Detection of Frequency Resonance Energy Transfer Pair on Double-Labeled Microsphere and *Bacillus anthracis* Spores by Flow Cytometry

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Development of an ultrasensitive biosensor for biological hazards in the environment is a major need for pollutant control and for the detection of biological warfare. Fluorescence methods combined with immunodiagnostic methods are the most common. To minimize background noise, arising from the unspecific adsorption effect, we have adapted the FRET (frequency resonance energy transfer) effect to the immunofluorescence method. FRET will increase the selectivity of the diagnosis process by introducing a requirement for two different reporter molecules that have to label the antigen surface at a distance that will enable FRET. Utilizing the multiparameter capability of flow cytometry analysis to analyze the double-labeling/FRET immunostaining will lead to a highly selective and sensitive diagnostic method. This work examined the FRET interaction of fluorescence-labeled avidin molecules on biotin-coated microspheres as a model system. As target system, we have used labeled polyclonal antibodies on *Bacillus anthracis* spores. The antibodies used were purified immunoglobulin G (IgG) molecules raised in rabbits against *B. anthracis* exosporium components. The antibodies were fluorescence labeled by a donor-acceptor chromophore pair, alexa488 as a donor and alexa594 as an acceptor. On labeling the spores with alexa488-IgG as a donor and alexa594-IgG as an acceptor, excitation at 488 nm results in quenching of the alexa-488 fluorescence ($E^q = 35\%$) and appearance of the alexa594 fluorescence ($E^* = 22\%$), as detected by flow cytometry analysis. The FRET effect leads to a further isolated gate (FL1/FL3) for the target spores compared to competitive spores such as *B. thuringiensis* subsp. *israelensis* and *B. subtilis*. This new approach, combining FRET labeling and flow cytometry analysis, improved the selectivity of the *B. anthracis* spores by a factor of 10 with respect to *B. thuringiensis* subsp. *israelensis* and a factor of 100 with respect to *B. subtilis* as control spores.

Development of an ultrasensitive biosensor for biological hazardous in the environment is a major need for pollutant control and for the detection of biological warfare (16, 22). The pathogen bacteria *Bacillus anthracis* is one of the biological agents likely to be used as a bioweapon (7); hence, it is the focus of a major effort in the field of rapid detection and identification (15). Fluorescence methods combined with immunodiagnostic methods are widely used for the detection and identification for biological hazards in the laboratory (8, 25, 28, 37; H. Kulaga, P.-E. Anderson, M. C. Cain, and P. J. Stopa, Proc. 1997 erdec Sci Conf. Chem. Biol. Defense Res. 1997) or the field (1, 30).

Philips et al. have shown that *B. anthracis* spores can be identified by fluorescence microscopy and flow cytometry after appropriate labeling of the sample using fluorescent labeled antibodies (24, 26, 28). Nevertheless, difficulties in the selective identification of *B. anthracis* in mixed population samples were previously encountered (27). Recently, Stopa (37) has shown that identification of *B. anthracis* spores by flow cytometry become more feasible and faster since only one step staining is required prior to the flow-cytometry analysis and no washing steps are needed. Stopa also showed that the light-scattering parameters of the spores are indicative spore viability.

However, due to the complexity of selective identification of *B. anthracis* in the environment, all experiments were performed on a laboratory prepared samples. Environmental samples included a large number of different species and debris that can lead, through nonspecific adsorption of labeled antibodies to their surface, to a high background signal and even false-positive results.

To minimize background noise arising from the nonspecific adsorption of the labeled antibodies, we have adapted the FRET (frequency resonance energy transfer) (41, 42) effect to the immunofluorescence method (10, 21). FRET is distance-dependent interaction between two fluorophores in which the excitation energy is transferred from the donor fluorophore to the acceptor. Hence, the result is a decreasing in donor emission and an enhancement of acceptor emission. FRET conditions are summarized in the Förster equation (41, 42); (i) the acceptor absorption spectrum must overlap the donor fluorescence emission spectrum; (ii) the donor and acceptor transition dipole orientations must be approximately parallel; and (iii) the donor and acceptor molecules must be in close proximity. The Förster equation defines R_0 as the distance at which FRET from the donor to the acceptor is at 50%. In most common cases R_0 is in the range of 10 to 100 Å. The proximity condition required for achieving FRET had led the technology into the field of molecular and macromolecular probes, which were utilized for oligonucleotide diagnosis (17), polymer folding (36), protein structure determination (34), protein-oli-

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gomer interaction (19), enzyme-substrate interaction (11), avidin-biotin (10) and antibody-antigen (32) complexation, interaction of cell surface receptors (5) and cell signaling (31).

Combining immunofluorescence methods with the FRET approach demands two reporter molecules, antibodies, or other recognition molecules, labeled with suitable fluorophores, such that one will serve as a donor and the other will serve as an acceptor. The reporter molecules must bind simultaneously to the biological target in a compactness that will enable the energy transfer from the excited donor to the acceptor, hence, to the enhancement of the acceptor fluorescence. Utilizing the multiparameter capability of flow cytometry analysis to analyze the double labeling/FRET immunostaining will lead to a highly selective and sensitive diagnostic method.

In recent years, the FRET effect has been introduced into diagnostic methods on microsphere surfaces. This method has been used for detection of cholera toxin (35) and peptides such as β -endorphin (3, 4). It was also introduced into research on ligand interactions with living cells (38–40).

