J Fluoresc (2010) 20:389–399
DOI 10.1007/s10895-009-0546-z

SHORT COMMUNICATION

Application of Fluorescent Nanocrystals (q-dots) for the Detection of Pathogenic Bacteria by Flow-Cytometry

Eran Zahavy · Vered Heleg-Shabtai · Yossi Zafrani · Daniele Marciano · Shmuel Yitzhaki

Received: 17 July 2009 / Accepted: 15 September 2009 / Published online: 14 October 2009

Abstract Fluorescent semiconductor nanocrystals (q-dots) benefit from practical features such as high fluorescence intensity, broad excitation band and emission diameter dependency. These unique spectroscopic characterizations make q-dots excellent candidates for new fluorescent labels in multi-chromatic analysis, such as Flow-Cytometry (FCM). In this work we shall present new possibilities of multi-labeling and multiplex analysis of pathogenic bacteria, by Flow-Cytometry (FCM) analysis and new specific IgG—q-dots conjugates. We have prepared specific conjugates against *B. anthracis* spores (q-dots585-IgGα*B. anthracis* and q-dots655-IgGα*B.anthracis*). These conjugates enabled us to achieve double staining of *B. anthracis* spores which improve the FCM analysis specificity versus control *Bacillus* spores. Moreover, multiplexed analysis of *B. anthracis* spores and *Y. pestis* bacteria was achieved by using specific antibodies labeled with different q-dots to obtain: q-dots585-IgGα*B. anthracis* and q-dots655-IgGα*Y. pestis*, each characterized by its own emission peak as a marker. Specific and sensitive multiplex analysis for both pathogens has been achieved, down to 10^3 bacteria per ml in the sample.

Keywords Fluorescent q-dots · Nanocrystals · Flow-cytometry · Bacteria

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Introduction

The recent use of biological hazardous *B. anthracis* spores as deadly agents has put back the threat of bioterrorism among other terrorist threats. Bioterrorism uses not only the deadly biological effects of the hazardous agents used, but also the panic from the unknown. A reliable and fast detection of the exposed agents is crucial for isolating of the incident area, treating it and protecting the remaining un-exposed population. For such requirements, one needs to develop fast and reliable means of detection of the possible agents that can be used. Those methods should be fast, sensitive, highly credible and suitable for simultaneous agent detection (multiplex analysis). In this manuscript we shall focus on Flow Cytometry (FCM) as a diagnostic tool for bacteria based on specific antibodies which are fluorescently labeled with fluorescent semiconductor nano-crystals (q-dots). It will be shown that such a combination can increase the diagnostic specificity and enables multiplex analysis of two different bacteria with high specificity and sensitivity. This new approach will improve both reliability and efficiency of the detection process of pathogens bacteria, which is a crucial point in the counterterrorism actions.

Immuno detection of bacteria by FCM have been described in the past by the Israel Institute for Biological Research—IIBR[1, 2] and others [3–7]. These works are based upon specific antibodies labeled with organic

fluorophores. Moreover, recent works [8,9] have shown the identification of bacteria by light scatter parameters only. However such criteria are still suffering from false positive results. Hence, the combination of FCM analysis with specific fluorophore labels (such as conjugated antibodies) is still crucial for specific identification of bacteria by FCM. However, organic fluorophores spectroscopy is characterized by a narrow excitation band and a broad emission band. Hence, the organic fluorophores require multi-wavelength excitation in order to observe several emission lines from different fluorophores, and this limits their use as simultaneous labels. As opposed to the organic fluorphores, q-dots are characterized by broad excitation band and narrow emission band. Such optical setting enables simple excitation source that simultaneously can excite several q-dots with distinctive emission bands, which can easily be separated by optical filters. Indeed, the common bench top Flow Cytometry include one or two excitation sources with set of optical filters and photo-multipliers for multiple detection, fitted to the q-dots optical requirements.

For accurate and selective multiplex analysis by FCM there is a necessity for new kind of fluorophore labels that will meet three basic conditions: The fluorophores should be covalently bounded to the antibodies without damaging their biological binding properties. The different fluorophores should be easily excited by the common laser lines available in bench FCM and they should emit light at distinctive emission lines. This will enable the possibility to perform multi-labeling on single target and use the multi-parameter analysis on a single laser FCM. Using such series of fluorescent labels will also make possible the development of multiplex analysis of several different targets, in a single measurement.

The q-dots spectral characteristics have been studied intensively as unique fluorescent markers [10–14]. Using a series of q-dots can form a series of labels with different emission bands depending on their diameter, and all can be excited by a single wavelength. However, fluorescent q-dots were used mainly in organic media and any attempt to dissolve them in water for biological conjugation ended with a major loss of fluorescence efficiency. Recently, several researchers overcame these obstacles [15–17] and prepared fluorescent nano-crystals in such a way that they were compatible to be linked to biological compounds without losing their spectroscopic characteristics. The potential of using q-dots as fluorescent labels for biological systems was studied by several research groups which are aiming for developing new tool for cell studying [18], cell staining [19], fluoro-immunoassays [16], pathogen immune-detection [20–22], in-vivo imaging [17], immuno-phenotyping by FCM [23] and multiplexed analysis for DNA analysis [24].

Therefore we will show in here that the use of the fluorescent q-dots can contribute to current immuno-fluorescence analysis of biological hazards in two ways:

1. Different conjugates can be used for multi-parameter analysis by FCM in order to achieve fast, sensitive and accurate analysis of a single biological threat, such as *B.anthracis* spores, Fig. 1a. While in previous work[1], we have shown specific FCM analysis of *Bacillus anthracis* spores using IgG fluorescent conjugates with an organic fluorophore such as Alexa488 and Alexa585 with increasing specificity by FRET effect. Here, we would like to present specific analysis of the spores by the combination of light scatter parameters and the double immuno-staining (with no FRET effect), using the Q-dots conjugates, Q-dots585-IgGα*B.anthracis* and Q-dots655-IgGα*B.anthracis*, for simultaneous double labeling. This will enable high selectivity gate selection for the target spores versus other common spores which are phyllogenetically close to *B. anthracis* [25].
2. Series of fluorescent q-dots that attached to different antibodies (or other recognition molecules), can be utilized for the development of multiplex analysis of two (or more) biological hazards in single measurements, Fig. 1b.

