic images taken at the excitation/emission conditions (fluorescent channels) optimized for each fluorochrome used. This approach, as opposed to the analysis of a single multi-color image, allows for the detection of cells secreting highly variable amounts of individual cytokines (or other analytes). Further, this eliminates both the necessity of employing complex color recognition algorithms and the possibility of spectral cross-bleeding from different fluorochromes.

At the first step of the analysis, individual monochromatic images for each fluorescent channel are collected and counted in exactly the same manner as has been established for single color ELISPOT analysis. Objective user-independent single-color analysis is facilitated by two main features of the ImmunoSpot® Software—SmartSpot™ and AutoGate™ [2, 3].

At the second step, spots from the monochromatic images for each individual channel are "paired" to identify double-, triple-, quadruple-analyte producing cells using the experimentally validated Center of Mass Distance (COMD) algorithm [4]. Different color spots (scanned trough individual fluorescent channels) form a multi-color event if each of their "centers of mass" is less than the maximal allowed COMD apart. These multi-color events are recorded the same way as in flow cytometry data analysis. Multiple measurements are extracted from each single or multi-color event, including spot sizes, max/average/total intensities, peak intensity values, XY coordinates, and more. In addition to the proprietary ImmunoSpot® data format, Fluoro-X™ software saves Flow Cytometry Standard (FCS) files, which can be further analyzed using the Fluoro-X™ Manage Data module, or with any commercial flow cytometry software to establish detailed statistical analysis of different analyte-producing/co-producing cell populations. In this chapter, we provide an overview of multi-color counting, quality control, and the advanced data management/presentation features of the ImmunoSpot® Fluoro-X™ Suite.

2 Materials

- Software: ImmunoSpot® Fluoro-X™ Software Suite Version 7.0 by CTL (Shaker Heights, OH).
- Hardware: ImmunoSpot® S6 ULTIMATE analyzer by CTL.
- Four-color FLUOROSPOT kit for human IFN-γ/IL-2/TNF-α/Granzyme B by CTL (http://www.immunospot.com/

immunospot-kits/human-interferon-gamma-il-2-tnf-a-granzyme-b-four-color-fluorospot).

- Human cryopreserved PBMC sample of HLA-A2-positive donor was obtained from CTL's commercial ePBMC® library. These PBMC had been previously HLA-typed at high resolution and characterized for T-cell reactivity to a variety of antigens (details are available at http://www.immunospot.com/ImmunoSpot-ePBMC).
- FLUOROSPOT assay shown was performed on a single HLA-A2 positive donor PBMC sample stimulated with HCMV HLA-A2 restricted peptide pp65 (495–503) (EZ Biolab Inc., Carmel, IN, USA) according to the test kit instructions.
- Throughout the article, "*single color spots*" refers to those spots that appear only on a single fluorescent channel, and "*multi-color spots*" to those appearing in multiple channels. When evaluating the results, it is important to distinguish objects that appear *exclusively* on a specific channel or combination of channels (*exclusive* results), from objects that are visible in other channels (or combinations of channels) as well (*inclusive* results).

3 Methods

3.1 Main Fluoro-X™ Switchboard

The main switchboard of the Fluoro-X™ Software Suite (Fig. 1) is designed to activate key software functions, including scanning multi-color fluorescence plates, counting multi-color T- and B-cell spots, quality control and data management of the counted results (*see* **Note 1**).

3.2 Scanning and Loading FLUOROSPOT Plates for Counting

As in conventional single-color ELISPOT analysis, FLUOROSPOT plates are first scanned on the ImmunoSpot® analyzer (*see* Subheading 2). For high-throughput assays, 384-well plates can be used in place of standard 96-well plates [5]. After loading a scanned plate (or multiple plates), the first step is to fine-tune parameters, including setting the spot-size gates for all analytes/channels before autocounting of the whole plate/plates. When scanning multi-color FLUOROSPOT plates, individual channels are organized as single-color plates. Once the counting parameters and spot size gates have been set for all channels, the autocount feature can be enabled to count all wells in multi-color mode. After the count is complete, it is possible to verify, quality control, visualize, and export the multi-count data. These steps are discussed and illustrated next.