In this work, FRET effect is applied on the particle (biotin-coated latex beads and live spores) surface by using fluorescence-labeled protein ligands (NeutrAvidin [NA] for the beads and polyclonal antibodies for the spores). By labeling two separate batches of the ligands with different fluorophores, donor-acceptor pair, we have made it possible to label the particle surface with a donor-acceptor mixture, (Fig. 1). We show that the high coverage of labeled NA on biotin coated microspheres, (Figure 1A), and the high coverage of the labeled polyclonal antibodies (immunoglobulin G [IgG]) on the *B. anthracis* spore surface (Fig. 1B) have resulted in the FRET effect between the neighboring labeled ligands (conjugates). The fluorescent tags used in this work are alexa488 and alexa594, for which an R_0 of 60 Å has been measured and calculated in their nonconjugated state (14).

MATERIALS AND METHODS

Materials. (i) Microspheres. We used biotin-labeled polystyrene microspheres (diameter, 1.0 μm), from Molecular Probes, Leiden the Netherlands (no. F-8769). The particle solution contain: 1% solid, with 1.8×10^{10} particles/ml; the polystyrene density is 1.055 g/cm³. The avidin capacity is 4.6 nmol/mg of microsphere.

(ii) NeutrAvidin. NA was obtained from Pierce (no. 31000).

(iii) Polyclonal antibodies. Polyclonal antibodies (anti *B. anthracis* exosporium) were raised in rabbits as described by Fisher et al. (M. Fisher, S. Weiss, D. Kobiler, H. Levy, and Z. Altboum, submitted for publishing). The resulting serum has been purified on protein G column (Pharmacia Biotech, Sweden).

(iv) Fluorescent probes. alexa488 (A-10235) and alexa594 (A-10239), from Molecular Probes, have been used for the protein (NA or polyclonal antibody) modification.

(v) Conjugates. NA and the IgG polyclonal antibodies were conjugated and purified with the fluorophores alexa488 and alexa594 by the method described by the fluorophore manufacture (Molecular Probes). The resulting conjugates, alexa488-NA, alexa594-NA, alexa488-IgG, and alexa594-IgG, have been characterized by spectroscopic means. The number of fluorophores per protein has been calculated by measuring the absorption spectra of the purified conjugates solutions. Knowing the absorption extinction coefficient the protein coefficient at 280 nm ($\epsilon_{\text{protein}}^{280\text{ nm}} = 203,000\text{ M}^{-1}\text{ cm}^{-1}$) and of the free fluorophores: alexa488 ($\epsilon_{\text{alexa488}}^{488\text{ nm}} = 70,000\text{ M}^{-1}\text{ cm}^{-1}$, $\epsilon_{\text{alexa488}}^{280\text{ nm}} = 7,000\text{ M}^{-1}\text{ cm}^{-1}$), alexa594 ($\epsilon_{\text{alexa594}}^{590\text{ nm}} = 73,000\text{ M}^{-1}\text{ cm}^{-1}$, $\epsilon_{\text{alexa594}}^{280\text{ nm}} = 36,000\text{ M}^{-1}\text{ cm}^{-1}$), one can measure the concentration of each component in the solution and hence the ratio of the fluorophore per protein in the conjugates. Values for two or three fluorophores per protein were measured in all the conjugates that have been prepared and used in this work.

FIG. 1. (A) FRET effect on a doubly labeled biotin-coated 1- μm microsphere. Labeling includes NA-alexa488 as a donor and NA-alexa594 as an acceptor. (B) FRET effect on a doubly labeled *B. anthracis* spore. Labeling includes IgG-alexa488 as donor and IgG-alexa594 as an acceptor.

(vi) Biotinylated IgG. Biotinylated IgG was prepared by using sulfo-NHS-biotin [sulfo-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce 21331] conjugated to the polyclonal antibody to form biotin-IgG. Addition of alexa594-NA results in the complex alexa594-NA/biotin-IgG. The number of biotins per antibody has been calculated by the HABA ([2-(4'-hydroxyazobenzene) benzoic acid] method (Pierce 28050) and shown to be on average four biotin molecules per antibody molecule.

(vii) Bacillus strains. *B. anthracis* $\Delta 14185$ is a nontoxigenic and nonencapsulated derivative of ATCC 14185 (6); *B. subtilis* BD 104 (18) and *B. thuringiensis* subsp. *israelensis* are from the Israel Institute for Biological Research collection.

Fluorescence measurements. Fluorescence measurements are performed on a PTI time master C-71 fluorimeter (Photon Technology International) with a sensitivity in the steady-state measurements of 1 nM fluorophore. The experiments were performed in emission scan mode with excitation at 488 nm.

Flow cytometry setup. Flow cytometry analysis was performed with a FACS-Calibur machine (Becton-Dickinson, Immuno Cytometry Systems) equipped with a 15-mW argon laser as the excitation light source. Analyses were performed using CellQuest (Becton-Dickinson) and FlowJo (FLOWJO) software. The positive events (beads or spores) were gated by forward-scatter (FSC) and side-scatter (SSC) parameters. For analysis of the donor emission, the FL1 detector ($\lambda_{\text{exc}} = 488\text{ nm}$; $\lambda_{\text{em}} = 530\text{ nm}$) was used, and for analysis of the acceptor emission, the FL3 detector ($\lambda_{\text{exc}} = 488\text{ nm}$; $\lambda_{\text{em}} = 660\text{ nm}$) was used. The instrument setting includes logarithmic amplifiers on all detectors. The instrument settings for the spores analysis were as follows: FSC = E01; SSC = 427 V; FL1 = 505 V; FL3 = 659 V. The instrument settings for the bead analysis were as follows: FSC = E00; SSC = 319 V; FL1 = 505 V; FL3 = 650 V.

