

UC Riverside

UC Riverside Electronic Theses and Dissertations

Title

Fluorescence-Enhancement Based Detection and Identification of Bacterial Contaminants

Permalink

<https://escholarship.org/uc/item/3pk05429>

Author

Upadhyayula, Srigokul

Publication Date

2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Fluorescence-Enhancement Based Detection and Identification of Bacterial Contaminants

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Bioengineering

by

Srigokul Upadhyayula

December 2012

Thesis Committee:

Dr. Valentine Vullev, Chairperson

Dr. Dimitrios Morikis

Dr. Hyle Park

Copyright by
Srigokul Upadhyayula
2012

The Thesis of Srigokul Upadhyayula is approved by:

Committee Chairperson

University of California, Riverside

Acknowledgements

I am extremely grateful to Dr. Valentine Vullev for providing me with the opportunity to join his lab as a graduate student, for imparting valuable knowledge & experience, and for his patience & advise. His mentorship to continually develop my abilities, his push to believe in my dreams, enabled me with the drive to reach my aspirations. His dedication, investment, and his expectations for excellence towards his graduate students leave me with a sense of gratitude that can only be expressed in the ways I have used and will use his teachings to further myself and the field as a whole. Thank you for being the kind of mentor that every graduate student prays for!

много благодаря!

I would like to thank Dr. Bahman Anvari, and his group members, for allowing me to use his lab's microscope and camera to produce amazing pictures of vegetative bacteria and endospores. I am thankful to Dr. Boris Hyle Park for helpful discussions on image analysis, for his advise as my graduate advisor, and for allowing me to attend and learn from his group's optics related meetings. I could not have accomplished any of this without the support of VullevGroup members. I would like to thank Vicente Nuñez for detailed discussions on an array of microbiology topics. I am grateful to Jaclyn Lock for inspiring and motivating me when I needed it the most.

I would like to thank the Dr. Marlon Thomas and Dr. Bing Xia for granting me permission to reprint the published journal articles. I would also like to acknowledge the undergraduate and graduate coauthors for their contributions to the chapters -- For chapter 1: Marlon Thomas, Vicente Nuñez, Elizabeth Zielins, Duoduo Bao, Jacob

Vasquez, and Baharak Bahmani; For chapter 2: Vicente Nuñez, Pavel Landsman, Samuel Lam, Harbani Malik, Sharad Gupta, Mohammad Sarshar, and Jingqiu Hu; For chapter 3: Samuel Lam, Alice Ha, and Harbani Malik; For chapter 4: Vicente Nuñez, Adam Lin, and Kenny Chau. This work would not have been as much fun with out all of you! Thank you!

I would like to thank and dedicate this thesis to:



My parents: Sridevi & Suryaprakash Upadhyayula

My brothers and Sister: Sriteja, Saikrishna & Sripriya Upadhyayula;

My uncle and aunt: Ramesh & Sujatha Upadhyayula;

My grandparents: Late Krishnamurthy Upadhyayula and Late VenkataRao Vadlamani (grandfathers), Lakshmi Upadhyayula and Lakshmibai Vadlamani (grandmothers);
and to:

My friends and family

For their unwavering love, support and confidence.

ABSTRACT OF THE THESIS

Fluorescence-Enhancement Based Detection and Identification of Bacterial Contaminants

by

Srigokul Upadhyayula

Master of Science, Graduate Program in Bioengineering
University of California, Riverside, December 2012
Dr. Valentine Vullev, Chairperson

Due to the considerable challenges for reliable and expedient detection of virulent vegetative bacterial, bacterial endospores and other hazardous biological agents, accidental exposure is a principal cause of fatality due to wound infection or slow diagnosis. Staining techniques, due to their simplicity, form a set of important tools for pathogen monitoring and control. The staining assays, such as immunoassays, however, provide only Boolean information: i.e., stain vs. does not stain. As a result, only the species being sought may have a chance to be identified, leaving key determining factors for the diagnosis quite susceptible to human error.

Our principle motivation for this project is to expand the current analytical techniques beyond their Boolean nature, and to address the apparent need for simple, expedient and inexpensive assays. We describe the development of an assay system based on the dynamics of fluorophore uptake that will serve as a robust platform for

detection of both vegetative and endospore bacterial contaminants. The kinetics of fluorescence staining depends on the coatings and protein content, which reflect the species phenotype. Hence, such kinetic measurements gain access to genetic information, in less than a minute, without conducting amplification procedures such as PCR.

We have demonstrated *for the first time* that the kinetics of emission enhancement, caused by cell uptake of fluorophores, provides statistically significant discernibility between different and closely related vegetative bacteria and bacterial endospore species respectively. We observed that the time course of the fluorescence signal provides a unique species-specific signature that can prove indispensable for the identification of dangerous and life-threatening spores.

Contents

Acknowledgements	<i>iv</i>
Dedication	<i>vi</i>
Abstract	<i>vii</i>
Contents	<i>ix</i>
List of Schemes	<i>xii</i>
List of Tables	<i>xiii</i>
List of Figures	<i>xiv</i>
Chapter 1	<i>p 1</i>
Chapter 2	<i>p 47</i>
Chapter 3	<i>p 101</i>
Chapter 4	<i>p 111</i>
<i>Curriculum vitae</i>	<i>p 128</i>

Chapter 1	<i>p 1</i>
------------------	------------

Kinetics of Bacterial Fluorescence Staining with 3,3'-Diethylthiacyanine

1.1	Introduction	<i>p 2</i>
1.2	Results & Discussion	<i>p 4</i>
1.2.1	Interaction between bacterial cells and THIA	<i>p 4</i>
1.2.2	Solvent-dependence of the spectral properties of THIA	<i>p 7</i>
1.2.3	Dynamics of bacterium-induced spectral changes	<i>p 9</i>
1.2.4	The effects of TWEEN® on the behavior of THIA	<i>p 12</i>
1.2.5	Kinetics of fluorescence enhancement for different species	<i>p 13</i>
1.2.6	Diffusion-driven dye uptake	<i>p 16</i>
1.3	Conclusions	<i>p 20</i>
1.4	Experimental	<i>p 21</i>
1.5	References	<i>p 25</i>
1.6	Schemes	<i>p 33</i>
1.7	Tables	<i>p 35</i>
1.8	Figures	<i>p 38</i>

Chapter 2 p 47

Amyloid Histology Stain for Rapid Bacterial Endospore Imaging

2.1	Introduction	p 49
2.2	Results	p 54
2.2.1	Staining of bacterial endospores with ThT	p 54
2.2.2	Spore-induced emission enhancement of ThT	p 56
2.2.3	Binding affinity of ThT for endospores	p 59
2.2.4	Imaging in the presence of contaminants	p 61
2.2.5	Signal to Noise of staining of bacterial endospores with ThT	p 62
2.2.6	Signal to Noise analysis of staining with contaminants	p 63
2.3	Discussion	p 64
2.4	Conclusions	p 69
2.5	Experimental	p 70
2.6	References	p 79
2.7	Schemes	p 89
2.8	Figures	p 90

Chapter 3 p 101

Dynamic Staining of Bacillus Endospores with Thioflavin T

3.1	Introduction	p 102
3.2	Results & Discussion	p 103
3.3	Conclusions	p 106
3.4	Experimental	p 107
3.5	References	p 108
3.6	Figures	p 109

Chapter 4 p 111

Dynamic Staining of Bacteria at a Single-Cell Level

4.1	Introduction	p 112
4.2	Results	p 115
4.2.1	DS of <i>E. coli</i> with 3,3'-diethylthiacyanine	p 115
4.2.2	DS of <i>P. aeruginosa</i> with 3,3'-diethylthiacyanine	p 116
4.2.3	Single-cell dynamic staining of <i>E. coli</i>	p 116
4.3	Conclusions	p 117
4.4	Experimental	p 118

4.5	References	<i>p 121</i>
4.6	Tables	<i>p 123</i>
4.7	Figures	<i>p 125</i>

List of Schemes

CHAPTER 1:

Scheme 1

p 33

Structural formula of 3,3'-diethylthiacyanine (THIA).

Scheme 2

p 34

Fluorescence enhancement due to migration of THIA from the aqueous surrounding into the fluorogenic cell-wall media.

CHAPTER 2:

Scheme 3

p 89

Thioflavin T (ThT).

List of Tables

CHAPTER 1:

Table 1 p 35
Fluorescence quantum yield of THIA, Φ_f , for solvents with different dielectric constants, ϵ , and different dynamic viscosity, η .^a

Table 2 p 36
Time constants, τ / s (represented in bold), for the enhancement of the fluorescence of THIA induced by four vegetative bacteria for different dye concentration, C_{THIA} , and cell count, CC , in the presence of TWEEN[®] surfactant.^a

Table 3 p 37
Results from one-factor ANOVA analysis comparing between the time constants obtained for the four investigated species (Table 2).^a

CHAPTER 4:

Table 4 p 123
Ensemble measurements of time constants for the fluorescence enhancement of THIA upon staining of *E. coli* cells at varying dye and cell concentrations.

Table 5 p 124
Time constants for *P. aeruginosa*, *B. sphaericus*, *B. subtilis* and *E. coli*. Cell concentrations are 2×10^7 cell / ml for *P. aeruginosa* and 5×10^7 cells / ml for the other species.

List of Figures

CHAPTER 1:

Figure 1

p 38

Fluorescence image of *E. coli* in 60 μM THIA solution, recorded with a confocal microscope ($\lambda_{\text{ex}} = 364 \text{ nm}$). The bacterial suspension was deposited on aminated glass slide with the THIA solution (60 μM), and allowed to incubate for 10 minutes prior the imaging.

Figure 2

p 39

Absorption and fluorescence of THIA (6 μM) in the presence and absence of bacterial cells (cell count, $CC = 5 \times 10^7 \text{ cell ml}^{-1}$). All samples were prepared with 2 mM aqueous Tris buffer, pH = 8.5, containing 0.5 mM TWEEN[®] 40. The spectra in the presence of bacteria were recorded after 15 min incubation of the cell suspension with THIA. (a) Absorption spectra. Baseline corrections, using cell suspensions with no dye, were applied. (b) Emission spectra ($\lambda_{\text{ex}} = 420 \text{ nm}$).

Figure 3

p 40

Interaction between THIA and carboxylate-terminated polystyrene beads (5 μm diameter). (a) Kinetic curve ($\lambda_{\text{ex}} = 420 \text{ nm}$, $\lambda_{\text{em}} = 480 \text{ nm}$) and emission spectrum ($\lambda_{\text{ex}} = 420 \text{ nm}$) of THIA (6 μM) in the presence of the beads ($1.5 \times 10^7 \text{ beads ml}^{-1}$). (b) Phase-contrast image of the beads immersed in THIA solution (600 μM). (c) Fluorescence image of the beads immersed in THIA solution (600 μM ; $\lambda_{\text{ex}} = 364 \text{ nm}$).

Figure 4

p 41

Absorption and emission spectra of THIA (6 μM) for water and different alcohols: (a) absorption spectra; (b) emission spectra ($\lambda_{\text{ex}} = 400 \text{ nm}$). Inset represents the same spectra plotted against logarithmic ordinate.

Figure 5

p 42

Kinetics of changes induced on the absorption and emission of THIA (6 μM) by *E. coli* ($2 \times 10^7 \text{ cell ml}^{-1}$) for aqueous media buffered with 2 mM Tris buffer, pH = 8.5, with no TWEEN[®] added, at 37 °C. (a) Top curve: normalized absorption change, monitored at 420 nm ($A_{\infty} = A_0 - \Delta A$, equation 1b). Middle curve: normalized fluorescence change, monitored at 480 nm ($\lambda_{\text{ex}} = 420 \text{ nm}$; equation 1a). Bottom curve: normalized fluorescence change, monitored at 600 nm ($\lambda_{\text{ex}} = 420 \text{ nm}$; equation 1a). (b) The same spectral changes plotted against logarithmic ordinate. The normalized emission rise features were inverted and represented as decays in order to discern the exponential components with different time constants ($F_{\infty} = F_0 + DF$, equation 1a).

Figure 6*p 43*

Spectral properties of THIA in the presence and absence of TWEEN[®]. **(a)** Absorption and emission spectra of THIA (5 mM; $\lambda_{\text{ex}} = 420$ nm) in the absence and presence of TWEEN[®] 40 (27 mM). **(b)** Kinetics of the THIA emission enhancement (6 mM; $\lambda_{\text{ex}} = 420$ nm; $\lambda_{\text{em}} = 480$ nm) induced by *E. coli* (5×10^7 cell ml⁻¹) in the absence and presence of TWEEN[®] 40 (0.5 mM). The blue markers, ×, represent the data points; the red solid lines, —, represent the corresponding exponential fits (equation 1a).

Figure 7*p 44*

Fluorescence-enhancement kinetic curves ($\lambda_{\text{ex}} = 420$ nm, $\lambda_{\text{em}} = 480$ nm, 37 °C) for *bacilli* samples injected into 6 mM THIA solution (at $t_0 \approx 5$ s, equation 1a). All samples were prepared with 2 mM aqueous Tris buffer, pH = 8.5, containing 0.5 mM TWEEN[®] 40. **(a)** Kinetic curve for 5×10^7 cell ml⁻¹ *B. sphaericus* (top and middle curves) and *B. subtilis* (bottom curve). (The fast rise prior to the slow intensity increase, asymptotically approaching the maximum fluorescence intensity, was characteristic for pretreatment with TWEEN[®] surfactant of *B. sphaericus*, *E. coli* and *E. aerogenes*.) For *B. sphaericus*, the monoexponential analysis yielded a time constant almost identical to the time constant corresponding to the slow component of the biexponential rise. **(b)** Kinetic curves for different cell counts of *B. subtilis* with the corresponding monoexponential fits ($n = 1$, equation 1). The error values represent the standard deviations yielded by the least square algorithms for each data fit.

Figure 8*p 45*

Simulated curves for fluorescence enhancement (equation 2 and 3) resultant from the diffusion-driven migration of weakly-fluorescent dye into the fluorogenic environment of cell walls (Scheme 2). The blue squares represent the calculated values for different cell counts, different cell-wall thickness, L , and different diffusion coefficients, D . The red solid lines represent the corresponding monoexponential data fits ($n = 1$, equation 1a) that produced the different time constants, t . For all calculations, $F_C = 0.2$, $F_W = 0.001$, $K = 2 \times 10^4$, $t_0 = 3$ s, $V_C = 1$ fl, $S_C = 6$ mm², and the number of exponential terms for the sum of (equation 2) was 10^3 .

CHAPTER 2:**Figure 9***p 90*

Epifluorescence microscopy images of bacterial endospores, **(a, b)** *B. atrophaeus* and **(c)** *G. stearothermophilus*. All endospore samples were imaged while suspended in 150 μM ThT aqueous solution (2 mM Tris buffer, pH 8.5, *exposure time* = 5 s). The spores were incubated in the ThT solution for 10 min prior to imaging.

Figure 10*p 91*

Gray-scale analysis ⁶⁵ of epifluorescence microscopy images of *B. atrophaeus* endospores suspended in solutions with various concentrations of ThT (2 mM Tris buffer,

pH 8.5). **(a, b)** Fluorescence images (*exposure time* = 5 s): **(a)** in the presence of 1 μM ThT and **(b)** with no dye present. **(c, d)** Traces from images of spores suspended in different concentrations of ThT recorded at *exposure time* = **(c)** 1 s, and **(d)** 5 s. **(e, f)** The same traces plotted against expanded ordinates, demonstrating the differences between the baselines of the images with and without dye, ΔI . ΔI represents the contribution of the fluorescence from the free ThT to the backgrounds of the microscope images, A_{DC} . (ΔI = 490, 240, 60 and <20 for 100 μM , 25 μM , 5 μM and 1 μM ThT at *exposure time* = 1 s; and ΔI = 4,100, 3,300, 460 and <50 for 100 μM , 25 μM , 5 μM and 1 μM ThT at *exposure time* = 5 s.) The peak signals corresponding to the spores labeled with ThT (i.e., to the dye taken up by the spores) exceed the background fluorescence, ΔI , by more than an order of magnitude: i.e., $(\langle\Delta h\rangle + \Delta I) / \Delta I > 10$. For 1-s *exposure time*, the baseline *RMS* = 35, 24, 32, 25 and 35 for 0 μM , 1 μM , 5 μM , 25 μM and 100 μM ThT; and for 5-s *exposure time*, *RMS* = 75, 65, 92, 96 and 120 for 0 μM , 1 μM , 5 μM , 25 μM and 100 μM ThT.