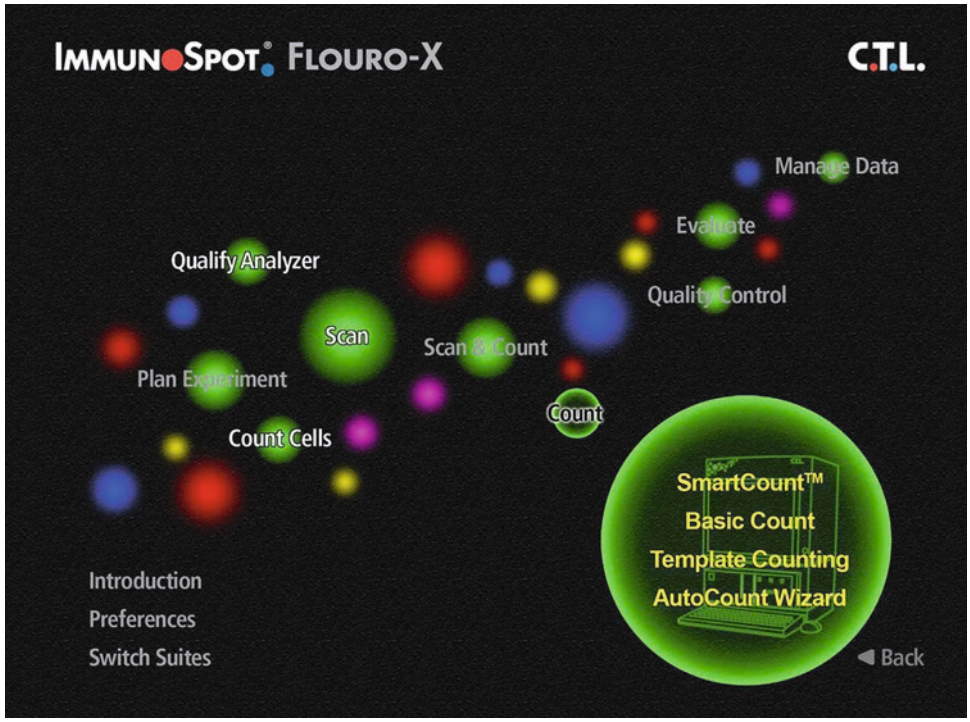

Fig. 1 ImmunoSpot® Fluoro-X™ Suite switchboard

3.2.1 Setting up Counting Parameters for all Channels Using SmartSpot™

Multi-color analysis starts with single-color (for each separate channel) counting, based on the established and validated ELISPOT counting algorithms implemented in the ImmunoSpot® Software. The input is a set of monochromatic images captured under conditions optimized for each individual fluorochrome/channel. This set of images records objects that may or may not appear in multiple channels, and the task is to identify and count objects of each individual fluorescent channel and all possible combinations of these.

To optimize parameters for the entire plate (or set of plates), it is recommended to set the counting parameters on a selection of typical wells representative of the type(s) of response(s) seen in the plate(s) (including both negative control and positive wells). It is also recommended to test the parameters on both sparsely and densely populated wells. Individual wells can be selected using the well navigation/selection interface (*see* red dot-marked well selection on Fig. 2) by clicking on the well of interest.

Since the scanned plates contain images for multiple channels, it is important to have single color counting fine-tuned for all channels. The channels for a currently selected well can be switched through the channel selection interface (*see* highlighted selection on Fig. 3).

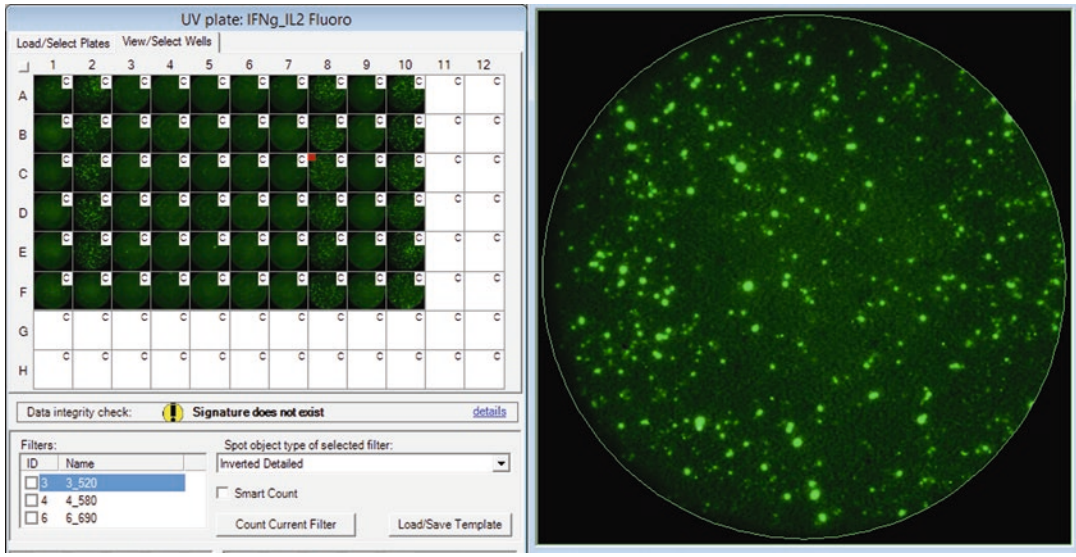

Fig. 2 Plate navigation and well selection interface

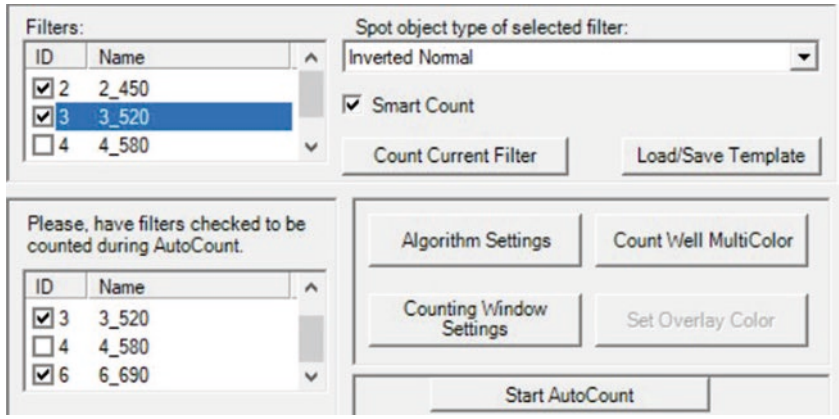

Fig. 3 Fluorescence channel selection window with all major function call buttons. In the upper-left "Filters" panel, the highlighted channel indicates the image to be displayed during a single well test count, checkboxes select which spot outlines will be shown on this image after it is counted during the setup. In the example shown, a 520 nm (green) channel image will be shown after counting with 520 nm (green) and 450 nm (blue) spot outlines. In the bottom left "Autocount" panel, checkboxes select channels to be included in the batch autocount

ImmunoSpot® software allows for the optimization of multiple counting parameters for ensuring accurate results (*see* below for list); however, the only parameter which may need to be adjusted in the majority of assays is the minimal spot intensity or "Sensitivity." ImmunoSpot® Software implements SmartCount™ mode for automatic recognition of spots of different morphologies on various backgrounds. SmartCount™ mode is based on the SmartSpot™ object/background recognition algorithm, and provides an

objective user-independent means for analyzing spot data (*see* **Note 2**). The accuracy, reliability, and reproducibility of SmartCount™ mode were confirmed during multiple studies including a multi-laboratory ELISPOT validation study [6, 7].