FIG. 2. Titration curve (curve a) and the first derivative (curve b) for biotin-coated 1- μ m latex microspheres (1.8×10^7 beads/ml) after staining with different concentrations of NA-alexa488 (0 to 240 nM). Fluorescence values are derived from the flow cytometry analysis of the stained beads.

Number of protein ligands per particle and average distance between proteins.

For the evaluation of the number of fluorescence-labeled ligands per particle (beads or spores), we used the titration method of Siiman and Burshteyn (33). Based on the equilibrium equation (equation 1) between the soluble ligands and the ligand adsorbed on the particle surface (K_a expression), one can derive the titration curve plot, which is a typical Langmuir adsorption curve:

$$\frac{\theta}{1 - \theta} = K_a \cdot C_{\text{solution}} \quad (1)$$

where θ is the fractional occupancy of the labeled particle at a known ligand (antibody or avidin) concentration, K_a is the association constant and C_{solution} is the nonbinding ligand concentration. Experimentally, the titration is performed by running a series of increasing concentrations of fluorescently labeled ligands with a fixed concentration of the particle and measuring the mean fluorescence intensity by the flow-cytometry analysis; measurement of each titration point is repeated three times. The point at which the fluorescence intensity per particle no longer changes or changes moderately due to nonspecific absorption effects (see Fig. 2) is defined as the saturation point. The saturation point can be defined visually on the titration curve or by the first derivative of the curve. The first derivative is maximal at the first point of the saturation state because the change in the fluorescence intensity for an increment at concentration change from the nonsaturate state to the saturation state is maximal. Beyond the saturation point, the derivative is zero, reflecting the saturation state (or close to zero for residual nonspecific absorption). θ can be derived from the ratio of the mean fluorescence intensity at certain fluorescent-ligand concentrations to the mean fluorescence intensity at saturation. The saturation point reflects the maximum number of labeled ligands per particle, assuming quantitative adsorption of the ligand to the particle. To ensure that the binding of the probes to the particle is quantitative, we should estimate the nonbinding probes concentration (C_{solution}). This can be done by putting the relevant values (K_a and θ for any ligand concentration) in the Langmuir equation (equation 1). For the avidin-biotin-coated bead system (Fig. 1A), where the association constant is 10^{15} M^{-1} , the resulting values (C_{solution}) of the nonbinding avidin (alexa488-NA or alexa594-NA) are less than 0.0001% of the overall avidin used. For the antibody-spore system (Fig. 1B) with association constant in the range of 10^8 to 10^9 M^{-1} , one can determine in the same way that the nonbinding antibody makes up ca ~5%. This value is within the error of the mean fluorescence-labeling measurements (10%) and hence will not affect the results.

The measurements are performed by the flow cytometer, gating on the particles by FSC and SSC parameters. The mean fluorescence intensity is being used to plot the titration curves. Knowing the number of proteins per particle surface and assuming spherical particles with a diameter of 1 μ m and a smooth surface, the free space per protein, S , can be calculated from equation 2, where r_{bead} is the radius of the bead and n is the number of proteins per bead (calculated from the saturation plot). The radius of the free space, r , is calculated from equation 3; the mean distance between two neighboring proteins is double that radius, r .

$$S = 4\pi r_{\text{bead}}^2/n \quad (2)$$

$$r = (S/\pi)^{1/2} = (4r_{\text{bead}}^2/n)^{1/2} \quad (3)$$

FRET measurements. FRET measurements have been performed both by flow cytometry analysis and by steady-state fluorescence. The main effect relating to the FRET phenomena is expressed in the decreasing of the donor emission and the enhancement of the acceptor emission double labeling of the particle with the donor-acceptor pair. For the biotin-coated beads, we have used as the donor-acceptor pairs the labeled proteins alexa488-NA and alexa594-NA, and for the spores we have used alexa488-IgG and alexa594-IgG.

To avoid depletion of the labeled protein (avidin or antibody) from the particle surfaces, the combined concentration of the fluorophores (as specified in each experiment) is kept below the total concentration of the free sites on the particles as measured by the titration method. FRET experiments have included all possible labeling permutations such as donor-only-, acceptor-only-, and donor-acceptor-labeled particles. For the flow cytometry analysis of the labeled spores, conjugated nonspecific goat anti-mouse antibodies were used as blanks for nonspecific adsorption. On conjugation to protein (IgG or NA) the dye alexa488 exhibits maximum absorption at 494 nm and maximum emission at 520 nm while alexa594 exhibits maximum absorption at 590 nm and maximum emission at 618 nm. When using a standard flow cytometer equipped with an argon laser as excitation source, $\lambda_{\text{exc}} = 488 \text{ nm}$, the donor is efficiently excitable analysis ($\epsilon_{488 \text{ nm}}^{488} = 70,000 \text{ M}^{-1} \text{ cm}^{-1}$). Under the same excitation conditions the acceptor absorption efficiency is less than 10% of the donor efficiency ($\epsilon_{488 \text{ nm}}^{488} = 6,000 \text{ M}^{-1} \text{ cm}^{-1}$). Moreover, at the donor maximum emission, the acceptor absorbs with a molar coefficient of $\epsilon_{520 \text{ nm}}^{520} = 25,000 \text{ M}^{-1} \text{ cm}^{-1}$. These spectral characteristics make alexa488 and alexa594 an excellent donor-acceptor pair due to their high overlap between donor emission and acceptor absorbance and due to the possibility of almost selective excitation of the donor at 488 nm, which is the most accessible light source for most flow cytometry users.