Figure 11

p 93

Epifluorescence microscopy images of: **(a)** *B. globigii* endospores; **(b)** *B. subtilis* endospores; **(c)** *B. subtilis* vegetative cells; **(d)** *B. subtilis* during sporulation; and **(e, f)** *E. coli* cells. All bacterial samples were imaged while suspended in 150 μM ThT aqueous solution (2 mM Tris buffer, pH 8.5). The bacteria were incubated in the ThT solution for 10 min prior to imaging.

Figure 12

p 94

Absorption and fluorescence properties of ThT (11 μM) dissolved in aqueous solutions (2 mM Tris buffer, pH 8.5, absorption and excitation optical path length = 0.5 cm) in the absence and the presence of different amounts bacterial endospores (*SC* = spore count): **(a)** absorption and emission ($\lambda_{\text{ex}} = 430$ nm) spectra of ThT samples containing *B. atrophaeus* with different *SC* (for *SC* exceeding 10^7 spore ml^{-1} the baseline of the absorption spectra in the blue and UV region shifts due to scattering); **(b)** dependence of the absorbance ($\lambda_{\text{abs}} = 415$ nm) and emission intensity ($\lambda_{\text{ex}} = 415$ nm; $\lambda_{\text{em}} = 490$ nm) on the spore count; and **(c)** emission spectra of ThT in the presence and absence of *B. globigii* endospores ($\lambda_{\text{ex}} = 430$ nm).

Figure 13

p 95

Fluorescence properties of ThT (1 μM) dissolved in aqueous solution (4 mM glycine buffer, pH 9.0) in the presence of different amounts of *B. subtilis* endospores: **(a)** excitation ($\lambda_{\text{em}} = 480$ nm) and emission ($\lambda_{\text{ex}} = 430$ nm) spectra; and **(b, c)** dependence of the emission intensity ($\lambda_{\text{ex}} = 430$ nm) on the spore count (*SC*). The circles represent the emission intensity at 480 nm, and the dotted lines are data fits. The residuals from the data fits are plotted against a separate ordinate above the data. **(b)** Linear analysis of the fluorescence dependence on the spore count: $F(SC) = F_0 + \Delta F SC$. **(c)** Nonlinear analysis employing a power-law function (equation 6) to fit the fluorescence dependence on *SC*. The appropriateness of the function used for data fits was examined using the Durbin-

Watson test statistics: $DW = \frac{\sum_{i=2}^n (\delta_i - \delta_{i-1})^2}{\sum_{i=1}^n \delta_i^2}$, where δ_i are the residual values at data points i , and n is the total number of data points.

Figure 14

p 96

Distribution coefficient, K_D (equation 7), of ThT for endospores of *B. globigii* and *B. thuringiensis*. **(a)** Absorption spectra of ThT dissolved in aqueous media (2 mM Tris buffer, pH 8.5, optical path length = 0.5 cm). The dotted line represents the spectrum of ThT recorded **before** any treatment ($C_{\text{ThT}}^{(0)} = 35 \mu\text{M}$). The solid line corresponds to a spectrum of the supernatant **after** the dye solution was incubated with *B. globigii* endospores (8×10^7 spores ml^{-1}) for 10 min and the spores were removed using centrifugation. The decrease in the absorbance corresponds to an $8\text{-}\mu\text{M}$ decrease in the ThT concentration: i.e., the concentration of ThT, which remained free in the solution after the spore uptake, was $28 \pm 2 \mu\text{M}$. (The error bar represents $\pm$ one single standard deviation.) Considering that the volume of a single *B. globigii* spore is about 8×10^{-13} ml, the average ThT concentration in the stained spores was 0.11 M, yielding distribution coefficient $K_D = 3.9 \times 10^3 \pm 0.4 \times 10^3$. **(b)** Contour plot depicting the dependence of ThT K_D for *B. thuringiensis* endospores on the initial dye concentration, $C_{\text{ThT}}^{(0)}$, and on the spore count, SC . The lines represent $K_D(C_{\text{ThT}}^{(0)}, SC)$ with the identical values as designated by the corresponding numbers tagged to the lines.

Figure 15

p 97

Imaging of endospores of *B. atrophaeus* suspended in solution of biological and environmental contaminants: **(a-e)** 1 mg ml^{-1} DNA extract from *E. coli*; **(f-j)** 50% v/v fetal bovine serum (FBS); and **(k-o)** 1 mg ml^{-1} humic acid (HA); dissolved in aqueous buffer (2 mM Tris buffer, pH 8.5; *exposure time* = 0.5 s). The ThT concentration for the stained samples **(c-e, h-j, and m-o)** was 150 μM . The heterogeneity and the pronounced fluorescence of HA (overlapping with the ThT fluorescence range) presented challenges for imaging of the added endospores. We do not have a proof if any of the structures depicted on the bright-field images **(k)** belong to the suspended spores. Staining the sample with ThT, however, allowed for visualization of the spores **(o)** and focusing the objective on them, provided means for observing them even in a bright-field mode **(n)**. Such fluorescence structures were not present in the HA samples without endospores **(m)**.

Figure 16

p 98

Solid cultures of *B. atrophaeus* plated from endospore suspensions (10^6 spore ml^{-1} , 2 mM Tris buffer, pH 8.5) in 7.5-cm Petri dishes. The endospores were suspended in a solution of **(a)** 0 μM , **(b)** 100 μM and **(c)** 3 mM ThT for > 10 min prior to plating. Similar plating with endospore-free buffer solutions containing: **(d)** no ThT **(e)** 100 μM and **(f)** 3 mM, did not produce detectable colonies.

Figure 17*p 99*

Signal-to-noise analysis of epifluorescence microscopy image of *B. atrophaeus* endospores suspended in a 100 μM solution of ThT (2 mM Tris buffer, pH 8.5). **(a)** Microscopy image with corresponding horizontal and vertical traces crossing through the fluorescent spores (*exposure time* = 4 s). **(b)** Designation of trace parameters: $A_{DC} = 7.7 \times 10^3$; $\langle h \rangle = 3.2 \times 10^4$; $\langle \Delta h \rangle = 2.4 \times 10^4$. **(c)** The trace baseline with average value of A_{DC} and $RMS = 90$. The data for the trace on **(b)** and **(c)** yield $S/N_{AC} = 69$ dB, and $S/N_{DC} = 12$ dB (equation 6).

Figure 18*p 100*

Dependence of signal-to-noise ratios, S/N , on exposure time and on dye concentration, C_{ThT} , for epifluorescence images of *B. atrophaeus* suspended in ThT solution (2 mM Tris buffer, pH 8.5). **(a)** Dependence of the amplitude of the signal, $\langle h \rangle$, and the amplitudes of the DC and AC noise, A_{DC} and RMS , respectively, on the exposure time for $C_{\text{ThT}} = 100$ μM . **(b)** Dependence of the AC and DC noise on the exposure time for $C_{\text{ThT}} = 100$ μM . **(c)** Dependence of the AC and DC noise on the ThT concentration for *exposure time* = 0.5 s and 5 s.

CHAPTER 3:**Figure 19***p 109*

Fluorescence staining of endospores of *B. atrophaeus* and *B. thuringiensis* with ThT. **(a, b)** Epifluorescence microscope images of endospores of **(a)** *B. atrophaeus* and **(b)** *B. thuringiensis* stained by mixing with a ThT aqueous solution ($C_{\text{ThT}} = 1$ $\mu\text{mol l}^{-1}$; 2 mM Tris buffer, pH 8.5, with 1 mM TWEEN[®] 40; $\lambda_{\text{ex}} = 482$ nm, $\lambda_{\text{em}} = 536$ nm, the band widths of the imaging filters were 25 nm; scale bar corresponds to 3 μm). **(c)** Kinetics of the enhancement of the emission of 1 $\mu\text{mol l}^{-1}$ ThT upon mixing with endospores, 1.5×10^5 spores ml^{-1} , at time t_0 , with the monoexponential data fits (2 mM Tris buffer, pH 8.5, with 1 mM TWEEN[®] 40; $\lambda_{\text{ex}} = 430$ nm, $\lambda_{\text{em}} = 480$ nm). Category plots of: **(d)** static, ΔF , and **(e)** dynamic, τ , characteristics of staining of *B. atrophaeus* and *B. thuringiensis* with ThT. The time constants, τ , and the change in the fluorescence intensity, ΔF , were extracted from least-square data fits of kinetic emission data using Equation 11 for different dye contraptions, C_{ThT} , and endospore densities of: A = 7.5×10^4 spores ml^{-1} , and B = 15×10^4 spores ml^{-1} . **(d, e)** The average values and standard deviations of τ and ΔF shown in the category plots were obtained from four repeats. ΔF values represent the maximum difference between the average final emission intensity (~ 120 s after the addition of endospores) and the average initial emission intensity (before the addition of endospores).

CHAPTER 4:

Figure 20

p 125

Fluorescence-enhancement kinetic curves ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 475$ nm) for THIA (6 μ M in 2 mM TRIS buffer, pH = 8.5) recorded for suspensions of three different bacterial vegetative cells. The solid lines represent the monoexponential data fits.

Figure 21

p 126

a) Fluorescence spectra of 6 μ M THIA versus 6 μ M THIA with 2×10^7 *P. aeruginosa* cells. b) DS of *P. aeruginosa* cells with 6 μ M THIA.

Figure 22

p 127

Sequential images showing the DS of single *E. coli* cells trapped on aminated surfaces, and stained with 100 μ M ThT. a) no dye present; b) imaged 1.7 s after 100 μ M ThT introduced; c) imaged 1.8 s after ThT addition; start of DS; d-i) imaged after ThT addition 2.0, 2.1, 3.7, 7.1, 13.3, 33.2 s respectively. The scale bar on the bottom left corresponds to 2 μ m.

Figure 23

p 128

Dynamic staining of individual *E. coli* cells based on the fluorescence intensity signal. a) comparison between intensity kinetics of background and single *E. coli* cell after the addition of 100 μ M ThT extracted from image sequence shown in figure 22. b) time constants derived for multiple single *E. coli* cells after addition of 100 μ M ThT; data plotted from multiple experiment image sets.

Chapter 1

Kinetics of Bacterial Fluorescence Staining with 3,3'-Diethylthiacyanine

ABSTRACT

For more than a century, colorimetric and fluorescence staining has been the foundation of a broad range of key bioanalytical techniques. The dynamics of such staining processes, however, still remains largely unexplored. We investigated the kinetics of fluorescence staining of two Gram-negative and two Gram-positive species with 3,3'-diethylthiacyanine iodide (THIA). An increase in the THIA fluorescence quantum yield, induced by the bacterial uptake, was the principal reason for the observed emission enhancement. The fluorescence quantum yield of THIA depended on the media viscosity and not on the media polarity, which suggested that the microenvironment for the dye molecules taken up by the cells was restrictive. The kinetics of fluorescence staining did not manifest statistically significant dependence on the dye concentration and on the cell count. In the presence of surfactant additives, however, the fluorescence-enhancement kinetic patterns manifested species specificity with statistically significant discernibility.

1.1 Introduction

This article describes the dynamics of fluorescence enhancement induced on 3,3'-diethylthiacyanine iodide (THIA) upon uptake by bacterial cells (Scheme 1). While it did not manifest statistically significant dependence on the dye concentration and on the bacterial cell count, the kinetics of fluorescence enhancement differed for the different bacterial species when pretreated with a surfactant additive, TWEEN® 40.

Bacterial infectious diseases remain one of the major health hazards nation- and worldwide. A quarter of worldwide deaths are a corollary of bacterial infections (4, 13, 45, 57). Annually, pneumococcal diseases, for example, claim close to a million children's lives worldwide (70). In the United States, as another example, food-borne bacterial infections cause millions of illnesses, thousands of which are fatal (67, 72, 95). The expedience of the detection and identification of such pathogens determines how early the diagnosis is, and hence, what the treatment and the outcome of the illness would be (48, 57, 62).

Due to their simplicity and operational expedience, colorimetric and fluorescence/luminescence staining are the basis of broadly used analytical techniques for biomolecules (9, 10, 16, 20, 35, 58, 64, 65, 75, 90), bacterial species (11, 24, 38, 43, 61, 66, 84), eukaryotic cells (5, 15, 21, 37, 86, 87), and tissue samples (26, 27, 30, 52, 82). In particular, because of its inherent sensitivity, fluorescence staining is a central component of bioanalytical assays and imaging methodologies (29, 44, 47, 54, 74, 76).

Ever since the first reports from Carl Friedländer and Hans Christian Joachim Gram, published in 1883 and 1884, respectively (19, 23), the identification of bacterial species using staining techniques has been based solely on the appearance of the stained cells (24, 66). Hence, the staining analyses yield Boolean outcomes: i.e., in principle, the reagents either stain or do not stain the analyzed bacteria. As a result, each analysis affords a classification of species only into two groups, based on the staining patterns. Morphology analysis, for example, is required for further identification (24, 43). Similarly, Boolean assays with improved specificity (such as immunofluorescence staining) (7, 46) are informative solely about species that are sought. Thus, analytes, which are present in the sample but not targeted by the particular stain, may remain undetected.

For more than a century, bacterial staining has been established as a benchmark for analytical and diagnostic techniques (24, 66). The dynamics of such staining processes, however, still remains largely unexplored. Recently we demonstrated that the dynamics of staining has the potential to discriminate between species that would be classified within the same group based on a “traditional” stain analysis (96).

Herein, we describe our investigation of the interaction of a cyanine dye, THIA, with two Gram-negative bacteria, *Escherichia coli* (*E. coli*) and *Enterobacter aerogenes* (*E. aerogenes*), and of two Gram-positive bacteria, *Bacillus subtilis* (*B. subtilis*) and *Bacillus sphaericus* (*B. sphaericus*). When taken up by bacterial cells, THIA manifested an increase in its fluorescence quantum yield. This fluorescence enhancement resulted in a significant contrast, allowing for imaging the stained cells without the removal of the

cyanine dye that remained free in the surrounding solution media (Figure 1). Investigation of the solvent-dependence of the photophysical properties of THIA revealed that the media viscosity caused the observed emission enhancement. The observed lack of concentration dependence of the species-specific staining kinetics was a principal feature of the dynamics of bacterial staining with THIA.

1.2 Results and Discussion

1.2.1 Interaction between bacterial cells and THIA

Similar to other cyanine dyes, THIA (Scheme 1) manifested a pronounced affinity for bacterial cells (Figure 1) (3, 59, 68). When taken up by bacterial cells THIA exhibited: (1) a decrease in the molar extinction coefficient and a change in the shape of the absorption spectrum (Figure 2a); (2) an increase in the emission intensity and about a ten-nanometer red shift of the emission maximum from ~ 470 to ~ 480 nm (Figure 2b); and (3) appearance of emission bands at wavelengths longer than the spectral maxima (Figure 2b). The relative intensities of the long-wavelength emission bands were sensitive to the conditions of staining. These long-wavelength emission bands were more pronounced in the absence of surfactant and in general, for cases exhibiting lesser fluorescence enhancement at 480 nm.

The observed changes in the THIA absorption spectra suggested for ground-state processes that perturbed the electronic structure of the dye upon bacterial uptake.

Ground-state isomerization from *trans* to *cis* could cause spectral red shifts of THIA upon bacterial uptake (85). While such isomerization could be the reason for the observed red shift of the principal fluorescence band of THIA (from ~ 470 to ~ 480 nm), the formation of the *cis* isomer would not be likely to account for the 600-nm emission band (i.e., implying for ~ 140 -nm Stokes shift of the *cis* isomer).

As prevalently observed for pyrene derivatives, for example, ground-state aggregation could be a plausible source for the observed metachromasy (32-34, 36, 56, 88, 89). Negatively charged microenvironment, combined with hydrophobic interfaces, such as the minor groove of the DNA double strand (8, 63, 80), binds thiacyanine dyes and propends their aggregation resulting in pronounced spectral changes (6, 25, 71, 73, 81, 94). Furthermore, the observed bacterium-induced spectral trends (Figure 2) were, indeed, in agreement with the reported metachromic behavior of THIA (49, 50). Thus, the 600-nm emission peak could be ascribed to a J-like aggregate of THIA taken up by the bacterial cells (50).