Materials and methods

Bacillus strains Bacillus anthracis Δ 14185, BA, is a non toxinogenic and non capsulated derivative of ATCC 14185

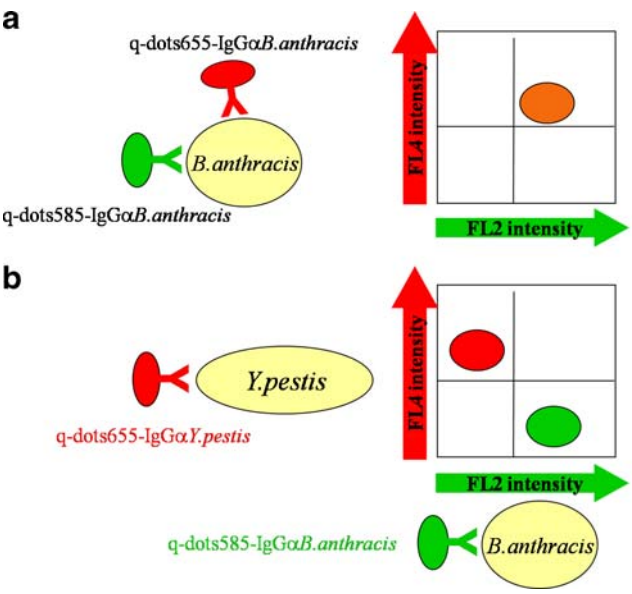


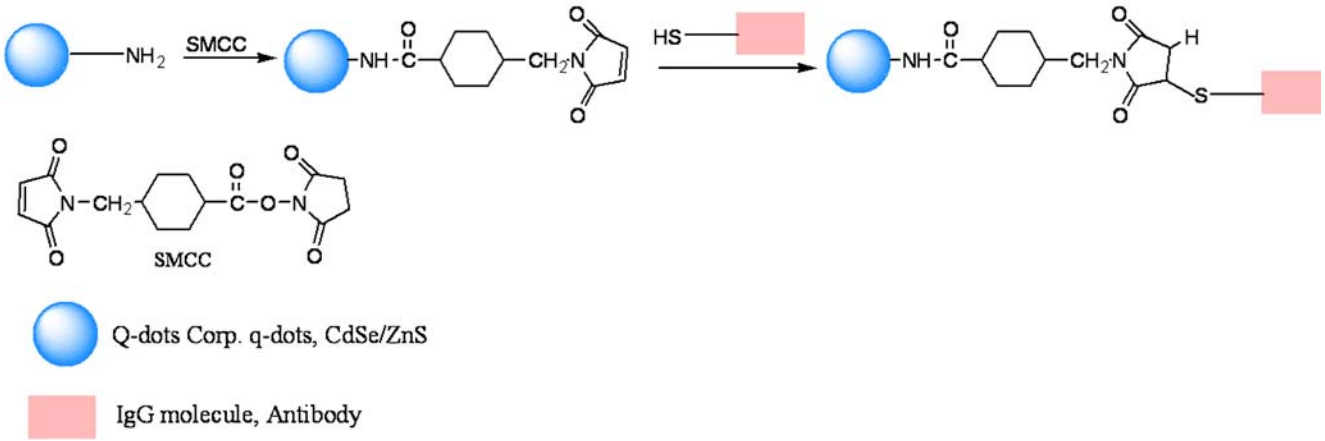
Fig. 1 **a** Double labeling of *B. anthracis* spores by two q-dots conjugates. **b** FCM concept for multiplex labeling and analysis of *B. anthracis* spores and *Y. pestis* bacteria for FCM analysis

[26]; *Bacillus subtilis* BD 104, B.sub [27]; *Bacillus thuringiensis subs. Israelensis*, BTI, and *Bacillus cereus thuringiensis*, BCT, are from IIBR collection.

For *Yersinia Pestis* (*Y. pestis*) the non-virulent vaccine strain EV76 is used [28]., and the bacteria were isolated on rich selective BIN agar [29].

Polyclonal Antibodies against *B.anthracis* spores were raised in rabbits after injection with *B.anthracis* exosporium [30]. Specificity of the antibodies is demonstrated in previous work, [1, 31]. Polyclonal Antibodies against *Y. pestis* bacteria were raised in rabbits after injection with formalin fixed *Y. pestis* bacteria. The resulting sera have been purified on protein-G column (from pharmacia/GE). The prepared antibodies were used as immuno-fluorescence labels to *Y. pestis* bacteria in FCM analysis (data not shown) as reported in previous work [32].

Fluorescent probes and fluorescence conjugates: CdS/ZnSe coated nanocrystals as: q-dots655 and q-dots585, with maximum emission at 650 nm and 585 nm respectively, were purchased from Q-Dots Corp. and later on from Invitrogen (cat # Q22021MP and Q22011MP respectively). The q-dots were obtained coated with polyethyleneglycol (PEG) with free amine group available for antibody conjugation through SMCC linker. Conjugation between the antibodies and fluorescent q-dots (IgG-q-dots) were performed according to protocol outlined by Invitrogen (q-dots antibody conjugation kit) and in the literature [33] using the hetero-bifunctional crosslinker, 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC). SMCC contains on one edge an active ester group (NHS) for amine modification, and a maleimide group on the other edge for thiol modification. The coupling reaction involves three steps. The PEG coated q-dots are modified at its amine groups via the NHS ester group in the SMCC compound to form amide linkages terminating in active maleimide groups, SMCC-q-dot. The SMCC—q-dots would react with the IgG molecules (from Sigma) to form the conjugate, Eq. 1 The IgG molecules must contain free sulfhydryl groups, which are obtained by pre-activation of the protein by reduction of disulfide bonds.