Fluorescence measurements were performed on the PTI fluorometer with 1-cm cuvette. Since in such an experimental setup there is no distinction between bound labeled proteins and soluble ones, if any, a separation procedure is necessary. Therefore, washing steps including three centrifugations (10 min at 1,400 rpm in an eppendorf centrifuge 5417) were performed to separate the labeled particle from the unbound reagents.

For the FRET analysis by flow cytometry, the FL1 detector was used for donor emission and FL3 was used for acceptor emission by itself and by the FRET. These detectors have enabled us to detect of the donor and acceptor separating with minimum compensation. Flow cytometry measurement are performed directly on the labeled samples, so that no washing steps are necessary. Gating by the light-scattering parameters on the particles avoided any interference from unbound reagents.

FRET efficiency. FRET efficiency in the doubly labeled particles has been calculated from the amount of donor emission quenched compare to the only donor labeled particle and from the enhancement of the acceptor emission compared to the only acceptor-labeled particle. Quantitation of the FRET efficiency (E) has been calculated based on measuring the donor quenching (E^q) and the acceptor enhancement (E^s) upon the double labeling (42) and by following Szöllösi's (39) method in his FRET measurements on live cell surface using single laser excitation by flow cytometry. The efficiency by the donor fluorescence quenching route, E^q is described in equation 4, where $FL1_D^0$ and $FL1_D$ are the donor fluorescence in the presence and absence, respectively, of acceptor. $FL1$ can be measured as the fluorescence peak (520 nm) by the fluorometer or by the mean FL1 detector ($\lambda = 530 \pm 15 \text{ nm}$) on the flow cytometer (9). The efficiency of the acceptor enhancement (E^s) is described in equation 5, where $FL3_A^0$ and $FL3_A$ are the acceptor fluorescence in the presence and absence, respectively, of donor, $FL3$ can be measured by the fluorometer at $\lambda = 620 \text{ nm}$ or by the mean FL3 detector ($\lambda = 660 \pm 15 \text{ nm}$) on the flow cytometer. ϵ and C are the molar extinction coefficient at the excitation wavelength and the concentration, respectively. The absorption factor $\left(\frac{\epsilon_A C_A}{\epsilon_D C_D}\right)$ is added to the equation to correct for the situation where the acceptor absorption overlaps with the donor absorption. Hence, part of the acceptor fluorescence measures would be a result of direct excitation of the acceptor and would not be caused by FRET.

$$E^q = 1 - FL1_D^0/FL1_D \quad (4)$$

$$E^s = \left(\frac{FL3_A^0}{FL3_A} - 1 \right) \cdot \frac{\epsilon_A C_A}{\epsilon_D C_D} \quad (5)$$

RESULTS

Double labeling on the microsphere surface. As a model system for the living spores, we first studied FRET on a microsphere system. The microsphere surface is biotin coated, simulating surface determinants on the spore. The labeled NA molecules, which bind tightly and specifically to the biotin molecules, are simulating the labeled antibodies (Fig. 1A).

Measuring the density of labeled NA molecules per bead. Figure 2 shows the titration curve of alexa488-NA with the biotin-coated 1- μm latex microspheres (1.8×10^7 beads/mL or 3×10^{-14} M). The fluorescence intensity per beads, measured by the mean FL1, increases with increasing ratios of labeled NA per bead, up to a point of saturation at 30 nM alexa488-NA. As described in Materials and Methods, the unbound labeled protein at that point makes up less than 0.001% of the used reagent. Hence, the unbound alexa488-NA is present at less than 10^{-14} M and was unmeasurable by spectroscopic means. The saturation point indicates the full capacity of labeled protein on the bead surface. Increasing the concentration of labeled protein beyond the saturation point does not increase the mean fluorescence intensity per bead, since there are no more spaces or acceptor sites available for the new labeled protein. From the saturation results, one can calculate that the average number of proteins per bead is $(1 \pm 0.2) \times 10^6$ proteins/bead. The number is consistent with the avidin loading measured by the manufacturer of the beads (Molecular Probes), which is 4.6 nmol/mg of beads (1.5×10^6 avidin molecules/bead).

Utilizing equations 2 and 3 for 1- μm -diameter beads and 10^6 proteins per bead, the free space available is $S = 390 \text{ \AA}^2$, with a radius $r = 11 \text{ \AA}$. This results leads to the conclusion that the NA molecules (molecular mass, 60 kDa) are very dense on the bead surface and that the distance between the centers of two neighboring molecules is 22 $\text{\AA}$. Such a density can still maintain the avidin-biotin recognition structure (20, 29), hence maintaining the protein structure. The maintained protein structure avoids self-aggregation and self-quenching of the tagged fluorophore (two or three fluorophores per avidin). However, the resulting distance should enable FRET between neighboring donor-acceptor-labeled NA conjugates.

FRET measurements on the bead surface. FRET measurements on biotin-coated beads were performed by labeling the beads as described in Fig. 1A with the donor NA-alexa488 (30 nM) and the acceptor NA-alexa594 (80 nM). The beads concentration is 1.8×10^8 beads/mL; knowing that there are about 10^6 sites per bead, the sites concentration would be 300 nM. Under such conditions, the double labeling would be free of competition between the different labeled proteins, and hence the effect would be due to FRET and not due to any depletion of the labeled protein from the surface.