We abstain, however, from using the terms H-aggregate and J-aggregate because they imply a prior knowledge of the relative orientation of the electric transition dipole moments of excitonically coupled dye molecules. While stacked parallel arrangements of the transition dipole moments, i.e., H-aggregation, results in blue-shifted spectral features, the head-to-tail arrangement, i.e., J-aggregation, results in red shifted spectral features (41, 42, 55). These chromophore arrangements, however, are not unique for producing such spectral shifts. Energy shifts in the electronic states of oblique aggregates,

for example, can accommodate transitions resulting J- and H-like (red and blue shifted) absorption and emission bands (41, 42, 55).

Due to lack of structural information of the binding environment for the staining fluorophores, the observed spectral shifts (Figure 2) could not be unambiguously ascribed to J and H structures of THIA aggregates. In fact, each absorption and emission spectrum of the thiacyanine dye in the presence of bacterial cells (Figure 2) represented overlapped spectra from THIA molecules in the environment of different chromotropes: e.g., spectra of monomeric and aggregated THIA bound to bacterial cells overlapped with the spectra of THIA remaining in the aqueous solution. Furthermore, while for most examined species we have observed 600-nm fluorescence bands of different relative intensities, which is in accordance with previously reported metachromatic behavior of THIA (50), *E. aerogenes* induced a fluorescence band at about 540 nm (Figure 2b).

To examine the phenomena, responsible for the cell staining and the observed imaging contrast (Figure 1), we investigated the interaction of THIA with negatively-charged polystyrene beads. Addition of bead suspension to the dyes solution did not cause emission enhancement (Figure 3a). Fluorescence images, however, revealed that THIA stains the beads producing moderate contrast ratios (Figure 3b).

This finding demonstrated that binding of the dye to the micrometer-size objects, such as cells and beads, was a necessary but not a sufficient condition for the observed fluorescence enhancement (Figure 2b). The bacteria induced an increase in the emission intensity (Figure 2a), which was accompanied by a decrease in the absorbance of the dye (Figure 2a). Therefore, the dye uptake by the bacteria caused an increase in its

fluorescence quantum yield, which along with the increase in the local dye concentration in the cells, was a principal reason for the imaging contrast for the stained bacteria (Figure 1).

1.2.2 Solvent-dependence of the spectral properties of THIA

Solvent-dependence measurements of the emission properties of THIA indicated that it is the viscosity, rather than the polarity, of the environment that induced the observed increase in the fluorescence quantum yields (Figure 4) (83). In fact, in comparison with aqueous media, the fluorescence quantum yield, Φ_f , of THIA increased one-to-two orders of magnitude when the dye was placed in solvents more viscous than water (Table 1): i.e., for viscous solvents with moderate polarity, $\Phi_f = 0.32$ and $\Phi_f = 0.012$ for glycerol and ethylene glycol, respectively. In comparison, the Φ_f of THIA did not manifest such a dependence on the polarity of the media: i.e., for polar media, water, $\Phi_f = 2.0 \times 10^{-3}$; and for a non-polar protic solvent, 1-butanol, $\Phi_f = 2.4 \times 10^{-3}$.

In addition, we observed a slight solvatochromism, which depended on the excitation wavelength. When excited at 420 nm, for example, THIA exhibited red-shifted fluorescence for relatively low-polarity solvents. Excitation at 410 nm did not produce the same effect. This observation suggested that the underlying effect resultant on the bacterium-induced fluorescence red shifts could be a corollary of a decrease in the polarity of the binding microenvironment for THIA. Such wavelength dependence of the

fluorescence spectra indicated for heterogeneity of the ground-state population of the THIA molecules.

The molecular structure of THIA elucidates the sensitivity of its fluorescence quantum yield to the viscosity of the environment. For cyanine dyes, and other molecular rotors with extended π -conjugation, the viscosity of the surrounding microenvironment modulates the kinetics of non-radiative decay, which is reflected by changes in the fluorescence quantum yields (1, 12, 18, 22, 28, 31, 39, 40, 77).

THIA has a symmetric molecular structure, in which extended π -conjugation, spanning over the bonds connecting the two ring systems, propends its planar conformation (Scheme 1). Rotational motions around these two bonds, connecting the ring systems, provide pathways for non-radiative decay. An increase in the media viscosity slows down such molecular motions that allow for exploration of the conformational space. As a corollary of the increase in the media viscosity, the decrease in the likelihood of conformational changes leading to non-radiative deactivation, results in an increase in the fluorescence quantum yield of the cyanine dye (1, 2, 18, 22, 28, 31, 39, 40, 69).

The observed viscosity dependence of the emission quantum yield suggested that the fluorescence stain, THIA, migrated from the relatively non-viscous aqueous media into the cell microenvironment with increased viscosity. Upon bacterial uptake, binding of THIA to cell components, which restricted its molecular motion (i.e., providing a microenvironment with relatively large effective viscosity), resulted in an increase in the dye emission quantum yield causing the observed fluorescence enhancement.

The spectral features of the fluorescence enhancement of THIA induced by bacteria (Figure 2) and by the solvent viscosity (Figure 4) manifested differences that indicated that the cell uptake of the cyanine dye involves more than just binding to microenvironment that restricts the molecular motions. Unlike bacterial cells, viscous solvents did not considerably perturb the absorption spectra of THIA: i.e., glycerol and ethylene glycol caused solely a slight red shift in the absorption maximum of THIA, rather than a decrease in the molar extinction coefficient (Figure 4a). Furthermore, neither a red shift of the principal band at 470 nm, nor an appearance of new fluorescence bands, accompanied the emission enhancement induced by the viscous solvents (Figure 4b).

1.2.3 Dynamics of bacterium-induced spectral changes

The appearance of the red-shifted emission bands was a feature that set apart the bacterium-induced from the solvent-viscosity-induced THIA fluorescence enhancement (Figure 2b and 4b). As we already discussed, such red-shifted fluorescence, along with the observed perturbation of the THIA absorption spectrum, upon cell uptake could be ascribed to ground-state aggregation (49, 50).

To elucidate the relation between the absorption and emission spectral features, we examined the dynamics of their alterations. The time constants, τ_i , representing the kinetics of the bacterium-induced fluorescence enhancement, monitored at wavelength, λ , were extracted from fit to exponential rise functions:

$$F(t)_{\lambda, t \geq t_0} = \Delta F \left(1 - \sum_{i=1}^n \alpha_i \exp \left(-\frac{t - t_0}{\tau_i} \right) \right) + F_0 \quad \text{Equation (1a)}$$

where F is the measured fluorescence intensity over time, t ; F_0 is the initial fluorescence intensity of the dye without bacteria; ΔF is the total increase in the fluorescence intensity; the pre-exponential factors, α_i , sum to unity, i.e., $\sum_{i=1}^n \alpha_i = 1$; and t_0 is the initial time of mixing.

Concurrently, we used a similar exponential decay function to analyze the bacterium-induced changes in the absorption of THIA, $A(t)$, monitored at λ :

$$A(t)_{\lambda, t \geq t_0} = \Delta A \left(-1 + \sum_{i=1}^n \alpha_i \exp \left(-\frac{t - t_0}{\tau_i} \right) \right) + A_0 \quad \text{Equation (1b)}$$

We recorded the changes in the absorption of THIA upon addition of *E. coli* (Figure 5). Under identical conditions, we recorded the THIA fluorescence enhancement induced by the bacterial cells (Figure 5). The evolution of the absorption and fluorescence kinetic curves exhibited multiexponential patterns. Biexponential functions ($n = 2$, equation 1), however, proved to be the simplest to produce satisfactory data fits (Figure 5). For the absorption decay at 420 nm and for the fluorescence rise at 600 nm, the slow components dominated the kinetics (with time constants ranging between 150 s

and 250 s). The fast components from the biexponential fits (with time constants of about 30 s) did not conspicuously contribute to the shapes of the kinetic curves and could be visualized solely in plots against logarithmic ordinates (Figure 5b). For the fluorescence enhancement monitored at 480 nm, on the other hand, the fast component dominated the kinetics (Figure 5, middle curves). The time constant of the predominant fast components for the 480-nm fluorescence enhancement did not exceed 10 s. It was several-fold faster than the fastest components for the 600-nm-emission and 420-nm-absorption kinetics.

The fluorescence enhancement, monitored at 480 nm, exhibits a fast increase within the first 10 – 20 s of the mixing, followed by an additional 25% intensity increase considerably slower than the initial jump. The absorption decay at 420 nm and the fluorescence rise at 600 nm, on the other hand, exhibited a steady change with kinetic patterns differing from the kinetic pattern observed for the 480-nm fluorescence enhancement (Figure 5). The bacterium-induced perturbation of the absorption spectra and the growth of the long-wavelength emission bands, hence, appeared related. The similar kinetics indicated that they could be a corollary of the same underlying process, reasonably ascribed to THIA aggregation (49, 50). The minor slow component of the 480-nm fluorescence enhancement could plausibly be ascribed to a similar concurrent aggregation of THIA exhibiting an H-like-aggregate emission (50).

The initial fast jump in the fluorescence intensity, monitored at 480 nm, represented the principal emission enhancement induced by the bacterium uptake. The time constant, representing the initial 480-nm fluorescence-intensity increase, was more than four-times shorter than the shortest time constants for the absorption decay (Figure

5). Therefore, the fast 480-nm fluorescence enhancement did not result from the same processes that caused the perturbation of the absorption spectra: i.e., the principal component of the 480-nm emission enhancement was not caused by dye aggregation. In accordance with the viscosity-dependence quantum-yield findings, we ascribed the fast increase in the THIA fluorescence intensity, at ~ 480 nm, to initial binding of the dye to cellular chromotropic microenvironment, which restricting the molecular motion (i.e., the chromotropes provide fluorogenic microenvironment for THIA.)

1.2.4 The effects of TWEEN[®] on the behavior of THIA

Additives, such as mild surfactants, modulated the spectral and kinetic features of the bacterium-induced emission enhancement. TWEEN[®] is a mild oligoethylene glycol amphipat that is regularly employed for preventing cell and protein adhesion without compromising their viability and integrity. In the presence of TWEEN[®], THIA exhibited spectral changes similar to the changes we observed for THIA induced by viscous solvents (Figure 4 and 6a): i.e., the addition of TWEEN[®] 40 to THIA solution caused: (1) a slight red shift of the absorption maximum without much other spectral perturbations; and (2) an increase in the fluorescence intensity without shifts of the 470-nm maximum (Figure 6a). The TWEEN[®] 40 surfactant also suppressed the appearance of the long-wavelength emission bands of THIA in the presence of bacteria (Figure 6b).

Furthermore, TWEEN[®] increased the rates of the fluorescence enhancement, monitored at 480 nm. In the presence of TWEEN[®], we also observed a fast, followed by a

slow, intensity increase (Figure 6b and 7a). In the presence of TWEEN[®] 40, however, the slow intensity increase was a principal component of the 480-nm kinetics. Monoexponential fits, limited to the time spans of the intensity increase that followed the few-second initial jump of the 480-nm fluorescence, yielded time constants similar to the time constant representing the slow components from the biexponential analyses (Figure 7a). *B. subtilis* in the presence of TWEEN[®], however, induced emission enhancement that exhibited solely monoexponential behavior (Figure 7b): i.e., a fast fluorescence intensity increase (in less than 10 s), which was not followed by a slow kinetic component.

1.2.5 Kinetics of fluorescence enhancement for different species

For samples containing 0.5 mM TWEEN[®] 40, we examined the kinetics of the fluorescence enhancement for dye concentration, C_{THIA} , and cell count, CC , spreading over two orders of magnitude: i.e., THIA concentrations, $C_{\text{THIA}} = 0.6, 6$ and $60 \mu\text{M}$; and cell counts, $CC = 5 \times 10^6, 5 \times 10^7$ and $5 \times 10^8 \text{ cell ml}^{-1}$. We focused on the 480-nm steady emission enhancement kinetics that followed the initial intensity jump upon mixing (Figure 7a). For each of the examined species, the obtained values of the emission-enhancement time constants did not significantly vary over the investigated dye-concentration and cell-count ranges (Table 2). The time constant, however, differed for the four investigated species (Table 2). The values of the time constants for *E. coli* were clustered around 30 s, for *E. aerogenes* – around 20 s, for *B. subtilis* – around 3 s, and for *B. sphaericus* – around 50 s (Table 2).

Analysis of variance (ANOVA) allowed us to examine the observed trends in the concentration and species dependence of the emission-enhancement time constants. For each species we employed two-factor ANOVA tests. The two null hypotheses for each of the tests stated that the time constants for the different cell counts and for the different dye concentrations were identical. The obtained p -values for the cells and for the dye were large enough to prevent from reasonably rejecting either of the null hypotheses (Table 2).

For examining the discernibility between the time constants for the different species, we employed one-factor ANOVA tests. Each test compared the emission-enhancement kinetics for two different species with the null hypothesis (H_0) that the time constants for these two bacteria are identical. Neither of the six p -values for the possible combination between the four investigated species exceeded 10^{-5} , corresponding to minute probabilities, i.e., $< 0.001\%$, for experiments to yield the values of τ , which we observed, if H_0 was true (Table 3). Therefore, we could confidently reject the null hypothesis for all comparisons between the bacterial species: i.e., the kinetics of staining with THIA in the presence of TWEEN[®] 40 was species specific.

One of the most important implications of these findings (i.e., important for assay developments) was that the staining kinetic curves for two closely related species, *B. subtilis* and *B. sphaericus*, were different (Figure 7). Furthermore, while *B. sphaericus* exhibited the slowest kinetic features, *B. subtilis* showed the fastest staining kinetics among the examined species (Table 2).

This discernibility between the staining kinetics for the different investigated bacterial species, however, was induced by the surfactant additive, TWEEN[®] 40. In the absence of a surfactant, the sub-10-second initial rise dominated the emission-enhancement kinetics at 480 nm (Figure 5, 6c). Analysis of this kinetics, obtained in the absence of TWEEN[®], revealed that for each species the time constants were not statistically dependent on the dye concentration and on the cell count. The values of the obtained time constants for the different bacteria, however, were not sufficiently segregated from one another to serve as a handle for species-specific kinetics (see Supporting Information). Therefore, the lack of the surfactant additive did not cause dependence of the staining kinetics on the dye concentration and on the cell count. The lack of TWEEN[®], however, compromised the kinetics-based discernibility between the species.

The species-specific concentration independence trends that we observed for the emission enhancement time constants (Table 1), were conducted for samples pretreated with TWEEN[®] 40. We observed similar trends when only the cells were pre-treated with TWEEN[®] 40 and added to THIA solution without any surfactant (96). Even under such different experimental conditions, we still observed concentration independent emission-enhancement time constants. For *B. sphaericus* these time constants were larger than the time constants for *E. coli*, which were larger than the time constants for *B. subtilis* (96). Our findings demonstrated that TWEEN[®] considerably modulates the dynamics of uptake of a cationic dye by bacterial cells to provide discernable kinetics for species as similar as the two investigated *bacilli* (Table 2).

The Gram-positive species, *B. subtilis* and *B. sphaericus*, have cell walls significantly thicker than the cell walls of the Gram-negative species. (The Gram-negative cell walls contain lipopolysaccharide-coated outer membrane enclosing the peptidoglycan-filled periplasmic space. The Gram-positive cell walls, on the other hand, contain only one membrane, i.e., the plasma membrane, and the periplasmic space is coated with a thick peptidoglycan layer.) The kinetic patterns for the two TWEEN® pretreated Gram-positive cells manifested the most drastic difference (Figure 4a, Table 2, 3). Therefore, the generic chemical composition and the thickness of the cell walls cannot account for the observed kinetic differences. The TWEEN® inhibition of the staining rates, observed for *B. sphaericus*, but not for *B. subtilis*, suggested that either (1) *B. subtilis* provided more binding sites for THIA, the access to which could not be visibly restricted by the surfactant, or (2) *B. sphaericus* had a stronger affinity for TWEEN® increasing its inhibiting potency against the staining dye uptake.

1.2.6 Diffusion-driven dye uptake

The mono- and biexponential data fits allowed for quantitative analysis of the emission-enhancement kinetics. Such mono- and biexponential treatments, however, were not informative about the lack of statistically significant dependence of the kinetics on the dye concentration and on the cell count.