Additionally, spot morphology may vary depending on the affinity of the capture antibodies and the kinetics of the analyte secretion by different cells [8]. To account for such morphological differences, ImmunoSpot® Software includes additional parameters chief among these being the following (*see* Fig. 4):

- Diffuseness (Diffuse Processing): This parameter handles differences in spot morphology and mainly reflects the relationship between spot sizes and densities. If the plate contains tight intense spots, it is best set at "normal" level. For large faint ("diffuse") spots, Diffuse Processing should be set to "Large" or "Largest," whereas for bright small spots the "Detailed" is the best choice.
- Background Balance: ELISPOT and FLUOROSPOT wells sometimes show uneven background staining. Among other possible reasons, "ELISA effect" (where an abundance of cytokine or other analyte is released into the culture medium during the assay incubation period, and then binds uniformly to capture antibody on the well surface) and leaking wells (if the assay is not performed in the optimal way) may create such strong uneven membrane coloration. This can be compensated for by the Background Balance feature, which detects unevenness of the background and normalizes (balances) it. This parameter is proportional to the size of the background area. It is important to note that setting this parameter too high may not sufficiently balance the background, whereas setting it too low may eliminate large spots of interest.

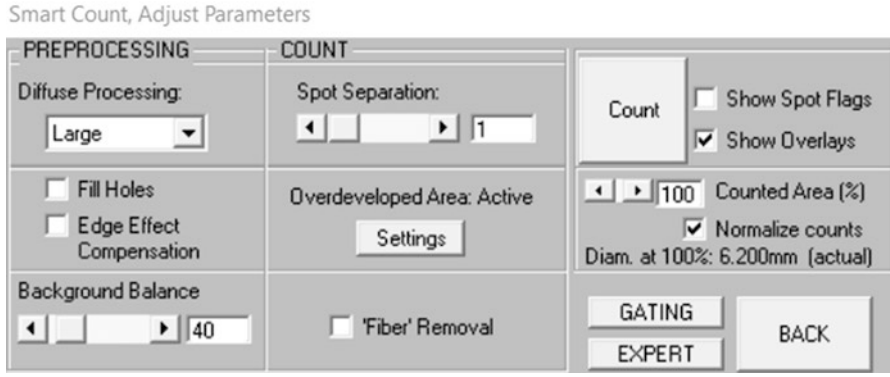

Fig. 4 SmartCount™ parameters window of ImmunoSpot® Fluoro-X™ suite

- Spot Separation: Closely situated or partially overlapping spots can potentially be mistakenly counted as a single object. To separate such touching or overlapping objects, ImmunoSpot® software implements intelligent spot separation function. The algorithm is based on detailed analysis of the spot-intensity profiles, and has proven to be robust even when separating large spot clusters in overcrowded wells. The parameter is proportional to the sizes of the spots to be separated, i.e., the smaller the spots, the lower this parameter should be set.

It must be noted that spot intensities and morphologies, along with differences in background staining, can be different for different analytes. Therefore, in multi-color analysis, counting parameters must be optimized for each fluorescent channel individually. However, as we emphasized earlier, in the majority of cases, the pre-set default settings for these parameters will be close to optimal and will not need adjustment. Additionally, ImmunoSpot® Fluoro-X™ Suite contains a standard set of counting templates (sets of parameters) optimized for each CTL multi-color B- and T-cell kit. For using pre-set, standard, or user-modified templates, the Load/Save Template function can be activated from the main channel/well selection interface (Fig. 3).

3.2.2 Setting Spot Size Gates for all Fluorescent Channels Using the AutoGate™ Function

As demonstrated previously, the range of spot sizes produced by antigen-stimulated T-cells uniformly (for different cytokines, cell donors and antigens) follows a Log Normal distribution [9]. Knowing the expected spot size statistics allowed the implementation of the AutoGate™ function (*see* **Note 2**). This facilitates the automatic discrimination between the relevant spots produced by stimulated cells, and irrelevant spots produced by bystander cells, which may be present either in both negative and positive wells (background production) or in positive wells only (bystander activation), such as when there is antigen-induced IFN-γ, IL-10, or IL-4 production by T cells, as well as background/bystander secretion of these cytokines by NK cells, monocytes, and basophils, respectively [3, 10, 11].

Background and bystander spots are usually smaller than antigen-induced spots and can be excluded from the final counts using automatic size gating. Gating options can be accessed from the counting parameters interface (Fig. 4) by pressing GATING button. The autogating interface (Fig. 5) prompts users to select first a few typical (for the specific cytokine/analyte) positive wells, containing stimulated and potentially bystander cell, then if applicable, select a few typical negative wells (background spots only).

After the selected wells are counted, and spot size statistics are calculated from these cumulative counts for both induced and background spots, the auto-adjust function can be executed to automatically set minimum and maximum size gates (Fig. 6).

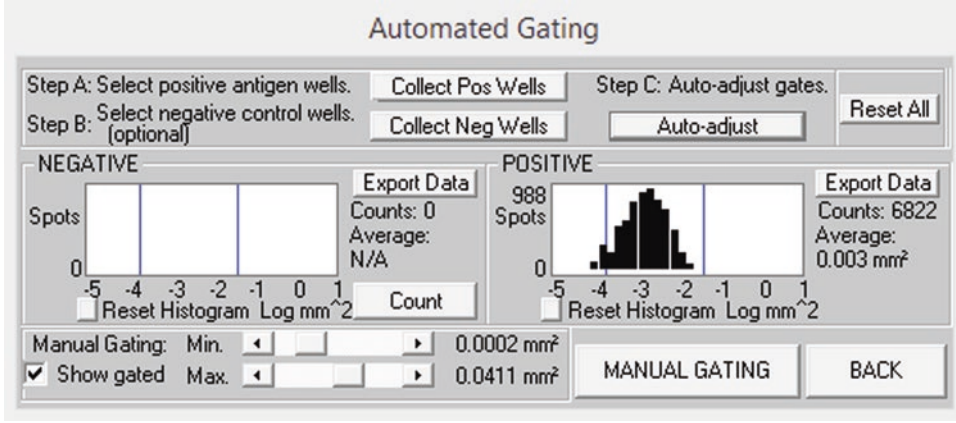

Fig. 5 AutoGate™ window. Main functions inlude selection of positive and negative (background) wells for autogating, execute autogating (Auto-adjust) button, and link to the manual gating window. In the example shown, autogating was done on positive wells only using Log Normal model

If negative wells are not available, or collectively do not have enough spots for statistical calculations, gates can be automatically set using positive wells only, using a Log Normal spot size distribution model (Fig. 5). It should also be mentioned that wells for autogating can be selected from different plates loaded for batch counting.