Fluorescence measurements of FRET on labeled beads. Figure 3 shows the fluorescence spectra of the beads labeled with both the donor (alexa488-NA) and acceptor (alexa594-NA) (curve b) compared to those of the donor-labeled beads (curve a) and the acceptor-labeled beads (curve c). The fluorescence of the doubly labeled beads shows a decreasing in the donor emission ($\lambda = 520 \text{ nm}$) due to quenching by the acceptor and an increase in the acceptor emission ($\lambda = 620 \text{ nm}$). By measuring the fluorescence intensities (table 1) and using equa-

FIG. 3. Fluorescence spectra ($\lambda_{\text{ex}} = 480 \text{ nm}$) of biotin-coated 1- μm latex beads (1.8×10^8 beads/mL) after labeling with NA-alexa488 (30 nM) (a), NA-alexa488 (30 nM) and NA-alexa594 (80 nM) (b). Curve c is the sum and normalization of the spectra of the beads labeled with NA-alexa488 (30 nM) and of the beads labeled with NA-alexa594 (80 nM).

tions 4 and 5, we have calculated the FRET efficiency to be $E^q = 45\%$ by the quenching process and $E^s = 23\%$ by the sensitization process.

Flow cytometry measurements of FRET on labeled beads. Figure 4 shows the flow cytometry histogram of the donor-only-, acceptor-only-, and donor- and acceptor-labeled beads. Histogram A (Fig. 4A) shows results for the FL1 detector, which follows the donor (alexa488-NA) emission (green line). In this histogram, the acceptor-labeled beads (pink line) have no significant fluorescence. The donor-labeled beads are highly fluorescent; in the doubly-labeled beads (blue line), the mean fluorescence intensity decreases. Histogram B (Fig. 4B) shows results for the FL3 detector, which follows the acceptor (alexa594-NA) emission. In this histogram, the donor-labeled beads (green line) have low fluorescence signal. The acceptor-labeled beads (pink line) are moderately fluorescent, while in the doubly labeled beads (blue line) the mean fluorescence increases. These results indicate that FRET effect has been recorded by flow cytometry.

Table 1 summarizes the FRET results from the flow cytometry measurements; the numbers presented in the table are average of three different samples. Using equations 4 and 5, we have measured the FRET efficiency to be $E^q = 38\%$ for the quenching process and $E^s = 18\%$ for the sensitization process.

We can see good correlation, within the error limits, of the results between the two measurement methods. However, the averaged quenching efficiency, $E^q = 42\% \pm 4\%$, is higher than the averaged sensitization efficiency, $E^s = 21\% \pm 3\%$. This implies a dark quenching process between the donor-acceptor pair along the FRET process that enhances the acceptor fluorescence by only half of the quenching efficiency.

Double labeling on the spore surface. Double labeling on spores has been performed using *B. anthracis* spores with fluorescence-labeled IgG polyclonal antibodies (Fig. 1B). The IgG molecules are labeled in two different ways to generate donor and acceptor molecules. For the donor molecules, we

TABLE 1. FRET experimental results, fluorescence, and flow cytometry for the differently labeled beads

Bead Type	Fluorescence measurements		Flow cytometry measurements	
	Fluorescence intensity (520 nm)	Fluorescence intensity (620 nm)	Mean FL1 intensity	Mean FL3 intensity
Unlabeled beads	$(2 \pm 0.2) \times 10^4$	$(0.9 \pm 0.1) \times 10^4$	1 ± 0.1	1.4 ± 0.1
Donor-labeled beads	$(13 \pm 1) \times 10^4$ ^a	$(0.9 \pm 0.1) \times 10^4$	13 ± 1	3 ± 1
Acceptor-labeled beads	$(2.2 \pm 0.2) \times 10^4$	$(1.9 \pm 0.1) \times 10^4$	1 ± 0.1	20 ± 2
Donor/acceptor-labeled beads	$(7.2 \pm 0.3) \times 10^4$	$(3.2 \pm 0.2) \times 10^4$	8 ± 1	31 ± 3
FRET efficiency (E)	45% $\pm$ 4%	23% $\pm$ 2%	38% $\pm$ 4%	18% $\pm$ 2%

^a Bold values are used for the efficiency (E) calculations.

have used alexa488-IgG, and for the acceptor, we have used alexa594-IgG.

Measuring the density of labeled polyclonal antibody molecules per spore. When using polyclonal antibodies, there is uncertainty concerning the exact proportion of the spore-specific IgG molecules in the overall IgG preparation. This parameter is vital for determination of the number of antibodies per spore. Using an exact concentration of fluorescent isothiocyanate (FITC)-labeled avidin (Sigma no. A-2901) with a known concentration of the biotin-IgG, we could determine the correct concentration of the active IgG antibodies in the polyclonal solution. To do this, we performed a two-step titration, as illustrated in Fig. 5. Figure 5A represents the first titration step, where to a sample of the spores (4×10^7 CFU/ml = 7×10^{-14} M) and an excess of FITC-avidin (300 nM), biotin-IgG is added (0 to 200 nM). The gradual addition of the biotin-IgG results in enhancement of the mean FL1, analyzed by flow cytometry, up to a saturation point. In the control experiment where different spores are being used (*B. thuringiensis* subsp. *israelensis*), the mean FL1 increases only to one-third the values of mean FL1 for to the *B. anthracis* spores. Saturation of the spore fluorescence is defined by the maximum in the derivative curve at 20 nM biotin-IgG. From the saturation point in the titration curve of the *B. anthracis* spores, we have determined the total number of biotin-IgG molecules needed per spore to be $(2.8 \pm 0.2) \times 10^5$.