Following on reports that, upon staining, the taken up cyanine dyes collect in the bacterial cell walls (3, 68), we modeled the dynamics of fluorescence enhancement as a

diffusion-driven migration of the dye from the aqueous environment into a thin sheet of permeable media (Scheme 2):(14)

$$M(t)_{t \geq t_0} = \frac{m(t)}{m(\infty)} = 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left(-\frac{D q_n^2 (t-t_0)}{L^2}\right) \quad \text{Equation (2)}$$

$$M(t)_{t < t_0} = 0$$

where the cell wall was modeled as a sheet of media with thickness L ; D , thus, is the THIA diffusion coefficient in the cell-wall media; $m(t)$ is the total mass of THIA in the cell wall at time t ; q_n is the n^{th} non-zero positive roots of $\tan(q_n) = -\alpha q_n$; and α represents the THIA distribution, i.e., $\alpha = A / KL$. In the latter, K is the partition coefficient of THIA between the cell-wall and the aqueous media; and A is a characteristic length of the bulk solution that can be expressed in terms of cell count, CC , cell volume, V_C , unit volume, V_1 , and surface area of a cell, S_C : i.e., $A = V_1 / (CC S_C) - V_C / S_C$.

The time-dependent normalized amount of the taken up dye, $M(t)$, was indicative for the emission-enhancement kinetics:

$$F(t) \propto \frac{M(t)}{\alpha+1} \Phi_C + \frac{\alpha+1-M(t)}{\alpha+1} \Phi_W \quad \text{Equation (3)}$$

where Φ_C and Φ_W are the fluorescence quantum yields of the dye in the cells and in the aqueous media, respectively. Equation 3 assumes that: (1) the fluorescence intensity is

linearly proportional to the fluorophore concentration (92); and (2) the molar extinction coefficient (at the excitation wavelength) for the solution-free and for the cell-bound dye are the same: i.e., the perturbation of the absorption spectrum of THIA (Figure 2a) is due to the relatively slow processes producing the emission at 600 nm, rather than to the initial dye-cell interaction ascribed to the enhancement at 480 nm.

Via the exponential terms of the mass-transport relation (equation 2), D and L determine the time constant of the fluorescence enhancement. These exponential terms, at the same time, do not depend on the dye concentration and only indirectly depend on the cell count via q_n (equation 2).

To analyze the diffusion-driven kinetics (equation 2 and 3) in a similar manner as the cell-induced emission enhancement, we fit the curves calculated from equation 3 to a monoexponential rise function ($n = 1$, equation 1a). The time constants, τ , obtained from the monoexponential fits, manifested a strong dependence on the sheet (i.e., cell-wall) thickness, L , and on the diffusion coefficient, D , rather than on the cell count (Figure 8).

For identical values of D and L , simulations of fluorescence enhancement, using (equation 2 and 3), indeed, revealed only about 10 % change in the time constant, τ , when the cell count was altered two orders of magnitude (Figure 8). Concurrently, the time constant of fluorescence enhancement, τ , manifested substantial dependence on the diffusion coefficient of the dye in the permeable media, D , and on the thickness of the permeable sheet, L (Figure 8). This model reveals the sensitivity of the staining kinetics to the type of species (via D and L) and to the type of staining agent (via D), rather than to their concentrations.

The diffusion-driven model of the fluorescence enhancement (equation 2 and 3) represented the observed lack of concentration dependence for the species-specific staining kinetics. This model, however, failed to provide physically feasible quantification for the diffusivity of the stain. A diffusion coefficient of THIA in the order of $10^{-13} \text{ cm}^2 \text{ s}^{-1}$, suggested for media with viscosity considerably exceeding the plausible viscosity of any material composing the bacterial cell wall. This finding implied for retention of the THIA migration, for example, due to binding processes.

A rough approximation of the characteristic diffusion time, τ_D , to an emission-enhancement time constant, τ , confirmed the unfeasibly small values of diffusion coefficients required for quantifying the observed cell-staining kinetics. (The characteristic diffusion time depends on the characteristic diffusion length, l , and on the diffusion coefficient: $\tau_D = l_D^2 / D(79)$). For cell wall thickness, $l_D \approx L$, ranging between 10 and 100 nm, to assure diffusion times as short as 1 s, for example, D had to range between 10^{-12} and $10^{-10} \text{ cm}^2 \text{ s}^{-1}$. For τ_D , compatible to the measured time constants in the order of 10 s, the values of D are even an order of magnitude smaller: i.e., 10^{-13} and $10^{-11} \text{ cm}^2 \text{ s}^{-1}$.

An important assumption for this diffusion-driven model was that the dye accumulated into the cell wall (3). Assuming that the THIA readily penetrated the intact cell membranes, 1- μm diffusion length for the dye to migrate into the cell, would require D ranging between 10^{-8} and $10^{-9} \text{ cm}^2 \text{ s}^{-1}$ for τ_D ranging between 1 and 10 s, respectively. These latter values of D still exceeded the reported diffusivities of ionic fluorophores in cytosol (53, 78).

Representing the complex structure of bacterial cell walls of Gram-negative and Gram-positive species as a homogeneous gel-like sheet (Scheme 2) was, indeed, an oversimplification. Furthermore, such a two-state model (i.e., the dye is either in the cell or in the solution, equation 3) failed to account for aggregation processes that we ascribed as the potential reason for the observed perturbations of the shapes of the absorption and emission THIA spectra (Figure 2).

Nevertheless, such a model allowed for demonstrating the negligible-to-no dependence of the staining kinetics on the cell count and on the dye concentration. The parameters L and D could be viewed as semi-quantitative characteristics for the species and for their interactions with the staining agents representing: i.e., as “effective thickness” and “effective diffusivity,” respectively.

1.3 Conclusions

The kinetics of fluorescence staining of bacteria with 3,3'-diethylthiacyanine iodide manifested a lack of dependence on the dye concentration and on the cell count. An oversimplified model, based on a diffusion-driven dye uptake, confirmed the lack of concentration dependence of the staining kinetics and suggested for species and dye specificity. A surfactant additive, TWEEN[®] 40, however, was required for obtaining kinetic patterns that were statistically different for the four different species we studied. We believed that the lack of statistically significant concentration dependence of species-

specific and dye-specific staining kinetics has the potential for bringing a broad range of easily administrable bioanalytical assays beyond their Boolean nature.

1.4 Experimental

The *E. coli* TOP 10 cells were obtained from Invitrogen (Carlsbad, CA). Propidium iodide and SYTO 9 were obtained from Invitrogen. LB broth, 3,3'-diethylthiacyanine iodide, eosin Y disodium salt, methylviologen dichloride hydrate, and alcohols (methanol, ethanol, 1-propanol, 1-butanol, ethylene glycol and glycerol: all spectroscopic grade) were purchased from Sigma-Aldrich. Tris(hydroxymethyl) aminomethane was obtained from Acros Organics. Sodium hydroxide was purchased from Fisher Scientific. For all aqueous solutions, we used Milli-Q water (from an in-house purification system).

The bacterial cultures were grown at 37 °C in sterilized LB broth liquid media, under aerobic conditions and constant shaking (at 200 rpm on Forma Orbital Shaker, Thermo), as we have previously described (91). For sample preparation, the bacterial cultures were subjected to a sequence of centrifugation/wash steps (using Eppendorf 5415D centrifuge) and finally suspended in 2 mM Tris buffer, pH = 8.5. Cell counting was performed using an improved Neubauer counting chamber (Paul Marienfeld GmbH & Co. KG) and a MicroMaster microscope (Fisher Scientific) equipped with a 40× (0.40 NA) objective.

To ensure that the bacterial cultures, which we used for the sample preparation, were in their exponential growth phase, we constructed growth curves for the investigated bacterial species. The curves revealed that the investigated bacteria, cultured for 18-24 hours, are still in their active growth phase.

We conducted viability tests by adding bacterial suspensions to a centrifuge tube containing a solution of 1 μM Propidium iodide and 5 μM SYTO 9. Propidium iodide is a DNA binding dye that is not membrane permeant, and hence, it stains only cells with permeabilized membranes. SYTO 9 is a membrane permeant nucleic acid stain that stains both live and dead cells alike. While all cells are stained green from SYTO 9, only nonviable cells are stained red from propidium.

The absorption spectra were recorded using a Varian Cary 50 UV/Visible spectrophotometer. The steady-state and kinetic fluorescence measurements were performed using a Horiba Jobin Yvon Fluorolog-3-22 spectrofluorometer (93).

The fluorescence quantum yields of THIA, Φ_f , were determined by comparing the integrated emission intensities of the samples with the integrated fluorescence of a reference sample with a known fluorescence quantum yield, Φ_{f0} :(17)

$$\Phi_f = \Phi_{f0} \frac{\int F(\lambda) d\lambda}{\int F_0(\lambda) d\lambda} \times \frac{1 - 10^{-A_0(\lambda_{ex})}}{1 - 10^{-A(\lambda_{ex})}} \times \frac{n^2}{n_0^2} \quad \text{Equation (4)}$$

where $F(\lambda)$ is the fluorescence intensity at wavelength λ ; $A(\lambda_{ex})$ is the absorbance at the excitation wavelength; n is the refractive index of the media; and the suffix “0” indicates the quantities for the reference sample used. For a reference sample we used a solution of 9-phenyl-coumarin 151 in ethanol ($\Phi_{f0} = 0.49$)(60).

For the kinetic measurements, cuvettes with four-polished sides, containing the dye solutions, were placed in the sample holder equipped with a magnetic stirrer and thermostated at 37 °C. Under constant stirring, small volumes of concentrated bacterial suspensions were added quickly, allowing for subsecond (i.e., < 100 ms) mixing and homogenization of the samples. The excitation wavelength was set at 420 nm. The recording at the emission wavelength (480 nm or 600 nm) was initiated before addition of the bacterial suspension and continued for at least 5 to 10 minutes.

To examine the contribution of rate of mixing toward the kinetics of fluorescence enhancement, we utilized the quenching of eosin Y with methyl viologen. Setting the excitation wavelengths to 490 nm, the change in the fluorescence intensity of 1 μM Eosin Y upon addition of methyl viologen (final concentration 100 μM) was monitored at 550 nm under identical stirring conditions.

Replicating the addition and mixing of the bacterial suspensions to the THIA solutions, we introduced methyl viologen (MV^{2+}) to solutions of eosin Y (EY^{2-}), under identical mixing conditions. The quenching of the fluorescence of EY^{2-} , due the formation of charge-transfer complexes with MV^{2+} (51), exhibited time constants smaller than ~ 100 ms. The measured time constants for the bacterium-induced fluorescence enhancement of THIA, on the other hand, were in the order of seconds (Tables 1 and 2). Therefore, the sample mixing and the diffusion of the cyanine dye to the bacterial exterior are not the rate-limiting steps of the observed fluorescence-enhancement processes.

The data fits, the statistical ANOVA analyses, and the mass-transport computations (equations 2 and 3) were performed using IgorPro v. 6 (WaveMetrics, Inc., Lake Oswego, OR) on Mac OS X workstations. The sum in equation 2 was carried for $n = 1$ to 1000.

Acknowledgments. This work was supported by the U. S. Department of Education, the National Science Foundation, and the U. C. Regents Faculty Development Award. We extend our gratitude to Profs. J. Schultz, V. Rodgers, and N. Schiller for the helpful discussions on the mass-transport models and the bacterial physiology. The *E. coli* cells (TOP 10, Invitrogen) were donated by Prof. J. Liao.

1.5 References

1. **Abdel-Mottaleb, M. S. A., A. M. K. Sherief, L. F. M. Ismaiel, F. C. De Schryver, and M. A. Vanderauweraer.** 1989. Photophysics of molecular rotors of functionalized surface-active styrylcyanine dyes. *Journal of the Chemical Society, Faraday Transactions 2: Molecular and Chemical Physics* **85**:1779-1788.
2. **Åberg, U., E. Åkesson, J. L. Alvarez, I. Fedchenia, and V. Sundström.** 1994. Femtosecond spectral evolution monitoring the bond-twisting event in barrierless isomerization in solution. *Chemical Physics* **183**:269-288.
3. **Anaya, C., N. Church, and J. P. Lewis.** 2007. Detection and identification of bacterial cell surface proteins by fluorescent labeling. *Proteomics* **7**:215-219.
4. **Anderson, J.** report 2007. Basic Research on Bacteria. The Essential Frontier. American Society for Microbiology and National Institute of Health.
5. **Benbow, R. M.** 1992. Chromosome structures. *Science progress* **76**:425-450.
6. **Benvin, A. L., Y. Creeger, G. W. Fisher, B. Ballou, A. S. Waggoner, and B. A. Armitage.** 2007. Fluorescent DNA Nanotags: Supramolecular Fluorescent Labels Based on Intercalating Dye Arrays Assembled on Nanostructured DNA Templates. *Journal of the American Chemical Society* **129**:2025-2034.
7. **Beutner, E. H., E. J. Holborow, and G. D. Johnson.** 1965. A new fluorescent antibody method; mixed antiglobulin immunofluorescence or labeled antigen indirect immunofluorescence staining. *Nature* **208**:353-355.
8. **Bostock-Smith, C. E., and M. S. Searle.** 1999. DNA minor groove recognition by bis-benzimidazole analogs of Hoechst 33258: insights into structure-DNA affinity relationships assessed by fluorescence titration measurements. *Nucleic Acids Research* **27**:1619-1624.
9. **Cable, M. L., J. P. Kirby, D. J. Levine, M. J. Manary, H. B. Gray, and A. Ponce.** 2009. Detection of Bacterial Spores with Lanthanide-Macrocyclic Binary Complexes. *Journal of the American Chemical Society* **131**:9562-9570.
10. **Cable, M. L., J. P. Kirby, K. Sorasane, H. B. Gray, and A. Ponce.** 2007. Bacterial Spore Detection by [Tb³⁺(macrocycle)(dipicolinate)] Luminescence. *Journal of the American Chemical Society* **129**:1474-1475.
11. **Chapin, K. C., and T.-L. Lauderdale.** 2007. Reagents, stains, and media: bacteriology. *Manual of Clinical Microbiology* (9th Edition) **1**:334-364.
12. **Comotti, A., S. Bracco, P. Valsesia, M. Beretta, and P. Sozzani.** 2010. Fast Molecular Rotor Dynamics Modulated by Guest Inclusion in a Highly Organized

- Nanoporous Organosilica. *Angewandte Chemie, International Edition* **49**:1760-1764.
13. **Cossart, P., and P. J. Sansonetti.** 2004. Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens. *Science* **304**:242-248.
 14. **Crank, J.** 1975. Diffusion in a Plane Sheet, p. 44-68, *The Mathematics of Diffusion*, 2nd ed.
 15. **Crissman, H. A., and J. A. Steinkamp.** 2001. Flow cytometric fluorescence lifetime measurements. *Methods in Cell Biology* **63**:131-148.
 16. **Deligeorgiev, T. G., S. Kaloyanova, and J. J. Vaquero.** 2009. Intercalating cyanine dyes for nucleic acid detection. *Recent Patents on Materials Science* **2**:1-26.
 17. **Demas, J. N., and G. A. Crosby.** 1971. Measurement of Photoluminescence Quantum Yields - Review. *Journal of Physical Chemistry* **75**:991.
 18. **Di Paolo, R. E., and J. O. Tocho.** 1997. Rotational diffusion parameters of cyanine dyes determined by depolarization of luminescence. *Journal of Luminescence* **72-74**:481-483.
 19. **Friedländer, C.** 1883. Die Mikrokokken der Pneumonie. *Fortschritte der Medizin* **1**:715-733.
 20. **Ghosh, A., A. Shrivastav, D. A. Jose, S. K. Mishra, C. K. Chandrakanth, S. Mishra, and A. Das.** 2008. Colorimetric Sensor for Triphosphates and Their Application as a Viable Staining Agent for Prokaryotes and Eukaryotes. *Analytical Chemistry* **80**:5312-5319.
 21. **Gill, M. R., J. Garcia-Lara, S. J. Foster, C. Smythe, G. Battaglia, and J. A. Thomas.** 2009. A ruthenium(II) polypyridyl complex for direct imaging of DNA structure in living cells. *Nature Chemistry* **1**:662-667.
 22. **Grachev, A. V., G. M. Esayan, A. N. Ponomarev, L. B. Rubin, and V. I. Yuzhakov.** 1991. Kinetics of fluorescence polarization for dyes in polymer matrixes. *Optika i Spektroskopiya* **70**:804-807.
 23. **Gram, H. C.** 1884. Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten. *Fortschritte der Medizin* **2**:185-189.
 24. **Green, L. H.** 2009. Diagnostic medical microbiology, p. 117-129, *Practical Handbook of Microbiology*, 2nd ed.
 25. **Hannah, K. C., R. R. Gil, and B. A. Armitage.** 2005. ¹H NMR and Optical Spectroscopic Investigation of the Sequence-Dependent Dimerization of a