The result of the AutoGate™ function can be seen in Fig. 6. Vertical blue lines show Min/Max gate positions on negative (top) and positive (bottom) spot size histograms for the corresponding selected wells (labeled on the well navigation interface with letters "N" and "P," respectively). Note the reduced count and white outlines marking undersized spots after gating was applied (Fig. 7).

ImmunoSpot® Software also allows the user to set minimum and maximum spot size gates manually by moving Min/Max sliders or by sampling the smallest and largest spots to be counted (Fig. 5). Manual gating can be used when there is not a sufficient amount of spots available for statistically significant results, or when a subpopulation of cells with a different cytokine productivity is to be evaluated separately. Spot-size statistics and gate settings can be reviewed/modified using the Verify Gates window of the Quality Control module (Fig. 6).

Because spot size distributions differ between cytokines/analytes, it is important to set spot size gates for all fluorescents channels individually. Well selections can be different between channels.

3.2.3 Setting Up Parameters for Multi-Color Spot Recognition

Once spot counting has been completed for all individual fluorescent channels, the next step is to establish the relationship between spots from the different channels using a pairing algorithm, which is based on spot coordinates within the well image. Spots of different colors occupying the same (or highly proximate) positions on

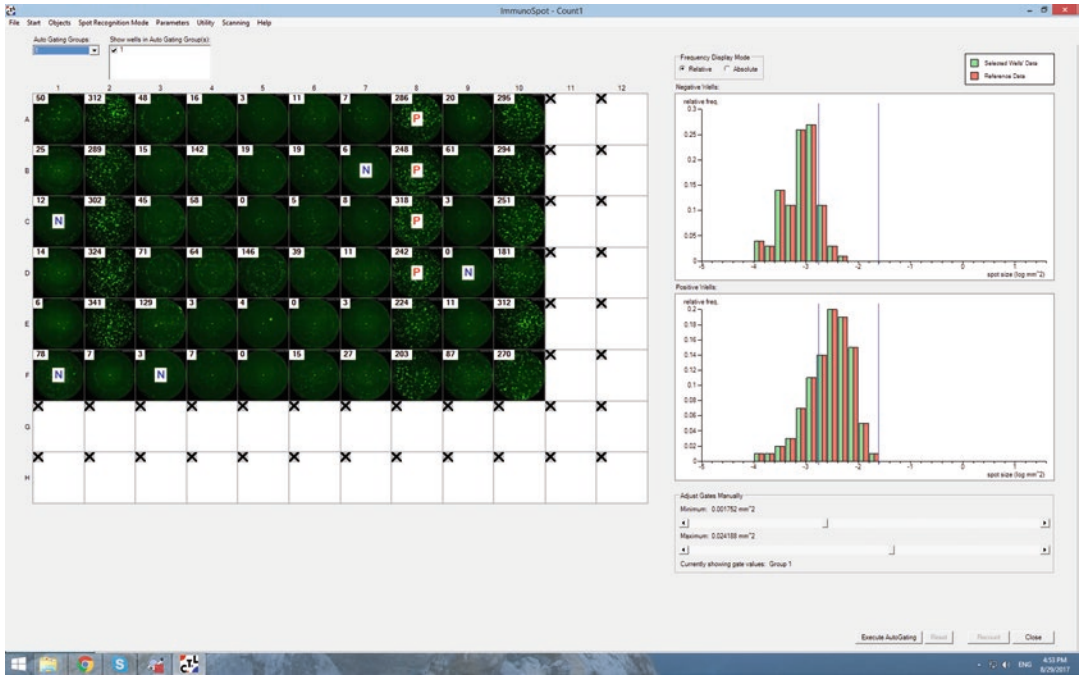

Fig. 6 Verify Gates window. On the left, wells selected for positive (P) and negative background (N) wells are shown in the plate navigation control. On the right, negative background (top) and positive (bottom) spot histograms calculated from the selected wells are shown with the auto-adjusted gates (blue vertical lines). Red histograms are calculated for reference (selected for autogating) wells. Green histograms are calculated for any other positive and negative wells chosen specifically for this gate verification process. In the example shown, no additional wells for gate verification are selected and both red and green histograms are the same

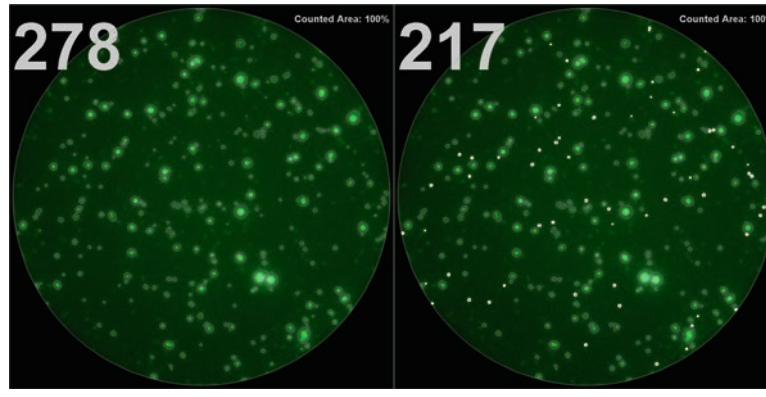

Fig. 7 Results of autogating on the spot counts in the 520 nm (green) fluorescence channel. Image on the left shows counting prior size gating, and image on the right shows counting with AutoGate™ enabled. Spots inside size gates are marked with purple outlines, and undersized background spots are shown with white outlines (these are excluded from the final count)

the well membrane are counted as multi-color. We tested multiple different algorithms, and found that pairing algorithm based on the Center of Mass Distance (COMD) is the most reliable among all of these to accurately determine co-localization of spots between different channels (*see* **Note 3**).