(1)

The procedure based on Invitrogen conjugation protocol is:

Quantum dot activation SMCC (final concentration 1 mM) was added to 125 μL of q-dots (4 μM), and reacted for 1 h at room temperature. Excess SMCC is removed by size exclusion chromatography (NAP-5 column, total volume should be approximately 400–500 μl).

Antibody (IgG) reduction Dithiothreitol (DTT, final concentration of 20 mM) is added to a solution of IgG molecules at 1 mg/ml in 300 μl of PBS and reacted for 0.5 h at room temperature. Excess DTT is removed by size exclusion chromatography (NAP-5, total volume should be approximately 500–600 μl).

Conjugation and quenching Column-purified activated quantum dots and column-purified reduced antibody are reacted for 1 h at room temperature (total volume ~1 ml). The reaction is quenched by β-mercaptoethanol (final concentration 100 μM) for 0.5 h at room temperature.

Conjugate purification The conjugate is concentrated by ultrafiltration and purified using size exclusion chromatography (superdex 200). Fractions of the conjugates, free proteins and free nanoparticles are separated on the column and distinguish by spectroscopic measurements of the collected aliquots of 200 μl each.

In order to determine the protein concentration in the conjugates solutions the Bradford assay was performed on

the conjugates. Bradford assay essentially measures arginine and lysine, hence is suitable for IgG molecules with appropriate controls to avoid charge effect on the results [34–36]. As a blank for these measurements we have prepared q-dots covalently blocked only with SMCC and with no proteins. For calibration curve of the Bradford assay, BSA protein and q-dots BSA conjugates were measured. Q-dots concentration is evaluated from absorption spectra using molar concentration coefficient provided by Invitrogen for QD585 $\left(\varepsilon_{QD585}^{488nm} = 530,000\mathrm{M}^{-1}\mathrm{cm}^{-1}\right)$ and for QD655 $\left(\varepsilon_{QD655}^{488nm} = 2,900,000\mathrm{M}^{-1}\mathrm{cm}^{-1}\right)$. From the UV spectra and the Bradford assays it was calculated 1–2 Q-dots per protein.

The fluorescence quantum yield for both q-dots before and after conjugation was measured and calculated by Birks method [37], in comparison to a standard fluorophore, Rhodamine 6G. The results show that the fluorescence quantum yield is not affected by the conjugation. For the q-dots585 it is 80%, and for q-dots655 is only 20–24%. Although, q-dots655 has a much larger cross section for absorbance its fluorescence efficiency is reduced. This might be attributed to the size and shape of the nanoparticles that might lead to quenching routes related to the chemical/biological environment and to the closed-shell structures of the nanocrystal [38, 39]. Electron microscopy (TEM) of the nanoparticles before and after the conjugation, data not shown, reveals highly spherical nanoparticles for the q-dots585 (r=4.9±0.5 nm), while for the q-dots655 an oval shape was revealed (r1=6.9±0.5 nm, r2=12±1 nm) , apparently with less perfect close shell, hence with higher percentage of existing "dark particles" [40] that reduce the quantum efficiency of the q-dots655.

The resulting conjugates obtained for this work are: Q-dots585-IgGα*B.anthracis* and Q-dots655-IgGα*B.anthracis* against *B.anthracis* spores and Q-dots585-IgGα*Y.pestis* and Q-dots655-IgGα*Y.pestis* against *Y. pestis*.

Flow—cytometry setup Experiments were performed on a Becton-Dickinson flow-cytometer, FACSCalibur model. Analyses were performed using CellQuest and Flow-Jo softwares. The positive events have been gated by forward-scatter (FSC) and side-scatter (SSC) parameters. For the analysis of q-dot585 conjugates FL2 detector has been used with the Ar laser excitation line, λexc=488 nm, and emission detection at λem=585/42 nm. For the detection of q-dot655 conjugated, FL4 detectors was used, with red diode laser excitation line at λexc=635 nm, and emission detection at λem=661/16 nm. Preliminary set up had shown the same efficiency for analyzing q-dots655 on FL3, with the Ar laser excitation line, λexc=488 nm, and emission detection at λem=670/30, however for multiplex application we found it easier to distinct between the two fluorophore on a FL1/FL4 channels.

The instrument setting includes logarithmic amplifiers on all detectors. Instrument setting for the spores analysis: FSC=E01; SSC=427V; FL2=572V; FL3=615V; FL4=639V.

Sample preparation for Flow-Cytometry analysis: Bacterial or spores samples were diluted to the desired concentration by PBS/BSA 1%/Tween 20 0.01% solution. To 1 ml of samples conjugates from stock solution was added to final concentration of 1 nM to 2 nM (1:500 to 1:1000 dilutions). The staining is complete within 15 min in rt, and ready for analysis. Washing steps found to be un-necessary.

Results

Flow cytometry specific analysis of *B. anthracis* spores by Q-dots conjugates staining

The high stability of the new conjugates in aqua solutions enables us to utilize them as immune-fluorescence labels for spore identification in FCM. However the gating strategy here includes pre-selection of the targets by light scatter parameters followed by the immuno-staining of the spores by the specific Q-dots conjugates. The contribution of the light scatter analysis is obtained from Figs. 2 and 3. Resulting dot plots for unstained spores are shown in Fig. 2, by light scatter parameters (Fig. 2a) and fluorescence (FL2) parameters (Fig. 2b). In the fluorescence analysis (B), one can see that the defined region for positive stained spores, upper right quadrant, is free of any event (less than 0.01 %), due to the lack of the immuno-staining. In the light scatter dot plot the spores are localized (more than 90% of the total events) in a defined region, R1, by FSC/SSC parameters. Moreover, from the 3D presentation of the light scatter analysis of the spores, Fig. 3, it is shown that the different spore populations are localized in similar channels position.