- Symmetrical Cyanine Dye in the DNA Minor Groove. *Biochemistry* **44**:15924-15929.
26. **Hofecker, J. L., T. L. Moss, and M. W. Becher.** 2007. Specialized histology for neuropathology. *Journal of Histotechnology* **30**:67-78.
 27. **Horny, H.-P., K. Sotlar, and P. Valent.** 2007. Diagnostic value of histology and immunohistochemistry in myelodysplastic syndromes. *Leukemia Research* **31**:1609-1616.
 28. **Humphry-Baker, R., M. Graetzel, and R. Steiger.** 1980. Drastic fluorescence enhancement and photochemical stabilization of cyanine dyes through micellar systems. *Journal of the American Chemical Society* **102**:847-848.
 29. **Jakobs, S.** 2006. High resolution imaging of live mitochondria. *Biochimica et Biophysica Acta, Molecular Cell Research* **1763**:561-575.
 30. **Jeans, A., and M. Esiri.** 2008. Brain histology. *Practical neurology* **8**:303-310.
 31. **Jee, A.-Y., H. Kwon, and M. Lee.** 2009. Molecular rotor dynamics influenced by the elastic modulus of polyethylene nanocomposites. *Journal of Chemical Physics* **131**:171104/171101-171104/171104.
 32. **Jones, G., II, V. Vullev, E. H. Braswell, and D. Zhu.** 2000. Multistep Photoinduced Electron Transfer in a de Novo Helix Bundle: Multimer Self-Assembly of Peptide Chains Including a Chromophore Special Pair. *Journal of the American Chemical Society* **122**:388-389.
 33. **Jones, G., II, and V. I. Vullev.** 2001. Contribution of a Pyrene Fluorescence Probe to the Aggregation Propensity of Polypeptides. *Organic Letters* **3**:2457-2460.
 34. **Jones, G., II, and V. I. Vullev.** 2001. Ground- and Excited-State Aggregation Properties of a Pyrene Derivative in Aqueous Media. *Journal of Physical Chemistry A* **105**:6402-6406.
 35. **Jones, G., II, and V. I. Vullev.** 2002. Medium Effects on the Stability of Terbium(III) Complexes with Pyridine-2,6-dicarboxylate. *Journal of Physical Chemistry A* **106**:8213-8222.
 36. **Jones, G., II, and V. I. Vullev.** 2002. Photoinduced Electron Transfer between Non-Native Donor-Acceptor Moieties Incorporated in Synthetic Polypeptide Aggregates. *Organic Letters* **4**:4001-4004.
 37. **Kaplan, D.** 2002. Flow cytometric analysis of cells from persons with HIV-1 disease by enzymatic amplification staining. *Cellular Aspects of HIV Infection*:351-369.

38. **Kaplan, M. L., and L. Kaplan.** 1933. The Gram Stain and Differential Staining. *Journal of bacteriology* **25**:309-321.
39. **Kasatani, K., M. Kawasaki, and H. Sato.** 1984. Short-wavelength fluorescence caused by sequential two-photon excitation of some cyanine dyes: effect of solvent viscosity on the quantum yields. *Chemical Physics* **83**:461-469.
40. **Kasatani, K., and H. Sato.** 1996. Viscosity-dependent decay dynamics of the S2 state of cyanine dyes with 3, 5, and 7 methine units by picosecond fluorescence lifetime measurements. *Bulletin of the Chemical Society of Japan* **69**:3455-3460.
41. **Kasha, M.** 1964. Classification of excitons. *Phys. Process. Radiation Biol., Proc. Intern. Symp., Mich. State Univ.*:17-19, discussion 20-12.
42. **Kasha, M., H. R. Rawls, and M. A. El-Bayoumi.** 1965. Exciton model in molecular spectroscopy. *Pure and Applied Chemistry* **11**:371-392.
43. **Kohlerschmidt, D. J., K. A. Musser, and N. B. Dumas.** 2009. Identification of Gram-negative bacteria. *Practical Handbook of Microbiology (2nd Edition)*:67-79.
44. **Kricka, L. J., and P. Fortina.** 2009. Analytical ancestry: "firsts" in fluorescent labeling of nucleosides, nucleotides, and nucleic acids. *Clinical Chemistry* **55**:670-683.
45. **Kumble, K. D.** 2006. Anti-bacterial drug discovery using systems biology. *Mini-Reviews in Medicinal Chemistry* **6**:1275-1283.
46. **Levin, P. A.** 2002. Light microscopy techniques for bacterial cell biology. *Methods in Microbiology* **31**:115-132.
47. **Ljosa, V., and A. E. Carpenter.** 2008. High-throughput screens for fluorescent dye discovery. *Trends in Biotechnology* **26**:527-530.
48. **Maloy, S., and M. Schaechter.** 2006. The era of microbiology: A golden phoenix. *International Microbiology* **9**:1-7.
49. **Mandal, A. K., and M. K. Pal.** 1997. Principal component analysis of the absorption spectra of the dye thiocyanine in the presence of the surfactant AOT: precise identification of the dye-surfactant aggregates. *Journal of Colloid and Interface Science* **192**:83-93.
50. **Mandal, A. K., and M. K. Pal.** 2000. Strong fluorescence emissions by H-aggregates of the dye thiocyanine in the presence of the surfactant aerosol-OT. *Chemical Physics* **253**:115-124.
51. **Marchioni, F., M. Venturi, A. Credi, V. Balzani, M. Belohradsky, A. M. Elizarov, H. R. Tseng, and J. F. Stoddart.** 2004. Polyvalent scaffolds. *Counting*

the number of seats available for eosin guest molecules in viologen-based host dendrimers. *Journal of the American Chemical Society* **126**:568-573.

52. **Masters, B. R.** 2009. Correlation of histology and linear and nonlinear microscopy of the living human cornea. *Journal of Biophotonics* **2**:127-139.
53. **Mastro, A. M., M. A. Babich, W. D. Taylor, and A. D. Keith.** 1984. Diffusion of a small molecule in the cytoplasm of mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **81**:3414-3418.
54. **McArt, D. G., G. McKerr, C. V. Howard, K. Saetzler, and G. R. Wasson.** 2009. Modelling the comet assay. *Biochemical Society Transactions* **37**:914-917.
55. **McRae, E. G., and M. Kasha.** 1964. The molecular exciton model. *Phys. Process. Radiation Biol., Proc. Intern. Symp., Mich. State Univ.*:23-42.
56. **Michaelis, L., and S. Granick.** 1945. Metachromasy of basic dyestuffs. *Journal of the American Chemical Society* **67**:1212-1219.
57. **Millar, B. C., J. Xu, and J. E. Moore.** 2007. Molecular diagnostics of medically important bacterial infections. *Current Issues in Molecular Biology* **9**:21-40.
58. **Miller, I., J. Crawford, and E. Gianazza.** 2006. Protein stains for proteomic applications: Which, when, why? *Proteomics* **6**:5385-5408.
59. **Miller, J. B., and D. E. Koshland, Jr.** 1978. Effects of cyanine dye membrane probes on cellular properties. *Nature* **272**:83-84.
60. **Nad, S., and H. Pal.** 2001. Unusual photophysical properties of coumarin-151. *Journal of Physical Chemistry A* **105**:1097-1106.
61. **Nybroe, O., K. K. Brandt, M. H. Nicolaisen, and J. Soerensen.** 2007. Methods to detect and quantify bacteria in soil. *Modern Soil Microbiology (2nd Edition)*:283-316.
62. **Overbye, K. M., and J. F. Barrett.** 2005. Antibiotics: where did we go wrong? *Drug discovery today* **10**:45-52.
63. **Patel, M. M., and T. J. Anchordoquy.** 2005. Contribution of hydrophobicity to thermodynamics of ligand-DNA binding and DNA collapse. *Biophysical Journal* **88**:2089-2103.
64. **Patton, W. F.** 2002. Background-free protein detection in polyacrylamide gels and on electroblots using transition metal chelate stains. *Protein Protocols Handbook (2nd Edition)*:273-286.

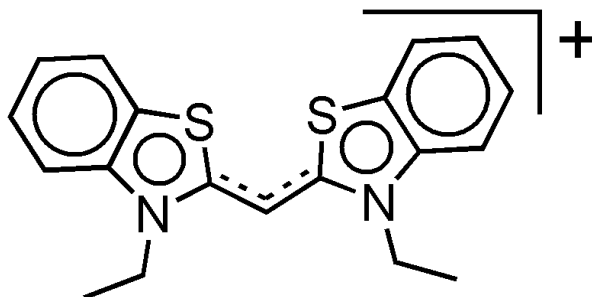
65. **Ploeger, L. S., H. F. J. Dullens, A. Huisman, and P. J. van Diest.** 2008. Fluorescent stains for quantification of DNA by confocal laser scanning microscopy in 3-D. *Biotechnic & Histochemistry* **83**:63-69.
66. **Popescu, A., and R. J. Doyle.** 1996. The Gram stain after more than a century. *Biotechnic & Histochemistry* **71**:145-151.
67. **Rose, J. B., P. R. Epstein, E. K. Lipp, B. H. Sherman, S. M. Bernard, and J. A. Patz.** 2001. Climate variability and change in the United States: potential impacts on water- and foodborne diseases caused by microbiologic agents. *Environmental health perspectives* **109 Suppl 2**:211-221.
68. **Roth, B. L., M. Poot, S. T. Yue, and P. J. Millard.** 1997. Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. *Applied and Environmental Microbiology* **63**:2421-2431.
69. **Sanchez-Galvez, A., P. Hunt, M. A. Robb, M. Olivucci, T. Vreven, and H. B. Schlegel.** 2000. Ultrafast radiationless deactivation of organic dyes: evidence for a two-state two-mode pathway in polymethine cyanines. *Journal of the American Chemical Society* **122**:2911-2924.
70. **Scott, J. A. G.** 2007. The preventable burden of pneumococcal disease in the developing world. *Vaccine* **25**:2398-2405.
71. **Seifert, J. L., R. E. Connor, S. A. Kushon, M. Wang, and B. A. Armitage.** 1999. Spontaneous Assembly of Helical Cyanine Dye Aggregates on DNA Nanotemplates. *Journal of the American Chemical Society* **121**:2987-2995.
72. **Shea, K. M.** 2000. Technical report: irradiation of food. Committee on Environmental Health. *Pediatrics* **106**:1505-1510.
73. **Smith, J. O., D. A. Olson, and B. A. Armitage.** 1999. Molecular Recognition of PNA-Containing Hybrids: Spontaneous Assembly of Helical Cyanine Dye Aggregates on PNA Templates. *Journal of the American Chemical Society* **121**:2686-2695.
74. **Stacey, D. W., and M. Hitomi.** 2008. Cell cycle studies based upon quantitative image analysis. *Cytometry, Part A* **73A**:270-278.
75. **Steinberg, T. H.** 2009. Protein gel staining methods: an introduction and overview. *Methods in enzymology* **463**:541-563.
76. **Straight, A. F.** 2007. Fluorescent protein applications in microscopy. *Methods in Cell Biology* **81**:93-113.

77. **Sutharsan, J., D. Lichlyter, N. E. Wright, M. Dakanali, M. A. Haidekker, and E. A. Theodorakis.** 2010. Molecular rotors: synthesis and evaluation as viscosity sensors. *Tetrahedron* **66**:2582-2588.
78. **Swaminathan, R., S. Bicknese, N. Periasamy, and A. S. Verkman.** 1996. Cytoplasmic viscosity near the cell plasma membrane: translational diffusion of a small fluorescent solute measured by total internal reflection-fluorescence photobleaching recovery. *Biophysical Journal* **71**:1140-1151.
79. **Thomas, M. S., J. M. Clift, B. Millare, and V. I. Vullev.** 2010. Print-and-Peel Fabricated Passive Micromixers. *Langmuir* **26**:2951-2957.
80. **Tolstorukov, M. Y., R. L. Jernigan, and V. B. Zhurkin.** 2004. Protein-DNA Hydrophobic Recognition in the Minor Groove is Facilitated by Sugar Switching. *Journal of Molecular Biology* **337**:65-76.
81. **Tomlinson, A., B. Frezza, M. Kofke, M. Wang, B. A. Armitage, and D. Yaron.** 2006. A structural model for cyanine dyes templated into the minor groove of DNA. *Chemical Physics* **325**:36-47.
82. **Travlos, G. S.** 2006. Normal structure, function, and histology of the bone marrow. *Toxicologic pathology* **34**:548-565.
83. **Vasquez, J. M., A. Vu, J. S. Schultz, and V. I. Vullev.** 2009. Fluorescence enhancement of warfarin induced by interaction with beta -cyclodextrin. *Biotechnology Progress* **25**:906-914.
84. **Veal, D. A., D. Deere, B. Ferrari, J. Piper, and P. V. Attfield.** 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. *Journal of Immunological Methods* **243**:191-210.
85. **Vogt, G., G. Krampert, P. Niklaus, P. Nuernberger, and G. Gerber.** 2005. Optimal Control of Photoisomerization. *Physical Review Letters* **94**:068305/068301-068305/068304.
86. **Vogt, R. F., Jr., A. Schwartz, G. E. Marti, W. E. Whitfield, and L. O. Henderson.** 2001. Quantitative fluorescence cytometry. *Methods in Molecular Medicine* **55**:255-273.
87. **Vogt, R. F., Jr., W. E. Whitfield, L. O. Henderson, and W. H. Hannon.** 2000. Fluorescence Intensity Calibration for Immunophenotyping by Flow Cytometry. *Methods (Orlando, Florida)* **21**:289-296.
88. **Vullev, V. I., H. Jiang, and G. Jones, II.** 2005. Excimer sensing. *Topics in Fluorescence Spectroscopy* **10**:211-239.

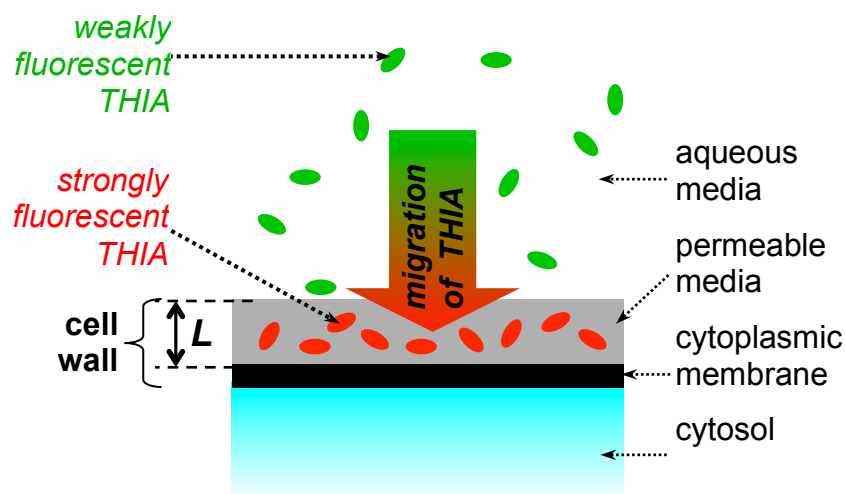
89. **Vullev, V. I., and G. Jones.** 2002. Photoinduced electron transfer in alkanoylpyrene aggregates in conjugated polypeptides. *Tetrahedron Letters* **43**:8611-8615.
90. **Vullev, V. I., J. Wan, V. Heinrich, P. Landsman, P. E. Bower, B. Xia, B. Millare, and G. Jones, II.** 2006. Nonlithographic Fabrication of Microfluidic Devices. *Journal of the American Chemical Society* **128**:16062-16072.
91. **Vullev, V. I., J. D. Wan, V. Heinrich, P. Landsman, P. E. Bower, B. Xia, B. Millare, and G. Jones.** 2006. Nonlithographic fabrication of microfluidic devices. *Journal of the American Chemical Society* **128**:16062-16072.
92. **Wan, J., M. S. Thomas, S. Guthrie, and V. I. Vullev.** 2009. Surface-bound proteins with preserved functionality. *Annals of biomedical engineering* **37**:1190-1205.
93. **Wan, J. D., M. S. Thomas, S. Guthrie, and V. I. Vullev.** 2009. Surface-Bound Proteins with Preserved Functionality. *Annals of biomedical engineering* **37**:1190-1205.
94. **Wang, M., G. L. Silva, and B. A. Armitage.** 2000. DNA-Templated Formation of a Helical Cyanine Dye J-Aggregate. *Journal of the American Chemical Society* **122**:9977-9986.
95. **Young, A. L.** 2003. Food irradiation: after 35 years, have we made progress. A government perspective. *Environmental science and pollution research international* **10**:82-88.
96. **Zielins, E., M. Thomas, and V. I. Vullev.** 2008. Bacterium-Induced Fluorescence-Enhancement Kinetics: Breaking 100-Year Old Traditions of Staining Bioanalyses. *UCR Undergraduate Research Journal* **2**:75-82.