The Center of Mass Distance is defined as a maximal allowed distance between centers of masses (in image analysis, it is often referred to as centers of gray) of two spots of different colors/channels. Extensions of this algorithm include resolving partial overlaps of spots in the same channel and processing of spot clusters.

The algorithm basically determines the precise coordinate location of the center of mass of a spot in one channel, and looks for spots in all other channels whose center(s) of mass are below the maximum allowed COMD. The spot and its multi-color pairs are removed from further tests. This step is repeated until all spots on all filters are processed. If a spot's center of mass appears within the COMD on multiple filters, it is considered a multi-color spot (or "event" using the analogy to flow cytometry).

The result of the multi-color pairing is two-/three-/multi-color spots (events) indicated on the counted images with double, triple, etc. concentric spot outlines of different colors (colors of outlines are selectable, Fig. 8). The pairing algorithm also returns inclusive and exclusive numbers of double-, triple-, multi-color spots for every possible filter combination. In the case of our four-color T-cell FLUOROSPOT, there are 11 combinations of multi-color spots: six double color, four three-color and one four-color. Inclusive double color results list all spots of two certain colors including three- and four-color spots; in contrast, exclusive results list only double color counts.

The COMD parameter depends primarily on the optics of an analyzer and is practically constant for each model of analyzer. However, if new fluorescent channels or different optical zoom factors are used, or if cells are moving during the incubation period (in case of asynchronous cytokine secretion by the same cell), it is recommended to fine-tune the COMD parameter using the Algorithm Setting option (Figs. 3 and 9). The unit for this tolerance parameter is defined as a percentage of the well bottom diameter. So, the zoom factor, or image scaling, will not affect this value (e.g., 1% of a 6 mm well would mean 0.06 mm tolerance regardless of zooming, *see* Fig. 9). With an image vertical resolution of 1000 pixels per well, the COMD equal to 1 is equivalent to 10 pixels. Integrated correction for "false" positive multi-color spots (by statistically determining the expected number of random co-localization events for a given number of spots in each channel) ensures that the final counts are accurate, even when the COMD is set higher then optimal [4].

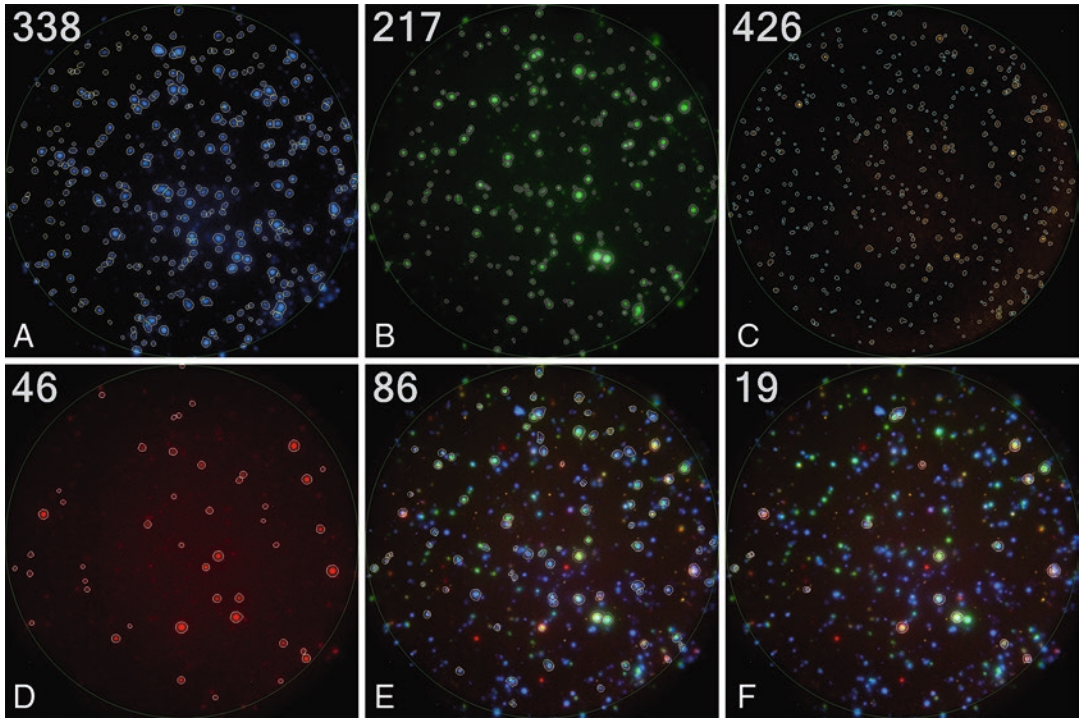

Fig. 8 Multi-color counting results: single-color spot outlines are shown for the individual fluorescence channels: GzB—450 nm blue (**a**), IFN-γ—520 nm green (**b**), TNF-α—580 nm yellow (**c**), and IL-2—690 nm red (**d**). Triple-color and four-color outlines are shown for GzB/IFN-γ/TNF-α triple-positive (**e**) and GzB/IFN-γ/TNF-α/IL-2 qudrouple-positive spots on the four-color merged image (**f**)