The second step is the titration of an exact known concentration of FITC-avidin (0 to 150 nM) with respect to the biotin-IgG (25 nM)-labeled spore (4×10^7 CFU/ml) (Fig. 5B). Saturation of the spore fluorescence is defined by the maximum in derivative curve at 30 nM FITC-avidin. From this titration, the number of FITC-avidin molecules per spore has been determined to be $(4.2 \pm 0.5) \times 10^5$. Since we have measured (by the HABA method) that there are four biotins per IgG molecule, we can determine the number of specific biotin-IgG molecules per spore to be $(1.1 \pm 0.2) \times 10^5$. Hence, the fraction of specific IgG molecules out side the polyclonal antibody solution is [(specific biotin-IgG) per (total biotin-IgG)] = $1.1/2.8 = 40\%$. Moreover, from the number of specific biotin-IgG molecules per spore, 1.1×10^5 , we can calculate the free space per antibody on the spore surface (equation 2). The results are area of $3,100 \text{ \AA}^2$ per antibody and a radius of $32 \text{ \AA}$. From this radius, the average distance between neighboring labeled antibodies is estimated to be $64 \text{ \AA}$, enabling the FRET effect to occur. We see that at maximum surface densities ($\sim 10^5$ antibodies/spore), the number of IgG molecules per spore is limited mainly by the protein dimension, where at the recognition site of the Fab the molecular radius is ca. $25 \text{ \AA}$ (12, 13). This implies that the IgG protein structure limits the number of proteins per spore and that there is no protein collapsing on the surface. The maintained protein structure protects its attached fluorophores (two or three per antibody) from self-

FIG. 4. Flow cytometry analysis. FL1 histogram (A) and FL3 histogram (B) of biotin-coated latex microspheres (1.8×10^8 beads/ml) reacted with NA-alex488 (30 nM) (green line), NA-alex594 (80 nM) (pink line); both reagents (blue line), and unstained (orange line).

FIG. 5. Titration curves of *B. anthracis* spores, as measured by calculating the mean FL1 of the stained spores. (A) Titration curves of the spores at 4×10^7 spores/ml + FITC-avidin (80 nM) with IgG-biotin (0 to 200 nM). Curve a, *B. anthracis*; curve b, *B. thuringiensis* subsp. *israelensis*; curve c, first derivative of curve a. (B) Titration curve (a) and first-derivative curve (b) of *B. anthracis* spores (4×10^7 CFU/ml) plus IgG-biotin (25 nM) with FITC-avidin (0 to 400 nM).

aggregation and self-quenching processes. However the proximity ($\sim 64 \text{ \AA}$) between the antibodies should lead to a FRET effect if the right donor-acceptor pair is chosen.

Flow cytometry measurements of FRET on doubly labeled spores. FRET measurements on the *B. anthracis* spores (4×10^8 CFU/ml) have been performed by labeling the spores with both the donor alexa488-IgG (20 nM) and the acceptor alexa594-IgG (40 nM), in contrast to the donor-only-labeled spores and the acceptor-only-labeled spores. By multiplying the spore concentration by the maximum spore site density as measured previously (10^5 IgG molecules per spore), the maximum site concentration is calculated to be 70 nM. Under such conditions, the double labeling would be free of competition between the different labeled antibodies; hence, the effect would be due to FRET, not to any depletion of the labeled antibodies from the spore surface. Figure 6 shows the flow cytometry histogram of the differently labeled spores. The labeled spores sample were analyzed immediately after the stain-

ing procedure (15 min) with no washing steps. Histogram A (Fig. 6A) shows results for the FL1 detector, which follows the donor (alexa488) emission. In this histogram, the acceptor-labeled spores (pink line) have no significant fluorescence. The donor-labeled spores (green line) are highly fluorescent; in the doubly labeled spores (blue line) the mean fluorescence (FL1) decreases. Histogram B (Fig. 6B) shows results for the FL3 detector, which follows the acceptor (alexa594) emission. In this histogram, the donor-labeled spores (green line) have low fluorescence signal. The acceptor-labeled spores (pink line) are moderately fluorescent, while in the doubly labeled spores (blue line), the mean fluorescence (FL3) increases. The main effect of the donor quenching and the acceptor enhancement indicates that the FRET effect has been recorded by the flow cytometry. The average mean fluorescence intensities, based on (5) sample repetition, are summarized in Table 2. These values are used for the FRET efficiency calculation using equations 4 and 5, as shown for the bead experiment. Efficiencies

FIG. 6. Flow cytometry analysis. FL1 histogram (A) and FL3 histogram (B) of *B. anthracis* spores (4×10^8 CFU/ml) reacted with IgG-alexa488 (20 nM) (green line) and IgG-alexa594 (40 nM) (pink line), doubly stained (blue line), and unstained (orange line).