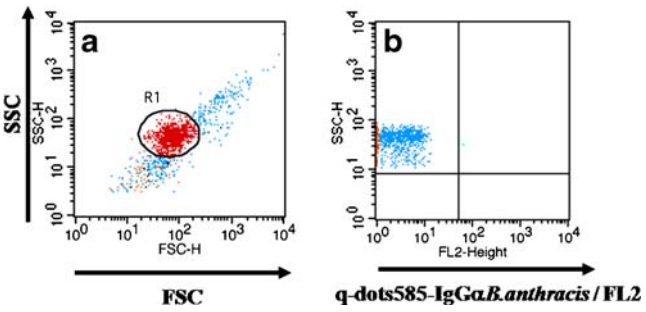
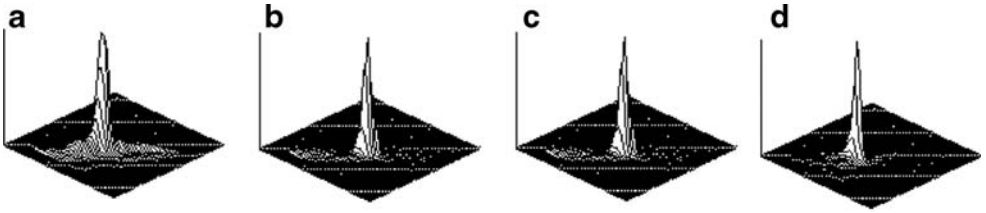


Fig. 2 Dot plot analysis of unstained *B. anthracis* spores. **a** Light scatter analysis of the spores, R1 represent the events reflected by the spores. **b** Fluorescence (FL2) analysis, *upper* right quadrant is the region of the positive labeled spores



e

	SSC	FSC
BA	42	74
BCT	54	122
BTI	20	66
Bsub	45	60

Fig. 3 3D presentation of the light scatter analysis of the spores: **a** *B. anthracis* spores, **b** *B. cereus* spores, **c** *B. thuringiensis Israelis* spores, **d** *B. subtilis* spores. **e** Table of the mean FSC and SSC values of each spore

The light scatter parameters are of course not sufficient to distinguish between different spores, however they are crucial for the initial gating and for preliminary filtration of the desired population. Herein, while analyzing quantitatively the light scatter parameters of the different spores, as outlined in the table, Fig. 3e, one can see small differences between the different species. Overall, those differences reduce undesired population by 10–40% by implying R1 (Fig. 2) as the primary gate selection for spores.

For single immuno-specific identification of the spores, samples were stained by the fluorescent conjugate, q-dots585-IgGα*B.anthracis* resulting in the fluorescence staining of the *B. anthracis* spores as can be observed in Fig. 4a. This staining is shown to be effective on more than 90% of the events related to spores as shown in the light scatter analysis (R1). The positive events, as *B. anthracis* spores, are localized on the upper right quadrant of the FL2/SSC dot plot, shown in Fig. 4a(I) and Fig. 2b as its negative control.

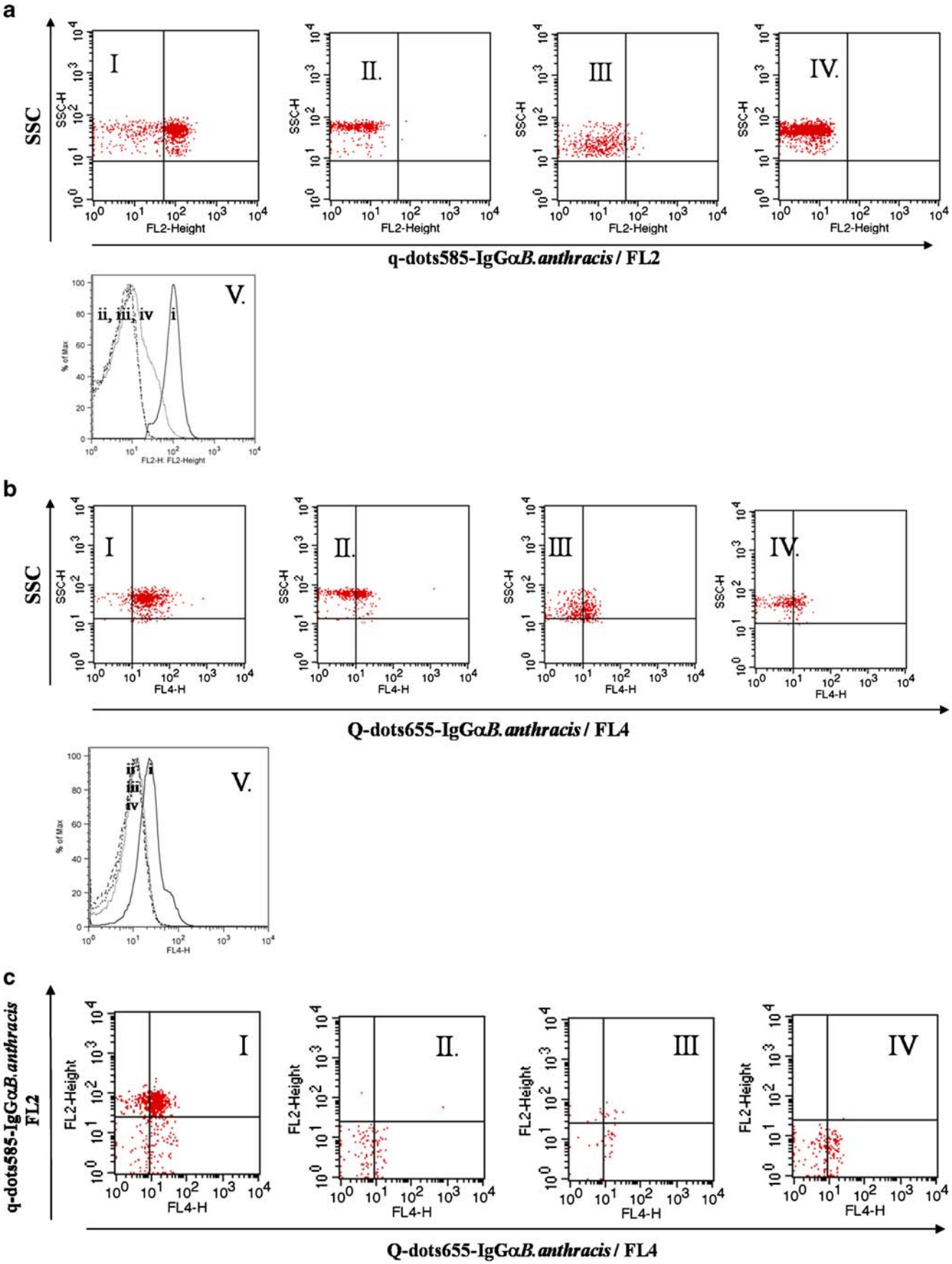
In order to evaluate the specificity of the immuno-staining, competitive spores were sampled, stained and analyze the same as the *B. anthracis* spores. Figure 4a shows the resulted fluorescence dot-plots for such analyses. One can see that while for the *B. anthracis* spores region R2 contain more than 90% of the events related to spores by the light scatter, for the competitive spores R2 (Fig. 4a: II, III and IV) is almost clear of any positive events. This is also shown in the fluorescence histogram, Fig. 4a(V), and can be calculated that the FL2 ration between the target spores to the competitive is more than one order of magnitude. Quantification of the specificity factor has been evaluated by measuring the percentage events in the upper right quadrant of the competitive spores compare to *B. anthracis* spores dot-plots normalized to 10,000 events. From the gating process we can see that in the specific region (upper right quadrant) for the stained *B. anthracis* spores the percentage of *B. cereus, B. subtilis* is in the range of 0.02–0.1% and higher for *B. thuriengensis I.* which is 1–2%, Table 1. This might be due to the high genetic resemblance between *B. thuringiensis I.* and *B. anthracis*.