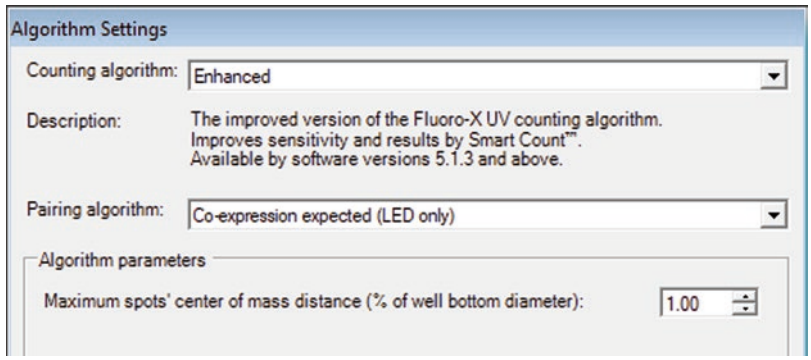

Fig. 9 Pairing parameters setup dialog for fine-tuning Center of Mass Distance

The object pairing algorithm can be tested on a well by well basis by pressing "Count Well Multicolor" (Fig. 3). Pairing results for each possible color combination can be easily visualized and verified by enabling/disabling corresponding channel selection checkboxes (top left on Fig. 3) (*see* **Note 4**). An example of a

triple-color pairing can be seen in Fig. 8. In this illustration, single-color spot outlines are shown on the individual channel images (A, B, and C) and triple-color spot outlines (objects that are present on all three filters) are shown on a merged multi-color image (D).

After all counting parameters, including min/max spot size gates and pairing parameters, are optimized, the Start Autocount command can be executed (Fig. 3). All wells for all pre-loaded plates will be counted, and the results will be written to Excel, XML, and Flow Cytometry Standard (FCS) files once the multi-color counting is completed.

3.3 Quality Control

The next step is to quality control and evaluate the results. The quality control (QC) module of ImmunoSpot® software is designed to provide maximal visual feedback on the levels of full plate overview as well as on the individual wells basis. The quality control process allows the user to verify or change counting parameters and gates (manually or automatically), remove artifacts and/or overdeveloped areas, and recount individual wells or batches of wells. It is also possible to adjust the COMD for the multi-color spot pairing. When the GLP (Good Laboratory Practice) Compliance Package is used, the software keeps a complete audit trail of all changes done to the original counts during QC using secure Code for Federal Regulations (CFR) Part 11 compliant database (*see* **Note 5**). The GLP Compliance Package also provides a role-based security feature, allowing pre-defined users and groups to perform certain individual tasks, like plate scanning, plate counting with locked parameters, changing counting parameters, or performing quality control, depending on the roles (permissions) set in the ImmunoSpot® Software.

3.4 Multi-Color Data Management

The evaluation of multi-color counting results is a complex task. The multi-dimensional nature of the counted data (multiple individual channel images plus multiple spot outlines for each possible color combination) makes it difficult to see the connections between the results. To this end, ImmunoSpot® Fluoro-X™ QC and Manage Data modules implement a Multi-Color View function which can be activated for each selected well (Fig. 10). The Multi-Color View function provides a tool that can combine (or merge) together and recolor any combination of the individual channel images and spot outlines into a single image, allowing visual cross-checking of the spot images and outlines.

The channel recolor tool is particularly helpful when fluorescent channels (600, 630, and 690 nm, for example) will generate similar red RGB images, which would be difficult to visually distinguish otherwise. All spot count outlines can be enabled, disabled, and recolored for better visual evaluation. The Multi-Color View tool also provides controls for automatic or manual image enhancement, and for weight/intensity control of individual selected col-

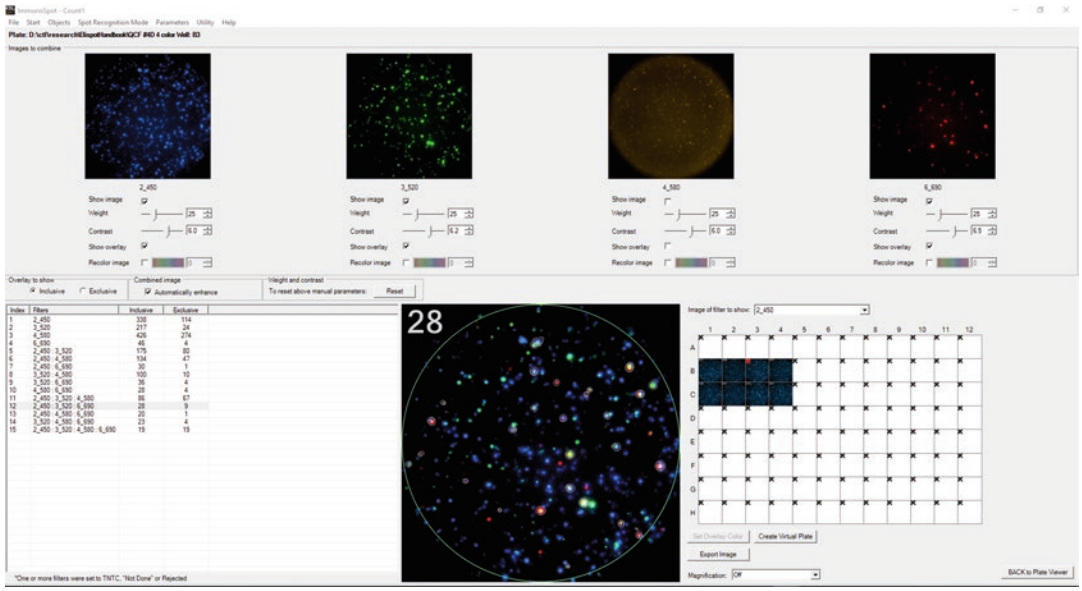

Fig. 10 Fluoro-X™ Multi-Color View window for Manage Data and Quality Control modules. Any combination of individual channel images and spot outlines can be created for any well (plate navigation is to the right of the well image) . Specific spot outline combinations can be generated for exclusive and inclusive counts using the selection list (bottom left panel)

ors in the resulting merged image. In the example shown in Fig. 10, three original well images (450, 520, and 690 nm channels) from well B3 are shown in the merged image, together with the triple color (exclusive) spot outlines for these three channels.