TABLE 2. FRET experimental results by flow cytometry analysis for the differently labeled *B. anthracis* spores

Spore type	Flow cytometry measurements ^a	
	Mean FL1 intensity	Mean FL3 intensity
Nonlabeled spores	1 ± 0.1	1.5 ± 0.1
Donor-labeled spores	54 ± 4	5 ± 0.2
Acceptor-labeled spores	1 ± 0.1	10 ± 2
Donor/acceptor-labeled spores	35 ± 3	21 ± 2
FRET efficiency (<i>E</i>)	<i>E</i> ^q = 35% ± 4% <i>E</i> ^s = 22% ± 2%	

^a Bold values are used for the efficiency (*E*) calculations.

are *E*^q = 35% for the quenching process and *E*^s = 22% for the sensitization process. The quenching efficiency is greater than the sensitization efficiency by ~1.5, which implies a dark quenching process, as discussed for the bead results.

Selective diagnosis of *B. anthracis* spores. The selective analysis of *B. anthracis* spores is based on the flow cytometry analysis and the FRET effect achieved by the double-label immunofluorescence method. Figure 7A' show the light scatter analysis dot plot for *B. anthracis*. The dot plot seems noisy due to the presence of debris and some spore aggregates. However, the subpopulation of the spores is clear in the three-dimensional presentation of the dot plot shown in Fig. 7A". Nevertheless, this light-scatter-based gate (R1) cannot be used as a selective gate for the *B. anthracis* spores but can be used only as primary filter for the targets spores. To achieve a specific diagnostic method, one must add the immunofluorescence staining. Figure 7A(I), B(I), and C(I) show the FL1/FL3 dot plots, after initial FSC/SSC gating on the *B. anthracis* spore cluster region of the target spores *B. anthracis* and the control spores *B. thuringiensis* subsp. *israelensis* and *B. subtilis*, respectively, at the same concentration, labeled with the polyclonal fluorescent antibodies alexa488-IgG and alexa594-IgG. While analyzing the labeled sample of *B. anthracis* spores 10,000 events were collected, gate R4 was set to contain 8,000 events, which by their parameters (*FL1* and *FL3*) were consider and positive for *B. anthracis* spores. For the control spores under the same gate conditions, concentration, and number of events collected, only 4 or 5 events are collected. Hence, we can evaluate the false-positive events in such analysis procedure to be only 0.06% for *B. thuringiensis* subsp. *israelensis* and *B. subtilis*.

For labeling only with alexa488-IgG [Fig. 7A(II), B(II), and C(II)], gate R2 (SSC/FL1) is also set to include 8,000 *B. anthracis* spores. Analyzing the control spores using the same staining and gating procedure, one can observe that there are still 5% *B. thuringiensis* subsp. *israelensis* and 0.3% *B. subtilis* spores. Comparing the number of unwanted events that have entered the positive gate of the donor-only (alexa488-IgG)-labeled spores (gate R2) to the number that have entered the gate for doubly labeled spores (gate R4), it is clear that the selectivity has improved by a factor of 80 (5%/0.06%) for the less genetically related spores (*B. subtilis*) and 5 (0.3%/0.06%) for the species genetically related to *B. anthracis* (*B. thuringiensis* subsp. *israelensis*). The FRET effect, by enhancing the acceptor (alexa594-IgG) emission, is clearly the main reason for the improvement.

Labeling the spores only with the alexa594-labeled antibodies (alexa594-NA/biotin-IgG or alexa594-IgG) [Fig. 7A(III), B(III), and C(III)] resulted in a poor selective gate R3 (SSC/FL-3) for the *B. anthracis* spores. In these experiments, the gate was also set up to include 8000 BA spores, but 50 to 60% the unwanted spores were found at the same gate when *B. thuringiensis* subsp. *israelensis* and *B. subtilis* spores were analyzed. The selective gate for the *B. anthracis* spores based on the FRET effect (FL1/FL3) dramatically improved the selectivity of the spores compare to the gate based on the alexa594-labeled antibodies alone. The selectivity improvement in this case is ~1,000 fold for *B. thuringiensis* subsp. *israelensis* and *B. subtilis*. The main reason is that the acceptor conjugate (alexa594-IgG) is poorly selective in the flow cytometry analysis, mainly due to its weak direct excitation at 488 nm.

The FRET effect achieved on the *B. anthracis* spore surface has resulted in doubly labeled spores with significant FL1 and FL3 signal. As shown in Fig. 7A(I) for gate R4, this effect has been used for the development of a selective gate for the *B. anthracis* spores based on the donor emission (FL1 detector) and the acceptor emission (FL3 detector). The FRET effect contributes to the enhancement of the FL3 signal, while the quenching in the FL1 signal is only 35%, enabling the creation of a selective gate for the labeled *B. anthracis* spores under the FL1/FL3 dot plot. Without the FRET effect, the acceptor fluorescence (*FL3*) would be too low to permit such a gate, since the acceptor (alexa594) absorption at the FACSCalibur excitation (laser argon, 488 nm) is less than 10% of its absorption at 594 nm or the donor absorption at 488 nm. Indeed, we observed that the signal-to-noise ratio in the FL3 gate is less than 2 when we compared the mean *FL3* of the acceptor-labeled *B. anthracis* spores to that of the control spores [*B. thuringiensis* subsp. *israelensis*, Fig. 7A(III), B(III)]. Due to the FRET effect, the signal to noise ratio at the mean FL3 is more than 4 and Figure 7(AI or BI). This is because the site coverage density on the *B. anthracis* spore surface is high enough to allow the FRET effect, thus enhancing the acceptor emission.