Following the same flow cytometry analysis on the target and competitive spores was performed by using the red shifted conjugate, q-dots655-IgGα*B.anthracis*. The resulting dot plots are presented on Fig. 4b. Here, the upper right quadrant of the FL4/SSC dot plot is related to FL4 stained *B. anthracis* spores by q-dots655-IgGα*B.anthracis*, Fig. 4b(I). Here in, labeling the competitive spores with the same conjugates revealed partially staining of the spores as revealed in the flow-cytometry analysis, Fig. 4b(II, III, IV). Quantification of such un-specific staining is performed by measuring the upper right quadrant events for the control spores (dot plots II, III, IV) compare to the target spores (dot plot I). The un-specific events ratio reaches the values of 11–26 %, Table 1. This can be observed by the attached FL4 histogram, Fig. 4(V), and can be calculated that the FL4 ratio between the target spores to the competitive is only 4 to 6.

B. anthracis spores diagnosis by FCM using Q-dots conjugates as double labeling

For increasing the immune-specific diagnosis of the *B. anthracis* spores by the FCM analysis, we have established the double labeling method as described in the introduction, Fig. 1a. Such diagnosis method would increase the specificity of the *Bacillus anthracis* spores vs. other Bacilli spores that are genetically related to *anthracis*.

The two conjugates, q-dots585-IgGα*B.anthracis* and q-dots655-IgGα *B.anthracis*, are used for double labeling of the *Bacillus anthracis* spores. Such labeling results in a dual channels (FL2/FL4) detection by FCM. This double channels analysis reflects in a new dot-plots analysis that includes both FL2 and FL4, Fig. 4c, after light scatter


a
SSC
SSC-H
FL2-Height
I
II.
III
IV.
q-dots585-IgGαB. anthracis / FL2
V.
ii, iii, iv
i
% of Max
FL2-H: FL2-Height
b
SSC
FL4-H
Q-dots655-IgGαB. anthracis / FL4
c
q-dots585-IgGαB.anthracis FL2
FL2-Height
IV

◀ **Fig. 4** Dot plots analysis of several spores stained with: **a** q-dots585-IgGα*B.anthracis*, **b** q-dots655-IgGα*B.anthracis* and **c** q-dots585-IgGαB.anthracis, q-dots655-IgGαB.anthracis. Dot plots (*I*) *B. anthracis* spores, (*II*) *B. cereus* spores, (*III*) *B. thuringiensis Israelensis* spores, (*IV*) *B. subtilis* spores, (*V*) Histogram overlay of the spores. All dot plots are "R1 gated, staining is with 1 nM conjugate, 15 min, rt, no washing

parameters gating as related to spores, Figs. 2 and 3. By presenting double labeled spores samples on double fluorescence channel dot plots(FL2/FL4), compared to non stained spores, we have defined new specific quadrant for the *B. anthracis* spores, Fig. 4c, dot plot I. From the competitive Bacilli spores samples we have measured the false events percentage in the same gate, to evaluate the contribution of the double labeling compare to the single labeling. As shown in table 1, the false positive events from the competitive spores by the double labeling is reduced to the levels of 0.01–0.2%.

Application of the Q-dots conjugates for multiplex analysis of *Y. pestis* and *B. anthracis* spores

The unique photo-physical properties of the Q-dots, as discussed in the introduction, facilitate them, after selective conjugation to the correct antibodies, as good candidates for multiplex analysis by FCM. Here we would like to present this methodology for simultaneous diagnosis of *B. anthracis* spore and *Y. pestis* bacteria. To obtain an analysis capabilities of the two pathogens simultaneously both the light scatter and the immuno-staining should established. The light scatter parameters were investigated (data not shown) and it was found that the FSC/SSC gate for both pathogens can be drawn in the same instrument setting with high proximity. For the immuno-labeling of *Y.pestis* we have prepared the relevant conjugates against *Y.pestis* bacteria: q-dots585-IgGα*Y.pestis* and q-dots655-IgGα*Y. pestis*, in the same method as described for the *B. anthracis* conjugates. The new prepared conjugates were found specific toward *Y. pestis* vs. variety of gram negative bacteria such as *P. tuberculosis, E.coli* (TG1), *S. aureus* , in a specificity factor range from six to ten respectively (data not shown) in both q-dots655 and q-dots585 conjugates. In order to achieve simultaneous analysis of both pathogens in a single FCM analysis the optimal conjugates combination needed for the task were studied. The main obstacle in achieving simultaneous detection is attributed to the possible cross reaction between the antibodies and the different pathogens. Moreover the conjugates should enable strong and selective labeling of its target bacteria, such as at least one magnitude higher (decade) than the background (un-labeled bacteria).