Both QC and Manage Data modules share the same well browsing interface for exporting well images from scanned/counted/quality controlled plates. The images may be arbitrarily arranged, and any individual filter or any combination of the filters can be exported (using image merging). In the example shown on Fig. 11 counted wells B2, B3, B4, C2, C3, and C4 are selected for the Well Survey using two rows by three columns format for the combination (merged image) of 450, 520, 580 and 690 nm fluorescent channels with exclusive quadruple color spots outlines. After evaluating the results, these Well Surveys can be exported as Power Point slides.

The Multi-Color View interface also allows the user to create a virtual plate (for presentation purposes) containing both original scanned well images merged from selected filters, and selected combinations of spot outlines (Create Virtual Plate button on Fig. 10).

The Data Management tool can open any saved data file format created by the counting and QC modules. The counted data are exported to different formats including Excel workbook, XML, and Flow Cytometry Standard format (FCS). The Excel and XML outputs also contain estimations for the probability of random sin-

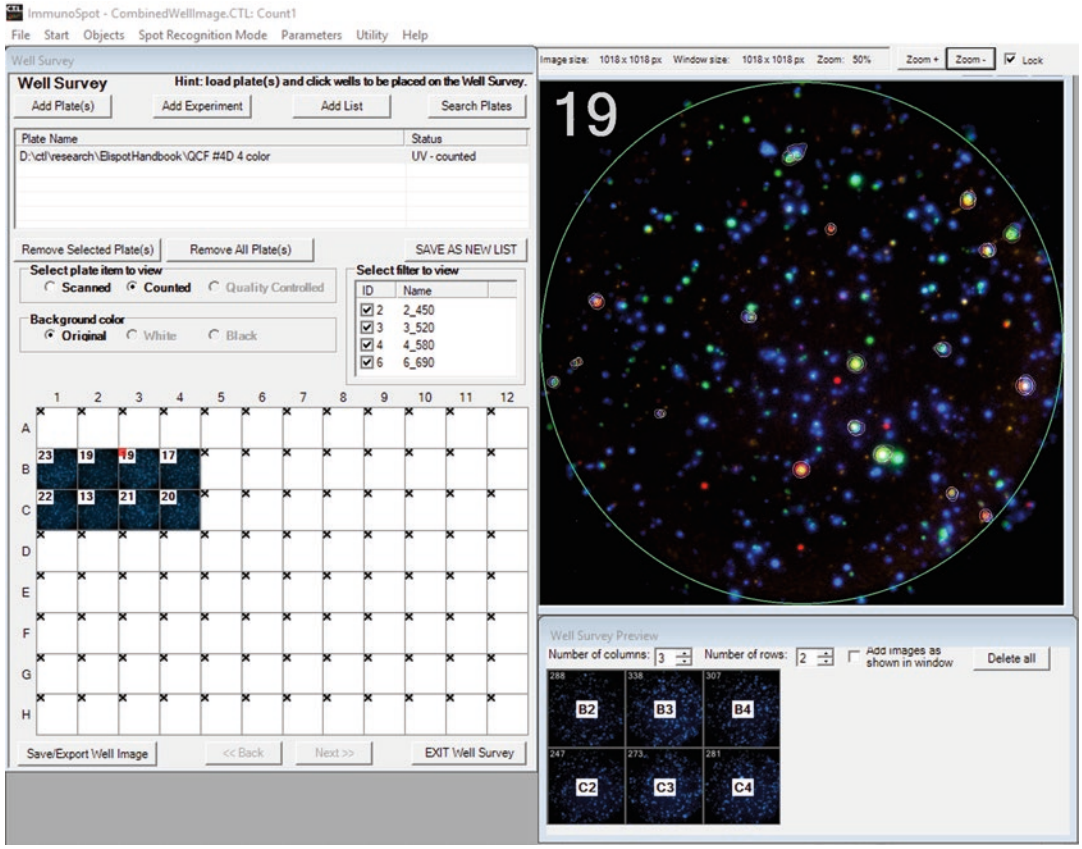

Fig. 11 Fluoro-X™ Well Survey window for exporting of individual images and arranged groups of images to a PPT presentation. In the example shown, a three by two image survey is created for wells marked in the plate navigator window

Inclusive Spot Counts					Probabilistic Inclusive Spot Counts				
	2_450 : 3_520	2_450 : 6_690	3_520 : 6_690	2_450 : 3_520 : 6_690		2_450 : 3_520	2_450 : 6_690	3_520 : 6_690	2_450 : 3_520 : 6_690
B1	167	25	34	24	B1	25	5	4	0
B2	156	34	46	28	B2	23	7	5	1
B3	175	30	36	28	B3	28	6	4	1
B4	170	27	35	27	B4	24	5	4	0

Fig. 12 A fragment of Excel workbook sections displaying numbers of double- and triple-spots for 450 nm (blue), 520 nm (green), and 590 nm (red) channels counted in wells B1, B2, B3, B4 (on the left) and numbers of calculated "false" positive random spots overlays (on the right)

gle-color spots overlays using statistically validated tools (Fig. 12 shows a sample of Excel output).

The Excel workbook stores results in separate sheets. The first two sheets store inclusive and exclusive multi-count results with filters in separate charts, while the next two sheets combine these results into a single chart (well ID versus filter combination). Additional sheets store probabilistic counts of random single-color