1.6 SCHEMES

Scheme 1. Structural formula of 3,3'-diethylthiacyanine (THIA).



Scheme 2. Fluorescence enhancement due to migration of THIA from the aqueous surrounding into the fluorogenic cell-wall media.



The cell wall is modeled as a sheet of permeable media over a membrane, which is not permeable for the dye. The thickness of the permeable sheet is L and the diffusion coefficient of the dye in it is D . For Gram-positive species the permeable is a simplified representation of the peptidoglycan coating of the periplasmic side of the cytoplasmic membrane. For Gram-negative species, this representation of the cell wall is, indeed, a further oversimplification approximating the periplasmic space with the peptidoglycan layer, the outer membrane, and the lipopolysaccharide layer as a homogeneous permeable sheet layer. Nevertheless, this simplistic model allows us to elucidate the kinetic trends of emission enhancement for THIA in the presence of bacterial species.

1.7 TABLES

Table 1. Fluorescence quantum yield of THIA, Φ_f , for solvents with different dielectric constants, ϵ , and different dynamic viscosity, η .^a

solvent	$\Phi_f \times 10^3$	ϵ	η / cP
water	2.0	81	0.89
ethylene glycol	12	41	17
glycerol	320	43	930
methanol	0.67	33	0.55
ethanol	1.0	24	1.2
1-butanol	2.4	18	2.5

^a Solution of coumarin 151 in ethanol was used as a standard ($\Phi_f^{(\text{coumarin 151})} = 0.49$; $\lambda_{\text{ex}} = 400$ nm).

Table 2. Time constants, τ / s (represented in bold), for the enhancement of the fluorescence of THIA induced by four vegetative bacteria for different dye concentration, C_{THIA} , and cell count, CC , in the presence of TWEEN[®] surfactant.^a

C_{THIA} / mM	Gram-negative, CC / cell ml ⁻¹			Gram-positive, CC / cell ml ⁻¹		
	5×10^6	5×10^7	5×10^8	5×10^6	5×10^7	5×10^8
	<i>Escherichia coli</i> ^b			<i>Bacillus subtilis</i> ^c		
0.6	34 ± 4	33 ± 6	22 ± 6	3.1 ± 1.7	2.2 ± 0.8	3.8 ± 1.4
6	27 ± 2	27 ± 6	29 ± 4	3.9 ± 1.0	3.9 ± 1.6	2.8 ± 1.1
60	31 ± 6	36 ± 5	26 ± 1	5.1 ± 1.1	2.6 ± 0.4	2.9 ± 0.3
	$p_{\text{cell}} = 0.07^{\text{d}}$, $p_{\text{cell}}^{(\text{NR})} = 0.30^{\text{e}}$ $p_{\text{dye}} = 0.08^{\text{d}}$, $p_{\text{dye}}^{(\text{NR})} = 0.67^{\text{e}}$			$p_{\text{cell}} = 0.03^{\text{d}}$, $p_{\text{cell}}^{(\text{NR})} = 0.40^{\text{e}}$ $p_{\text{dye}} = 0.45^{\text{d}}$, $p_{\text{dye}}^{(\text{NR})} = 0.77^{\text{e}}$		
	<i>Enterobacter aerogenes</i> ^b			<i>Bacillus sphaericus</i> ^b		
0.6	21 ± 7	19 ± 5	20 ± 10	52 ± 9	59 ± 6	58 ± 7
6	16 ± 3	17 ± 2	22 ± 3	28 ± 13	52 ± 8	38 ± 12
60	20 ± 3	17 ± 4	20 ± 6	54 ± 7	52 ± 8	51 ± 3
	$p_{\text{cell}} = 0.30^{\text{d}}$, $p_{\text{cell}}^{(\text{NR})} = 0.30^{\text{e}}$ $p_{\text{dye}} = 0.53^{\text{d}}$, $p_{\text{dye}}^{(\text{NR})} = 0.50^{\text{e}}$			$p_{\text{cell}} = 0.01^{\text{d}}$, $p_{\text{cell}}^{(\text{NR})} = 0.35^{\text{e}}$ $p_{\text{dye}} = 0.08^{\text{d}}$, $p_{\text{dye}}^{(\text{NR})} = 0.10^{\text{e}}$		

^a Aqueous Tris buffer (2 mM, pH = 8.5) with 0.5 mM TWEEN[®] 40; 37 °C; $\lambda_{\text{ex}} = 420$ nm, $\lambda_{\text{em}} = 480$ nm; the bacterial samples were from cultures in their exponential growth phase.

^b The time constants represent the slower components from biexponential fits (Figure 2a).

^c The time constants were obtained from monoexponential fits.

^d The p -values were obtained from two-factor ANOVA analysis of the time constants for the same species with THIA considering all measurement repeats.

^e The non-repeat p -values, $p^{(\text{NR})}$, were obtained from two-factor ANOVA considering solely the average values for each concentration / cell density data point.

Table 3. Results from one-factor ANOVA analysis comparing between the time constants obtained for the four investigated species (Table 2).^a

	<i>E. coli</i>	<i>E. aerogenes</i>	<i>B. subtilis</i>
<i>E. aerogenes</i>	$p = 5.1 \times 10^{-12}$ ($F = 79$) ^b $p^{(NR)} = 8.7 \times 10^{-6}$ ($F = 41$) ^c	—	—
<i>B. subtilis</i>	$p < 10^{-16}$ ($F = 730$) ^b $p^{(NR)} = 7.8 \times 10^{-12}$ ($F = 300$) ^c	$p < 10^{-16}$ ($F = 510$) ^b $p^{(NR)} = 1.4 \times 10^{-13}$ ($F = 510$) ^c	—
<i>B. sphaericus</i>	$p = 1.7 \times 10^{-10}$ ($F = 63$) ^b $p^{(NR)} = 5.6 \times 10^{-5}$ ($F = 29$) ^c	$p < 10^{-16}$ ($F = 160$) ^b $p^{(NR)} = 1.5 \times 10^{-7}$ ($F = 78$) ^c	$p < 10^{-16}$ ($F = 390$) ^b $p^{(NR)} = 2.8 \times 10^{-10}$ ($F = 190$) ^c

^a For the comparison of the species from each column with the species from each row, the p -values were estimated from the F -ratios (shown in parentheses), obtained from one-factor ANOVA analysis.

^b For the p -values comparing between each two species, the time constants for all measured repeats, for all THIA concentrations, and for all cell counts were considered.

^c For the non-repeat p -values, $p^{(NR)}$, comparing between each two species, solely the average time constants, for all THIA concentrations, and for all cell counts were considered.

1.8 FIGURES

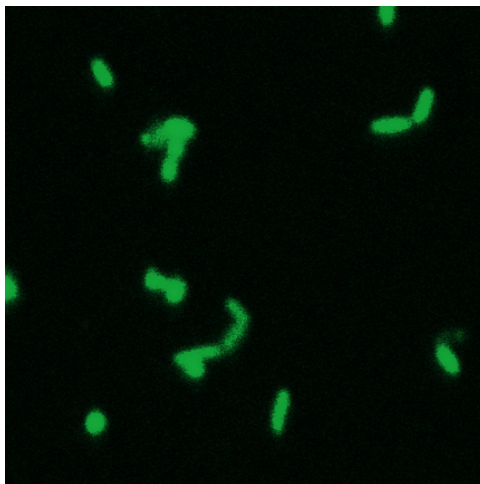


Figure 1. Fluorescence image of *E. coli* in 60 μM THIA solution, recorded with a confocal microscope ($\lambda_{\text{ex}} = 364 \text{ nm}$). The bacterial suspension was deposited on aminated glass slide with the THIA solution (60 μM), and allowed to incubate for 10 minutes prior the imaging.

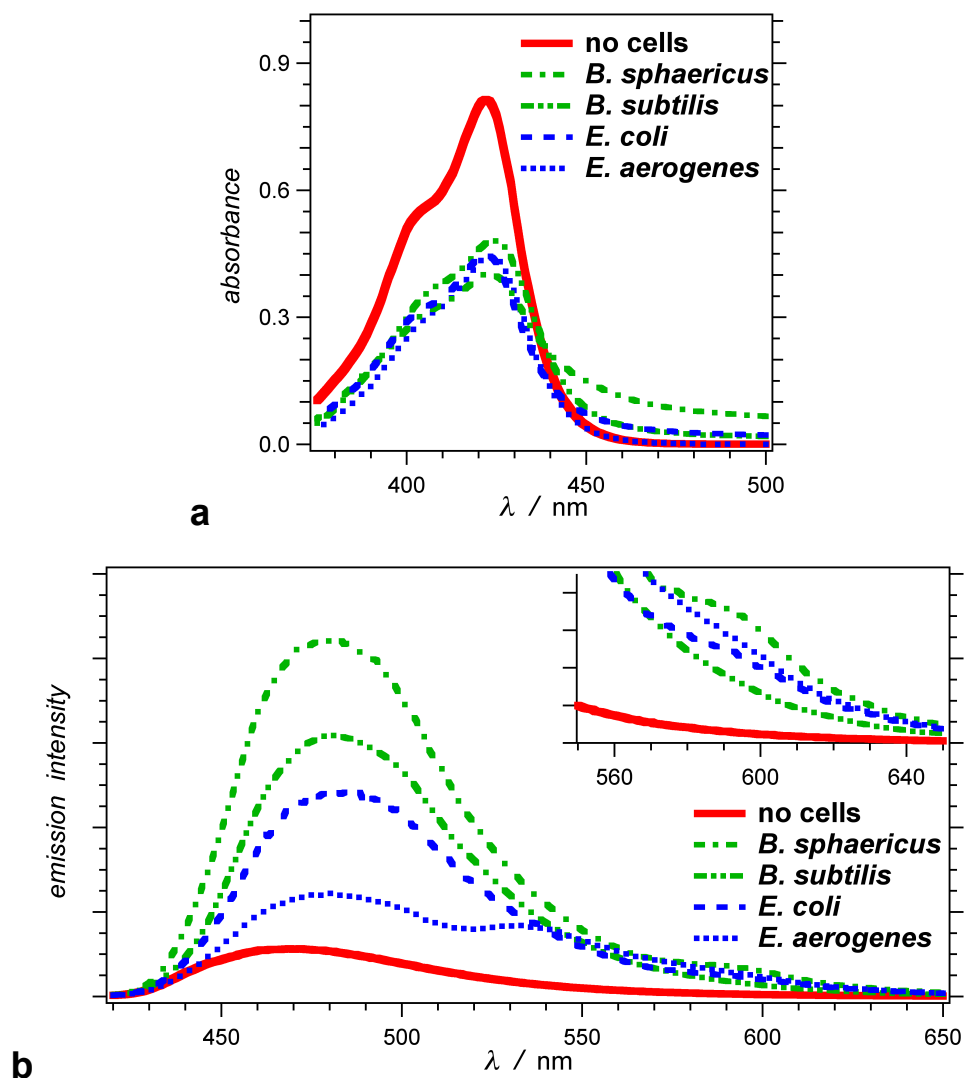


Figure 2. Absorption and fluorescence of THIA (6 μM) in the presence and absence of bacterial cells (cell count, $CC = 5 \times 10^7$ cell mL^{-1}). All samples were prepared with 2 mM aqueous Tris buffer, pH = 8.5, containing 0.5 mM TWEEN[®] 40. The spectra in the presence of bacteria were recorded after 15 min incubation of the cell suspension with THIA. **(a)** Absorption spectra. Baseline corrections, using cell suspensions with no dye, were applied. **(b)** Emission spectra ($\lambda_{\text{ex}} = 420$ nm).

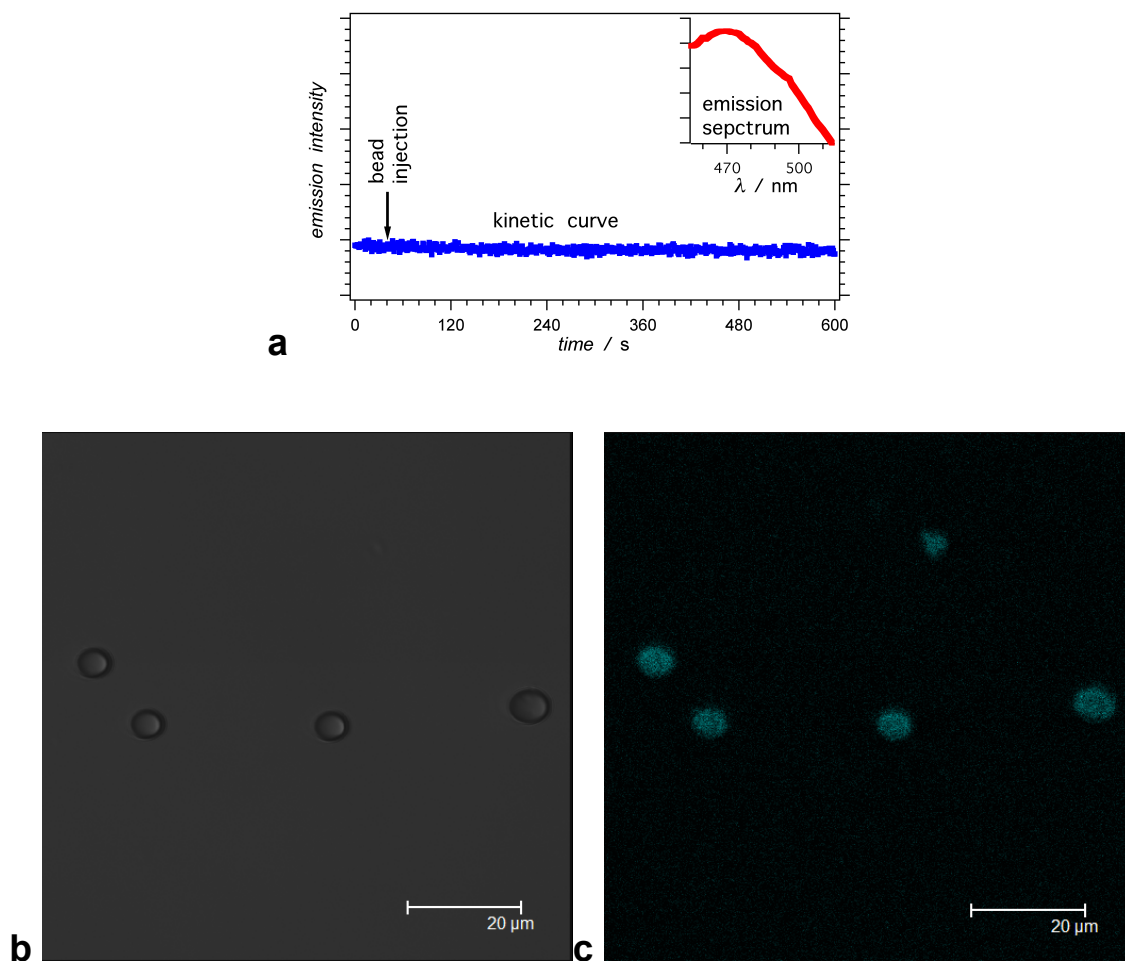


Figure 3. Interaction between THIA and carboxylate-terminated polystyrene beads (5 μm diameter). **(a)** Kinetic curve ($\lambda_{\text{ex}} = 420 \text{ nm}$, $\lambda_{\text{em}} = 480 \text{ nm}$) and emission spectrum ($\lambda_{\text{ex}} = 420 \text{ nm}$) of THIA (6 μM) in the presence of the beads ($1.5 \times 10^7 \text{ beads ml}^{-1}$). **(b)** Phase-contrast image of the beads immersed in THIA solution (600 μM). **(c)** Fluorescence image of the beads immersed in THIA solution (600 μM ; $\lambda_{\text{ex}} = 364 \text{ nm}$).