For measuring cross reaction between the conjugates and the different bacteria, we performed a series of control experiments, where sample of each species was labeled with all possible conjugates. Figure 5 summarize the results of the experiments by showing set of fluorescence histograms of the different bacteria species, *B. anthracis* and *Y. pestis* in solid and dashed lines respectively, and with different staining. In Fig. 5a, the samples were stained with q-dots585-IgGα*Y.pestis*, and accordingly were detected by the FL2 channel in the FCM. In Fig. 5b the samples were stained with q-dots655-IgGα*Y.pestis*, and accordingly were detected by the FL4 channel in the FCM. One can see that both conjugates facilitate selective staining of the *Y. pestis*. In fact by measuring the FL2 or FL4 mean values of the *Y. pestis* to the *B. anthracis* we obtained a ratio of 10, which reflects a specific staining and good terms for further simultaneously staining. Figure 5c and D, refers to samples stained with q-dots585-IgGα*B.anthracis* and with q-dots655-IgGα*B.anthracis* respectively. From the data shown in Fig. 5c a selectivity factor of ca ~18 is derived which indicates high specific staining by the q-dots585-IgGα*B.anthracis* conjugate. However, Fig. 5d, shows poor selectivity staining of the q-dots655-IgGα*B.anthracis* conjugate, with selectivity factor of only 3 (less then one decade). Following these results we decided to use the conjugates**: q-dots585-IgGα*B.anthracis*** and **q-dots655-IgGα*Y.pestis*** for multiplex analysis of both pathogens. These conjugates are selective, have no cross reaction between the two pathogens and their labeling intensity is one order of magnitude higher than the background.

For multiplex diagnosis of both pathogens it is required to assemble them into one dot plot where each population is in a distinctive gate/quadrant, as described schematically in Fig. 1b. This schematic fluorescence dot-plot represents events related to both pathogens after light scatter gating. It was found (data not shown) that both events related to *B. anthracis* spores and *Y.pestis* are overlapping in their light scatter analysis and therefore they both gated with the same gate.

Table 1 Effect of simultaneous labeling on the reduction of non-specific signals

The numbers are percent of gated events, per 10,000 events of *B. anthracis* spores obtained from each channel (FL-4, FL-2 and their combination)

Conjugate/channel	Bacillus spores		
	B.thuringiensis I.	B. cereus	B. Subtillis
Q-dots655/FL4	11%	20%	26%
Q-dots585/FL2	2%	0.02%	0.02%
q-dots655+585/FL4+FL2	0.2%	0.01%	0.01%

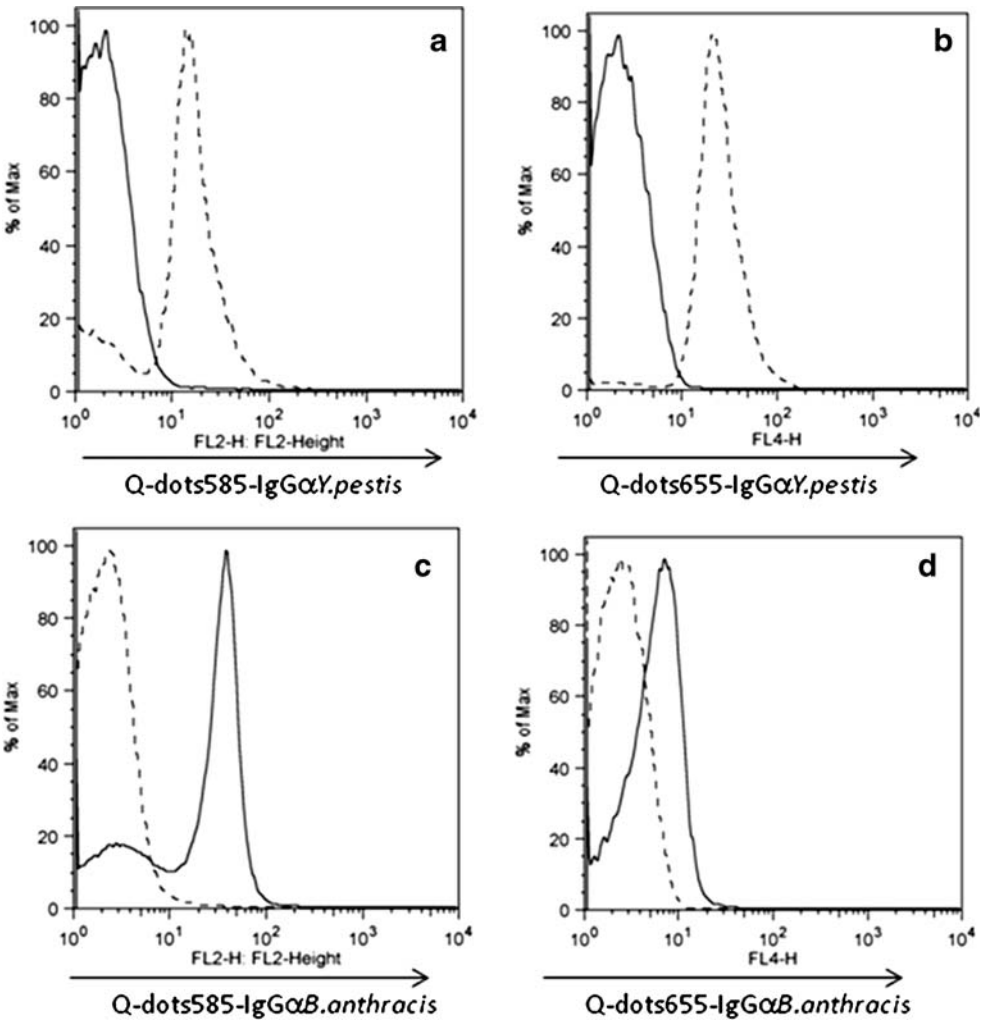


Fig. 5 Flow cytometry fluorescent histograms of *Y. pestis* (in *dashed line*) and *B. anthracis* spores (in *solid line*). A. FL2 histograms after staining with q-dots585-α*Y.pestis*. B. FL4 histograms after staining with q-dots655-α*Y.pestis*. C. FL2 histograms after staining with q-dots585-α*B.anthracis*. D. FL4 histograms after staining with q-dots655-α*B.anthracis*