DISCUSSION

This work has implemented the titration method by using flow-cytometry analysis to quantitate fluorescence-labeled proteins on a particle surface. We have measured and calculated the number of NA molecules per 1-μm latex bead to an average value of 10⁶ ligands per bead. Such ligand compactness leads to an average distance between neighboring NA molecules of 22 Å, which is within the FRET *R*₀ (Förster radius) value of the nonconjugated alexa488 and alexa594 dyes. The resulting compactness also implies that the protein structure of avidin is maintained on the bead surface and the ligand density is inhibited mainly by the protein structure. Hence, with two or three fluorophores per protein and with the shielding of the protein matrix, it can be assumed that on the surface there is no fluorophore aggregation that might lead to self-quenching.

The FRET effect, measured by flow cytometry and fluorescence spectroscopy, is observed by comparing the fluorescence signals of the donor-labeled, acceptor-labeled, and doubly labeled beads. In the doubly-labeled beads, one can observe quenching of the donor emission and enhancement of the acceptor emission. This is different from self-quenching or

FIG. 7. Flow cytometry dot plots of samples (4×10^8 CFU/ml) of *B. anthracis*, *B. thuringiensis* subsp. *israelensis*, and *B. subtilis* after several staining processes. (A') Light-scatter parameter dot plots of unstained *B. anthracis* spores. (A'') Three-dimensional presentation of panel A'. (A to C) Flow cytometry dot plots of immunostained *B. anthracis* (A), *B. thuringiensis* subsp. *israelensis* (B), and *B. subtilis* (C) spores; 10,000 events were collected. The presented dot plots (A to C) are the subset of the FSC/SSC gate related to the *B. anthracis* spore cluster. Entry (I), double labeling of the spores by using IgG-alexa488 (20 nM) plus IgG-alexa594 (40 nM). Entry (II), single labeling of the spores by the donor using IgG-alexa488 (20 nM). Entry (III), single labeling of the spores by the acceptor using IgG-alexa594 (40 nM).

screening effects in two-color analysis, where no enhancement is observed. The FRET efficiency was calculated by the quenching process, $E^q = 42\% \pm 4\%$, and by the acceptor fluorescent enhancement, $E^s = 21\% \pm 2\%$. The finding that the enhancement is smaller than the quenching process implies a dark quenching process, such as energy transfer into the form of vibration of the surrounding protein structure. The reproducibility of the FRET efficiency value measured by flow cytometry and fluorescence suggests the use of highly stable reagents (avidin-biotin) and a highly efficient donor-acceptor pair (alexa488 and alexa594).

The titration method has been modified to a double titration step to measure and calculate the number of the polyclonal IgG molecules per *B. anthracis* spore. This was successfully achieved and results in the finding of 10^5 IgG molecules per spore, with an average distance of 64 Å between neighboring antibodies. The average distance of 64 Å implies that the conjugate structure is preserved after adsorption to the spore surface. Such configuration avoids fluorophore aggregation and self-quenching and permits FRET only under the distance limitation between the donor-acceptor conjugates. Flow cytometry measurements of the doubly labeled *B. anthracis* spores (alexa488-IgG and alexa594-IgG) revealed the FRET effect with efficiency of $E^q = 35\% \pm 4\%$ for the quenching and $E^s = 22\% \pm 2\%$ for the sensitization processes, implying that there are dark quenching processes, as mentioned for the bead-NA system. In previous work by Batard et al. (2), a FRET efficiency of 10% was measured; the FRET pair used consisted of protein embedded dyes, phycoerythrin and allophycocyanin, tagged on antibodies. In our study, the higher efficiency (22%) was obtained because the fluorophores used are small organic molecules tethered to the protein matrix by flexible linker and there are two-three dye molecules per protein. In such a configuration, the probability of having a donor-acceptor pair from neighboring conjugates, at both the right distance and orientation, is increased. Comparing our FRET results for the beads to the spore system, one can observe that the resulting efficiencies are similar although the average distances found on each particle surface are different. This can be explained by several factors. The distance measurements are an average between the proteins on the surface. The actual FRET effects might occur only in certain positions where the distance and dipole orientation allow it. In each case, the labeled protein is different; this can affect mainly on the dark quenching process or the resulting dipole orientation of the dye. Hence, the two systems cannot be studied as distance-dependent FRET since the other parameters are not fixed.

The FRET effect is used for the development of highly selective gate for the immunodiagnosis of *B. anthracis* spores by flow cytometry. This has been achieved due to the significant FL3 signal, enhanced by the FRET effects, that enables us to create an isolated FL1/FL3 gate for *B. anthracis* spores versus different spores such as *B. thuringiensis* subsp. *israelensis* and *B. subtilis*.

The data show a 5- to 80-fold reduction of background noise from the control spores by using FRET immunolabeling compared to donor-only (alexa488-IgG) labeling of the spores, and ca. 1,000-fold reduction compared to acceptor-only (alexa594-IgG) labeling. Alexa594-IgG by itself shows poor selectivity. The main reason for this effect is its weak excitation (10% of its

absorption coefficient at 594 nm) at the flow cytometry light source (488 nm). Under such condition, without any FRET enhancement of its emission, the conjugate signal is difficult to resolve from the background. Using two different IgG molecules against different determinants of the spore might result in more significant improvement of the selectivity. This can be achieved by further developing monoclonal antibodies against different spore epitopes of *B. anthracis* (23). Such reagents, labeled with a donor-acceptor pair, may produce a even more isolated gate for the target spores.

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