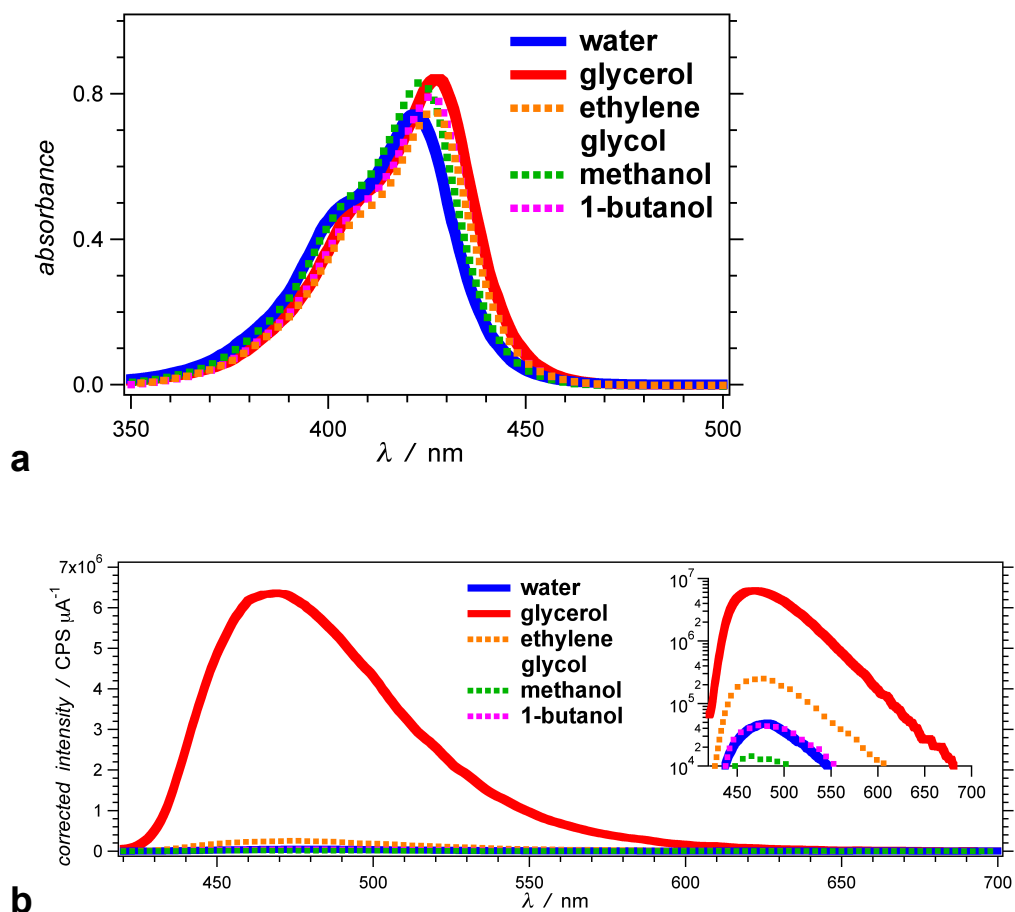


Figure 4. Absorption and emission spectra of THIA (6 μM) for water and different alcohols: **(a)** absorption spectra; **(b)** emission spectra ($\lambda_{\text{ex}} = 400$ nm). Inset represents the same spectra plotted against logarithmic ordinate.

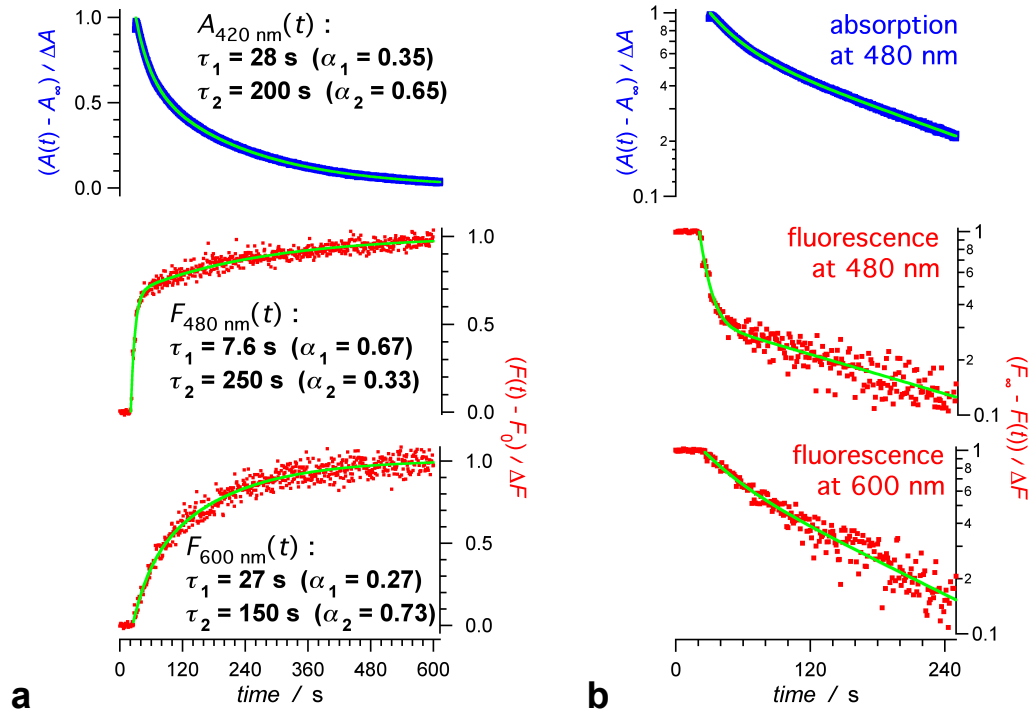


Figure 5. Kinetics of changes induced on the absorption and emission of THIA (6 μM) by *E. coli* ($2 \times 10^7 \text{ cell ml}^{-1}$) for aqueous media buffered with 2 mM Tris buffer, pH = 8.5, with no TWEEN[®] added, at 37 °C. **(a)** Top curve: normalized absorption change, monitored at 420 nm ($A_\infty = A_0 - \Delta A$, equation 1b). Middle curve: normalized fluorescence change, monitored at 480 nm ($\lambda_{\text{ex}} = 420 \text{ nm}$; equation 1a). Bottom curve: normalized fluorescence change, monitored at 600 nm ($\lambda_{\text{ex}} = 420 \text{ nm}$; equation 1a). **(b)** The same spectral changes plotted against logarithmic ordinate. The normalized emission rise features were inverted and represented as decays in order to discern the exponential components with different time constants ($F_\infty = F_0 + \Delta F$, equation 1a).

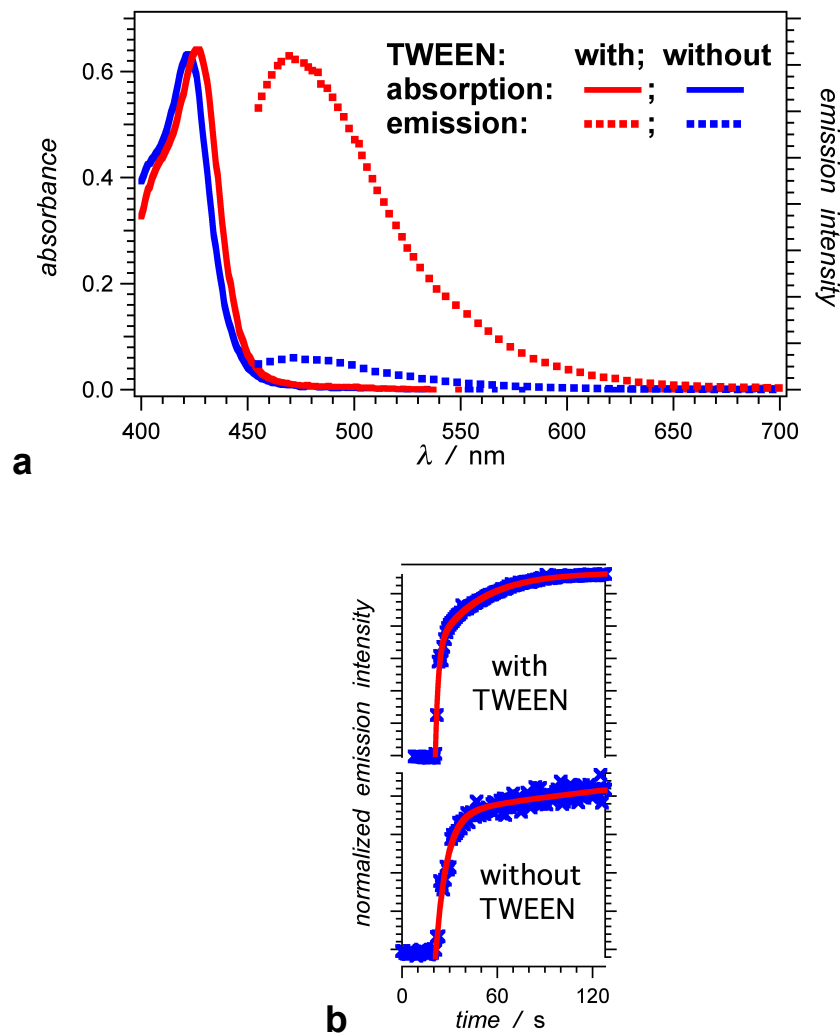


Figure 6. Spectral properties of THIA in the presence and absence of TWEEN[®]. **(a)** Absorption and emission spectra of THIA (5 mM; $\lambda_{\text{ex}} = 420$ nm) in the absence and presence of TWEEN[®] 40 (27 mM). **(b)** Kinetics of the THIA emission enhancement (6 μM ; $\lambda_{\text{ex}} = 420$ nm; $\lambda_{\text{em}} = 480$ nm) induced by *E. coli* (5×10^7 cell ml^{-1}) in the absence and presence of TWEEN[®] 40 (0.5 mM). The blue markers, $\times$, represent the data points; the red solid lines, —, represent the corresponding exponential fits (equation 1a).

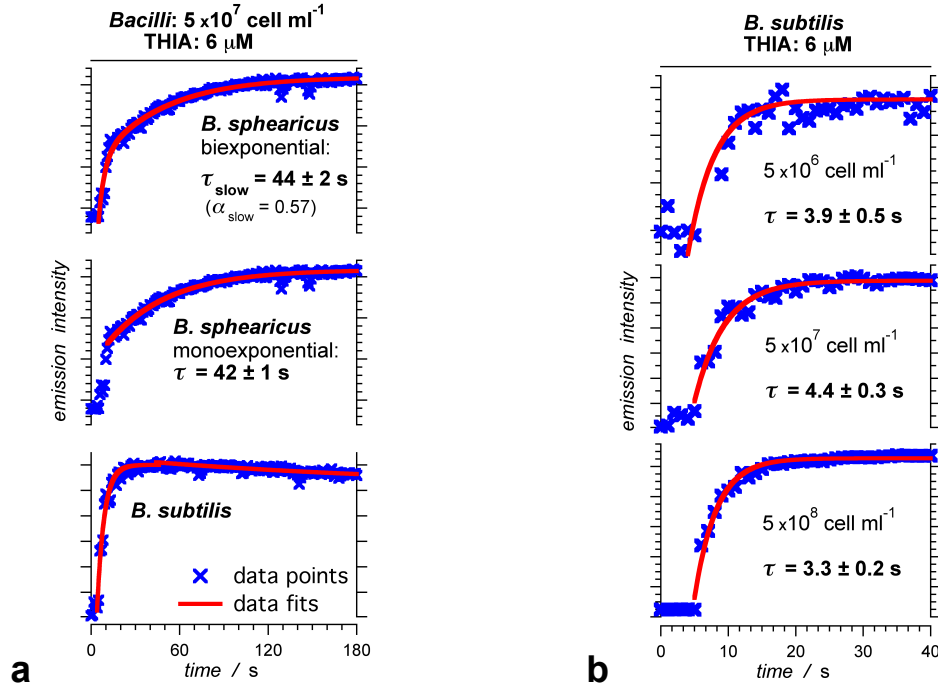
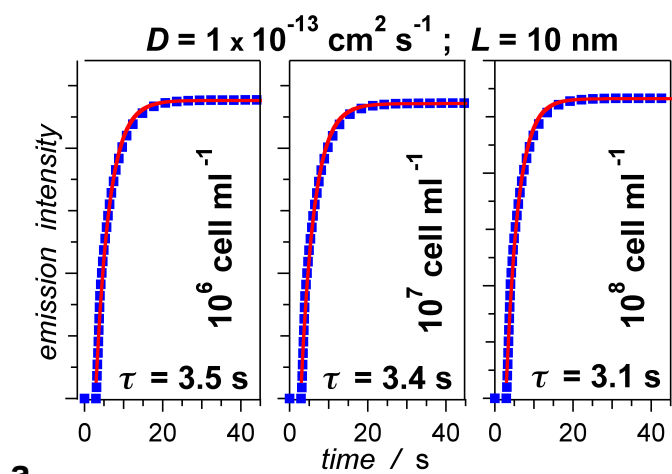
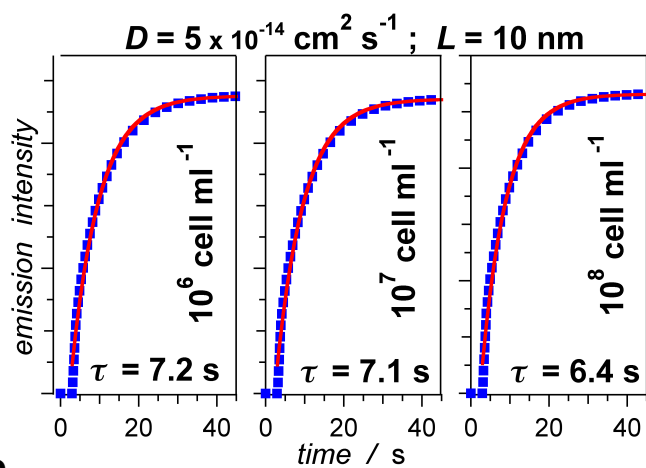


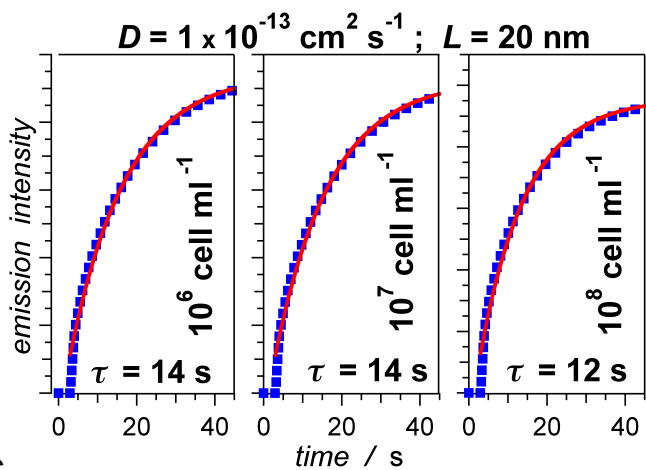
Figure 7. Fluorescence-enhancement kinetic curves ($\lambda_{\text{ex}} = 420$ nm, $\lambda_{\text{em}} = 480$ nm, 37 °C) for *bacilli* samples injected into 6 μM THIA solution (at $t_0 \approx 5$ s, equation 1a). All samples were prepared with 2 mM aqueous Tris buffer, pH = 8.5, containing 0.5 mM TWEEN[®] 40. **(a)** Kinetic curve for 5×10^7 cell ml^{-1} *B. sphearicus* (top and middle curves) and *B. subtilis* (bottom curve). (The fast rise prior to the slow intensity increase, asymptotically approaching the maximum fluorescence intensity, was characteristic for pretreatment with TWEEN[®] surfactant of *B. sphearicus*, *E. coli* and *E. aerogenes*.) For *B. sphearicus*, the monoexponential analysis yielded a time constant almost identical to the time constant corresponding to the slow component of the biexponential rise. **(b)** Kinetic curves for different cell counts of *B. subtilis* with the corresponding monoexponential fits ($n = 1$, equation 1). The error values represent the standard deviations yielded by the least square algorithms for each data fit.



a



b



c

Figure 8

Figure 8. Simulated curves for fluorescence enhancement (equation 2 and 3) resultant from the diffusion-driven migration of weakly-fluorescent dye into the fluorogenic environment of cell walls (Scheme 2). The blue squares represent the calculated values for different cell counts, different cell-wall thickness, L , and different diffusion coefficients, D . The red solid lines represent the corresponding monoexponential data fits ($n = 1$, equation 1a) that produced the different time constants, t . For all calculations, $F_C = 0.2$, $F_W = 0.001$, $K = 2 \times 10^4$, $t_0 = 3$ s, $V_C = 1$ fl, $S_C = 6$ mm², and the number of exponential terms for the sum of (equation 2) was 10^3 .