The selective analysis of the different bacteria species is possible thanks to the specific fluorescent conjugates that we have prepared and carefully examined. The fluorescent dot plot consists of the specific channel (FL2) for the *B. anthracis* based on the conjugate q-dots585-IgGα*B.anthracis*, and a specific channel (FL4) for the *Y. pestis* based on the conjugate, q-dots655-IgGα*Y.pestis*. Such configuration enables us to identify separately the *B. anthracis* spores on FL2 channel and *Y. pestis* on the FL4 channel.

The fluorescence dot-plots shown in Fig. 6, represent a series of experiments where in each sample we introduced either one of the two pathogens in different concentrations. In all samples both conjugates, q-dots655-IgGα*Y.pestis* and q-dots585-IgGα*B.anthracis,* were present. It is clearly shown by the dot-plots that each bacteria is lightning the expected channel for its identification. We can see the events related to *Y. pestis* filling the lower right quadrant on the channel FL4 and the events related to the *B. anthracis* spores filling the upper left quadrant on the channel FL2. Moreover, while using mixed samples of both pathogens we have revealed both populations distinct in their designated FL2/FL4 gates (data not shown). The number of events related to each bacterium is correlated to the concentration of the samples. From the number of events for each bacteria (gate), the flow rate (v, μl/min) and the time of the sampling (t, min), we can calculate the bacteria concentration in the samples by Eq. 2. The concentration relation to the events is shown in Fig. 6c. The graph shows high correlation between the actual pathogen concentrations in the samples and the concentration calculated from the events number as it appear in the FCM analysis. This correlation is firm from 10^3 up to 10^7 cfu/ml for both pathogens.

$$\mathrm{C(n/ml)} = \frac{gate}{t \times v} \times 1000 \tag{2}$$

C	bacteria concentration (cfu/ml)
gate	number of events in the selected gate
t	time of analysis(min)
v	flow rate (microL/min)