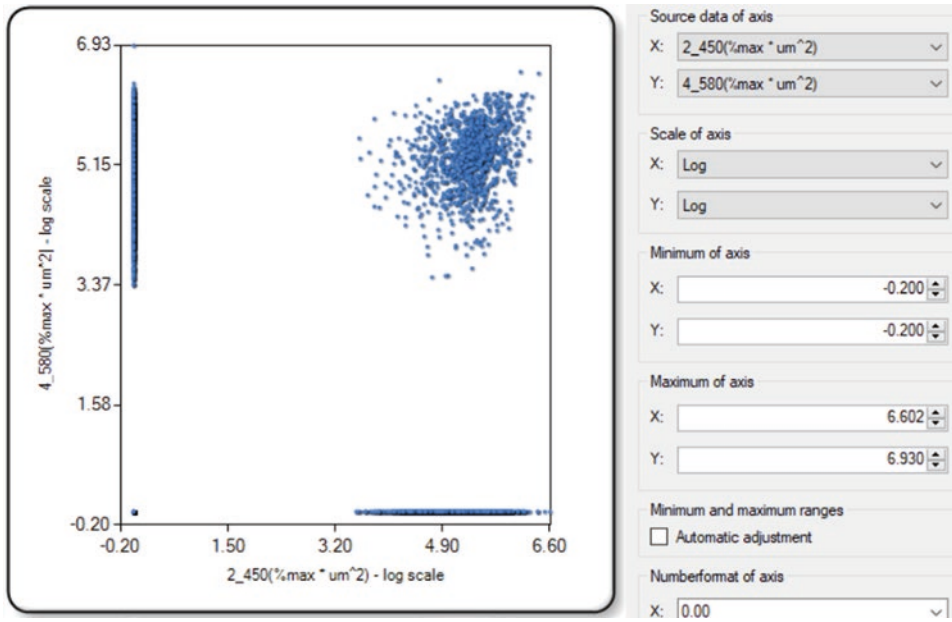

Fig. 13 A Flow Cytometry Standard (FCS) file opened using Fluoro-X™ Data Management module. A standard dot-plot graph is shown as total spot fluorescence for TNF-α 580 nm (yellow) and GzB 450 nm (blue) spots in Log scale. Single-color GzB and TNF-α spots are aligned along *X* and *Y* axis, respectively, and double color spots form a cluster in the upper-right quadrant of the plot. Average total intensities of these two cytokines in single color spots are practically the same as in double color spots

spots overlays using different methods. The last sheet stores all parameters used for counting.

The XML format provides a bridge between the ImmunoSpot® Software and customer databases using Common Laboratory System.

For more detailed analysis of sub-populations of T cells producing different combinations of cytokines, and High Content analysis of T- and B-cell produced spots based on the spot morphologies [8] ImmunoSpot®, Fluoro-X™ Suite generates Flow Cytometry Standard (FCS) files (*see* **Note 6**). The individual fluorescence channel data that is exported to the FSC file include maximal, mean and total spot fluorescence, spot size, circularity, and image-based $X\Upsilon$ center of mass. FCS files can be opened by either Fluoro-X™ Manage Data module as a standard dot-plot (Fig. 13) or in any commercial Flow Software. In Fig. 13, the total spot intensities in 450 nm (GzB) and 580 nm (TNF-α) fluorescent channels are shown as a dot-plot in Log scale. Single color TNF-α and GzB spots are aligned along the *X* and Υ axis (no compensation required), with the double positive population in the upper-right quadrant. For more detailed analysis including gating on different populations, 2D and 3D dot-plot statistics, histogram statistics, and cluster analysis, any commercial Flow Cytometry analysis software can be used. In most cases, the number of spots

in a single well may not be sufficient for building a good quality high-resolution dot-plot or histogram. Fluoro-X™ Manage Data module provides a function to merge multiple similar (repetitive) well data to a single FCS file.

Although Fluoro-X™ software contains multiple functions and features, the user interface is intuitive and easy to use (*see* **Note 1**). As we reported earlier, single-color algorithms used for individual color spots recognitions are robust, user error-free and guarantee objective counts over a wide range of spot numbers per well (*see* **Note 2**). The multi-color spot detection algorithm is based on the maximal allowed distance between spot centers and contains only a single parameter—COMD (*see* **Note 3**). This parameter depends primarily on the configuration of the optics of the analyzer used, and does not need to be adjusted between assays if the same fluorescent channels are used. To maximize the accuracy of multi-color counting, Fluoro-X™ software automatically calculates numbers of false positive multi-color spots resulting from random overlays of single color spots of different channels for each well (*see* **Note 7**). An integrated quality control system provides data security and validation by authorized QC-users (*see* **Note 5**). Extended data output includes Excel workbooks, Power Point graphical output, Common Laboratory System compatible XML files, and Flow Cytometry Standard files for detailed analysis of different sub-populations and High Content analysis (*see* **Note 6**). Built-in image and data export features help to prepare publications, reports, and Power Point presentations (*see* **Note 8**).

Although we demonstrated the Fluoro-X™ Suite using a four-color T-cell FLUOROSPOT, the same principles and procedures are applicable to any numbers of the fluorescent channels. The current ImmunoSpot® 7.0 platform supports scanning and counting of up to twelve different analytes in both T- and B-cell modes.

4 Notes

1. ImmunoSpot® Fluoro-X™ Suite is an integrated data acquisition, analysis, and management solution for high-throughput processing of multi-color T- and B-cell FLUOROSPOT plates.
2. ImmunoSpot® Software utilizes SmartCount™ mode, which is based on the SmartSpot™ automatic object recognition algorithm and statistically validated AutoGate™ function for objective user-independent data analysis.
3. Fluoro-X™ multi-color spot detection algorithm is based on the maximal allowed distance between centers of masses of spots, the Center Of Mass Distance (COMD), in individual fluorescent channels.
4. The Software provides options for setting parameters for the major steps of multi-color counting (counting, size-gating, and

multi-color spot detection) and has a built-in validation tool for evaluation and visualization of multi-color counting results.

5. The integrated quality control (QC) module with an Optional GLP and CFR Part 11 compliance package enables multi-color counting data review, validation, and approval with complete audit trail and data security.
6. Multiple counted data output formats include Flow Cytometry Standard (FCS) files for extended High Content spot morphology analysis and XML files for Laboratory Information Management System (LIMS) compatibility.
7. For maximal accuracy of true multi-color spots counting, the Fluoro-X™ Software calculates frequencies of "false" positive multi-color spots (random spots overlays) using two independent statistical methods.
8. Built-in data and image exporting tools assist in composing high quality data presentations and reports.

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