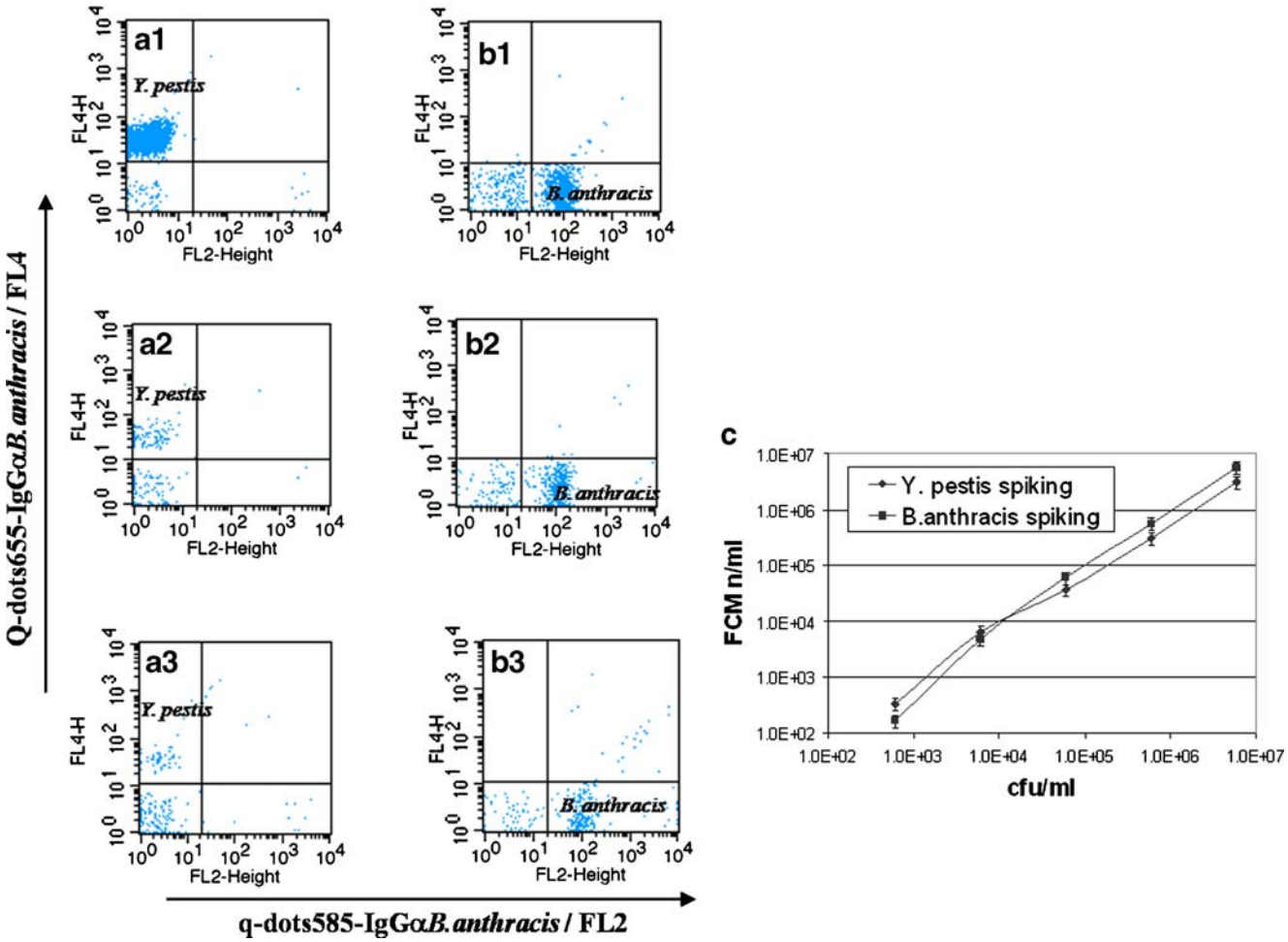


Fig. 6 Fluorescent (FL4/FL2) dot plots analysis of multiplex setup for *Y. pestis* in **a1** 10^6 cfu/ml, **a2** 10^5 cfu/ml and **a3** 10^3 cfu/ml. **b** anthracis spores in B1. 10^6 cfu/ml, **b2** 10^5 cfu/ml and **b3**. 10^3 cfu/ml. All events were light scatter gated. **c** Correlation graph between the bacteria concentration in the samples to the FCM analysis and counting

Discussion

This work demonstrates the use of fluorescent q-dots conjugates for Flow-Cytometry analysis of pathogens bacteria such as *B. anthracis* spores and *Y. pestis*. Although the use of fluorescent for q-dots had been shown previously by flow-cytometry analysis on the immune cell for immunophenotyping [23], here we are demonstrating for the first time substantive improvement in the field of bacterial analysis in term of specificity and multiplexity.

The advantages of using q-dots as fluorescence labels above standard organic dye such as PE (compare to q-dots585) or APC (compare to q-dots655) is in the high photostability of the q-dots, bright fluorescence and mainly in the high excitation efficiency of the q-dots in a simple FCM layout with only 488 nm (Ar laser) and 633 nm (HeNe laser) excitation lines. This is due to the q-dots broad absorption line compared to organic dye where the absorptions lines are close to the emission lines and usually narrow, hence they are not compatible to the light sources in simple commercial Flow-Cytometers.

By using q-dots585 and q-dots655 we have prepared two conjugates (q-dots585-IgGα*B.anthracis* and q-dots655-IgGα*B.anthracis*) against the *B. anthracis* spores to achieve single and double immuno-gating of the targeted spores. We have measured the cross reactivity of the *B. anthracis* spores designated gate with control *Bacilli* spores, *B. cereus*, *B. subtillis* and *B. thuringiensis I.*. The results, are summarized in Table 1, and allow comparison of the selectivity between the different labeling methods.

It is shown that using q-dots585-IgGα*B.anthracis* revealed better selectivity than using q-dots655-IgGα*B. anthracis*. These results imply that the selectivity factor for the q-dots655 conjugates by itself wouldn't be sufficient for the identification of the *B. anthracis* spore in complex environment. This can be attributed to two

effects: Due to the relatively low fluorescence quantum yield (ϕ=20%) of q-dots655- IgGα *B.anthracis*, compared to the q-dots585- IgGα *B.anthracis* (ϕ=80%), as described and explained in the material and methods section. However, since this did not repeat with the gram negative bacteria group (*Y. pestis* and its control group) it is more likely that the reason for the high background arises from affecting the antibody specificity by the attachment to the q-dots655 as shown for other fluorophores [41].

The double labeling method using both q-dots655- IgGα *B.anthracis* and q-dots585- IgGα *B.anthracis* results in the lowest ratio of false positive events (0.01–0.2%), and increases of the selectivity factor by 2 to 10 compare to the single q-dots585- IgGα*B.anthracis* labeling, and by 100 compare to q-dots655- IgGα*B.anthracis*. Overall, the double labeling staining combined with FCM multiparameter analysis, both light scatter (FSC/SSC) and immunofluorescence (FL2/FL4), result in highly selective gate toward the *B. anthracis* spores versus phyllogenetically closed species. By comparing these results to double labeling with organic fluorophores conjugates, as shown in our previous work [1], one can see that by using q-dots as fluorescent labels for double labeling the signals are brighter almost in one order of magnitude. This is mainly due to the superior spectroscopic characteristics of q-dots and due to the FRET effect exhibits between the two organic fluorophores conjugates on the same bacteria cell wall.

This was also partially shown previously[20, 23] on different type of bacteria (*E. coli*), however with an assistant of DNA staining (PI) and in higher bacteria concentration.

The same q-dots conjugates were prepared against *Y. pestis* bacteria, q-dots655-IgGα*Y.pestis* and q-dots585-IgGα*Y.pestis*, and its staining characterization were measured by FCM and compared to the *B. anthracis* spores staining. It was found that the conjugates couple, q-dots655-IgGα*Y.pestis* and q-dots585-IgGα*B.anthracis*, is suitable for multiple staining of both pathogens, as shown in the results, Fig. 6. These conjugates did not give any cross reaction between the two pathogens and their labeling intensity was one order of magnitude higher than the background. We have used those conjugates to develop a new method for the multiplex detection of *B. anthracis* spores and *Y. pestis*. However, the main rationale for multiplex analysis is to detect the presence of one pathogen out of several existing threats. Existing of different pathogens in one sample is not feasible, hence is not the main route for this work. The detection was found to be specific and sensitive with a limit of detection of 10^3 cfu/ml for both micro-organisms. Such an advanced limit of detection is shown previously in the detection of spores[1, 5] or bacteria [42, 43], and it is mainly due to the unique capabilities of single cell detection by FCM and its great coordinate to the spectroscopic characteristics of the q-dots. Such good match leads to the highly resolved fluorescence signals, intense double labeling and finally to multiplex pathogen analysis.

The new data presented in this manuscript will enhance the application of bacterial detection by Flow Cytometry in general with a sensible utilization of q-dots as fluorophores.

Acknowledgments This work was partially supported by the Technical Support Working Group (TSWG).

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