Chapter 2

Amyloid Histology Stain for Rapid Bacterial Endospore Imaging

ABSTRACT

Bacterial endospores are some of the most resilient forms of life known to us, with their persistent survival capability resulting from a complex and effective structural organization. The outer membrane of the endospores is surrounded by the densely packed endospore coat and exosporium, containing amyloid or amyloid-like proteins. In fact, it is the impenetrable composition of the endospore coat and the exosporium that makes staining methodologies for endospore detection complex and challenging. Therefore, a plausible strategy for facile and expedient staining would be to target components of the protective surface layers of the endospores. That is, instead of targeting endogenous markers encapsulated in the spores, here we demonstrated staining of these dormant life entities that target the amyloid domains: i.e., the very surface components that make the coats of these species impenetrable. Using an amyloid staining dye, thioflavin T (ThT), we examined this strategy. Short incubation of bacilli endospore suspensions with ThT, under ambient conditions, resulted in: (i) an enhancement of the fluorescence of ThT; and (ii) accumulation of ThT in the endospores, affording fluorescence images with excellent contrast ratios. Fluorescence images revealed that ThT tends to accumulate in the surface

regions of the endospores. The observed fluorescence enhancement and dye accumulation, coupled with the sensitivity of emission techniques, provide an effective and rapid means of staining endospores without the inconvenience of pre- or post-treatment of samples.

2.1 Introduction

This chapter describes the use of a benzothiazole dye, thioflavin T (ThT) (Scheme 3), for expedient and facile fluorescence staining of bacilli endospores. Using fluorescence microscopy and spectroscopy, we examined the capabilities of ThT to stain the endospores of four bacterial species. Our findings showed that the observed efficient staining resulted from: (i) an increase in the emission quantum yield of ThT upon its uptake by endospores; and (ii) a high propensity of ThT to accumulate in the endospores.

Bacterial endospores are some of the most resilient living entities known (8, 14, 59). Although the majority of the sporulating species are not virulent, spore-forming bacteria produce the most potent toxins known to humans, such as botulinum toxin (2, 57). This potential threat of highly virulent biohazards, along with their resistance to treatment, places a demand for rapid and simple bioanalytical methods for detection of bacterial endospores. Such robust bioanalytical methods are not only essential for biodefense, but also relevant to clinical and microbiological applications (1, 31, 69, 73).

The classical technique of endospore detection uses malachite green dye to stain the endospore coat via thermal treatment or long incubation periods (74). Genetic bioassays have since evolved utilizing polymerase chain reaction (PCR) to provide the specificity required for identification of certain sporulating pathogens (5, 21, 54, 71). Employing these techniques for detection of bacterial endospores, however, is a multistep process and requires prior sequence knowledge for the design of the primer (32, 45, 92).

Other signal-amplification techniques, such as enzyme-linked immunosorbent assay (ELISA), provide sensitivity for detection of endospore pathogens with potentially high specificity, without tedious sample pretreatment (81, 84). Endospore immunoassays, however, require: (i) identification of a protein or another biomarker at the surface of the endospore particles; and (ii) development of immunoglobulins (i.e., antibodies) for that surface biomarker (32, 67, 92). In order to reliably identify endospores, the immunoassays often require several fold higher concentration than the infectious dose or several distinct antibodies to detect one of several strains of the same sporulating pathogenic species (20, 49, 67, 101).

A variety of optical and mass-spectrometry techniques have been explored for detection of bacterial endospores without requiring signal-amplification biochemical reactions. For example, surface-enhanced Raman spectroscopy (SERS) brings the detection to relatively low spore count, and even to a single-spore level (66, 106). Mass spectrometry provides means for identification of macromolecular markers based on their exact masses (6, 82). The specialized equipment needed, along with the complexity of the data analysis, has proven prohibitive for the wide deployment of such bioanalytical methodologies into the clinical field.

For the last decade, dipicolinic acid (DPA) has gained considerable popularity as a marker for detection of bacterial endospores (17, 24, 27, 61, 68, 87, 98, 104). Emission-enhancement assays for DPA and bacterial endospores, utilizing lanthanide ions, were developed in Army Research Laboratory in the end of the 20th century (61, 70) and recently improved by a research team in the Jet Propulsion Laboratory (9, 10). Up to

about 20% of the weight of bacterial endospores can be attributed to calcium dipicolinate: i.e., DPA is a natural product observed solely in sporulated bacterial species (79, 80). Furthermore, DPA has high affinity for chelating lanthanide metal ions, such as terbium (III) (37, 38). Excitation of the DPA ligands at about 270 nm to 280 nm, at which the lanthanides do not have allowed spectral transitions, permits selective photoexcitation of the DPA-chelated ions. This selective photoexcitation of DPA, followed by a fast energy transfer from the DPA ligands to the chelated ions, results in luminescence predominantly from the lanthanides that are in a complex with DPA.

These lanthanide emission assays are general for all bacterial endospores and do not provide discernibility between virulent and benign sporulated species. The simplicity, cost efficiency and relative sensitivity, however, make these emission-enhancement techniques a preferred choice for the first line of defense: i.e., for monitoring the environmental endospore content. Abnormal fluctuations, i.e., an increase in the spore count in the environment would call for deployment of more costly bioanalytical technologies with higher specificity. Due to the expedience and relative simplicity, lanthanide emission-enhancement approaches are also deployed for assaying alternative biomarkers with clinical importance (17).

The use of DPA as an endogenous biomarker requires sample pretreatment to lyse the endospores for extracting their content without chemically deteriorating it. The resilience of bacterial endospores, however, makes the DPA extraction step, which requires heat treatment, surfactant additives, or microwaving, a source of potential irreproducibility in the quantification of the results. Furthermore, the lysing of the

endospores, as a pre-treatment for extraction of the DPA, makes this bioanalytical approach unfeasible for imaging applications.

Alternative biomarkers, which are in abundance on the surface of the endospore particulates, will provide venues for facile detection without requirement for lysing pretreatment of the samples. High-resolution scanning-probe images, for example, revealed significant abundance of β -sheet amyloid-like proteins on the surface of bacterial endospores (62-65). Amyloid folds are, indeed, intricate components of bacterial cell walls and of endospore coatings (19, 22, 25, 43, 62-65).

Amyloid fibrils are aggregated filamentous protein structures found in more than 20 diseases, including the amyloidosis and several neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (13, 25, 33, 53, 103). The structural properties of amyloid fibrils and the kinetics of their formation have been objects of intensive investigation. Among techniques developed in the field, amyloid fluorescence stains served as the dominant method. Among the amyloid stains, thioflavin T (ThT) is the most widely used (7). Furthermore, ThT does not interact with folded or partially folded monomeric proteins, soluble oligomers, or amorphous aggregates.(50) Changes in ThT fluorescence are imperceptible even if interactions with such impurities in the sample occur (44, 46, 50, 55, 58, 60, 94).

Due to the relatively high inherent sensitivity of emission techniques, fluorescence staining and imaging are the foundation for widely preferred bioanalytical methodologies (48, 51). Herein, we explored the possibility for fluorescence staining of bacterial endospores with ThT (Scheme 3). For this study we focused on *Bacillus subtilis*

(*B. subtilis*) and *Bacillus globigii* (*B. globigii*), which are phylogenetically related organisms and are frequently explored as models for virulent bacilli. In addition, we also used *Bacillus atrophaeus* (*B. atrophaeus*) and *Geobacillus stearothermophilus* (*G. stearothermophilus*), which form relatively large endospores, suitable for optical imaging.

For the staining, we relied on the specificity of ThT for amyloid folds as endogenous markers occurring on bacterial endospore surfaces and in the cell walls of vegetative bacteria. The lack of requirements for sample pretreatment not only provided expedience, but also allowed for preserving the bacterial entities intact, which is essential for imaging. We observed high contrast between the ThT-stained bacterial endospores and the free dye remaining in the surrounding solution (Figure 9). Therefore, sample wash was not required after the staining and prior to imaging, which was another important factor contributing to the expedience of this approach. Our analysis revealed that the reasons for the observed high contrast of the fluorescence images of the bacterial species are: (i) an increase in emission quantum yield of ThT upon binding to bacterial endospores; and (ii) high-affinity of ThT for components of the examined bacterial species, leading to accumulation of the dye in the imaged bacteria: i.e., the concentration of ThT in the stained endospores was more than three orders of magnitude larger than the concentration of ThT in the surrounding staining solution.

2.2 Results

2.2.1 Staining of bacterial endospores with ThT

Treatment of bacterial samples with ThT allows for visualization of the endospores via fluorescence microscopy, employing excitation wavelength shorter than about 450 nm (Figure 9). For the staining, which tends to complete in less than five minutes as we previously reported (90), we incubated the bacterial samples in dye solutions with micromolar concentrations. The background fluorescence from the ThT (free in solution) was significantly less intense in comparison with the fluorescence from the stained endospores (Figure 10). Therefore, no washing of the staining dye, which was left over in the sample solution, was required in order to attain good quality images.

In fluorescence microscope images, the noise has direct current, DC, and alternative current, AC, components. The AC and DC contributions to S/N were estimated separately using the noise amplitudes and the average heights, $\langle h \rangle$, of the peaks on the traces crossing over imaged spores (Figure 10):

$$S/N_{DC}[\text{dB}] = 20 \lg \left(\frac{\langle h \rangle}{A_{DC}} \right) \quad \text{Equation (5a)}$$

$$S/N_{AC}[\text{dB}] = 20 \lg \left(\frac{\langle \Delta h \rangle}{RMS} \right) \quad \text{Equation (5b)}$$

where the multiple of 10 in front of the logarithms converts the units to dB; and

$$\langle \Delta h \rangle = \langle h \rangle - A_{DC}.$$

Signal-to-noise analysis revealed key trends in the quality of the images recorded under different settings and staining conditions. As expected, S/N increased with the prolongation of the exposure time, i.e., $S/N \propto \sqrt{\text{exposure time}}$. A five-fold increase in the exposure time doubled S/N_{DC} and caused about a 30% increase in S/N_{AC} of images of spores suspended in 100 μM ThT.

Imaging of relatively large bacterial endospores (i.e., endospores larger than about 1 μm) revealed their contours and provided information about their shapes and dimensions (Figure 9). For organisms that form endospores with sub-micrometer dimensions, however, the size of the imaged endospores approached the diffraction-limit of optical microscopy and their shapes and dimensions could not be always extracted with sufficient precision (Figure 11).

Examination of the fluorescence images of the stained bacterial endospores, which were large enough for visualizing their shapes via optical microscopy, revealed (Figure 9): (i) consistently bright pixels following the contours of the endospores; and (ii) bright spots distributed throughout the endospore images. The latter observation suggested a heterogeneous distribution of the endogenous biomarkers (e.g., β -sheet proteins) that are targeted by the amyloid-stain. The former finding suggested that the coating layers of the endospores had a pronounced propensity for accumulating the staining dye. Our observation for surface accumulation of ThT was consistent with the abundance of β -sheet proteins found on the endospore surfaces (25, 62-65).

Amyloid type proteins, however, are native not only to the endospore surfaces but also to the cell walls of vegetative bacteria (including the Gram-positive spore-forming

species) (19, 22, 25, 43). While ThT provided an excellent basis for staining bacterial endospores with considerable ease (at ambient temperature and with no sample pre-treatment or post-treatment), it also stained vegetative bacteria: Gram positive (Figure 11c) and Gram negative (Figure 11e and f). The morphologies of vegetative bacterial cells and their endospores, however, are quite different and they can be readily discerned from each other by comparing their shapes and sizes even when they are in the same sample (Figure 11d, sporulating bacteria).

2.2.2 Spore-induced emission enhancement of ThT

The brightness of a fluorescence image depends on the number of dye molecules at the focal plane (or in the vicinity of the focal plane), and on the emission quantum yield of the dye. The number of molecules within the focal volume of illumination (i.e., the local concentration of the dye) determines how much of the excitation light is absorbed. Concurrently, the emission quantum yield determines how much of the absorbed light is emitted by the dye (at wavelengths longer than the excitation wavelength, λ_{ex}). Therefore, potential reasons for the observed fluorescence staining of the bacterial endospores with ThT include: (i) an increase in the emission quantum yield of ThT upon binding to the endospores; and (ii) accumulation of ThT in the endospores.

Upon addition of endospores to ThT solution, we observed an increase in its emission intensity without significant change in its optical density at the excitation wavelength (Figure 12). This finding was indicative that an increase in the ThT emission

quantum yield was one of the factors governing the observed emission enhancement upon endospore uptake of the dye.

The observed emission enhancement phenomenon, induced by the endospore uptake, is consistent with the sensitivity of the photo-physical properties of ThT to the viscosity of the environment: i.e., the emission quantum yield of ThT increases with the increase in the medium viscosity (76, 78, 85, 86, 93, 95). Due to the single carbon-carbon bond between the two ring systems and the single carbon nitrogen bond between the phenyl ring and the dimethyl amine (Scheme 3) ThT is a molecular rotor(26, 77, 78, 85). Similar to other chromophores that are molecular rotors (3, 4, 30, 40), within the lifetime of the lowest singlet-excited state of the dye, viscous microenvironment slows down and/or completely restrains the attainment of ThT conformers (i.e., rotamers) that have the density of vibrational states allowing efficient internal conversion to the ground state. Such viscosity-induced suppression of efficient non-radiative deactivation, indeed, results in an increase in the emission quantum yield of ThT, as demonstrated by photophysical studies of this staining agent for solvent media with varying viscosity (86). Binding of ThT to protein aggregates (or other macromolecular assemblies) has restrictive effect on its modes of molecular motion that is similar to the effect of increased medium viscosity: i.e., the binding sites provide microenvironment with relatively large effective viscosity and causes an increase in the fluorescence quantum yield of ThT (86).

For μM dye concentrations, the extent of emission enhancement steadily increased with the increase in the spore count (*SC*) in the suspension (Figure 13). This *SC*-dependence of the emission intensity was consistent with an increase in the

concentration of the spore-bound ThT, which has larger fluorescence quantum yield than the free ThT in the aqueous media. The power law representing the relation between fluorescence intensity and fluorophore concentration (100) provided an excellent fit for the dependence of the observed emission enhancement on the spore count (Figure 13c):

$$F(SC) = \Delta F(1 - 10^{-\alpha SC}) + F_0 \quad \text{Equation (6)}$$

where $F(SC)$ is the emission intensity of ThT for spore count SC ; F_0 is the emission intensity of ThT in the absence of endospores; and ΔF and α depend on the photo-physical properties of the dye. As depicted by the Bouguert-Lambert-Beer law (also known as Beer's law), while absorbance at the excitation wavelength, $A(\lambda_{\text{ex}})$, is linearly proportional to the fluorophore concentration, $A(\lambda_{\text{ex}})$ is logarithmically related with the intensity of the absorbed light. Therefore, broadly used linear correlations between fluorescence intensity and fluorophore concentration are approximation of a power law: i.e., $F(SC) \propto \alpha SC$ is an approximation of equation 6. The fluorescence intensity, $F(SC)$, depends on the concentration of ThT taken up by the endospores. Hence, for the spore count range, in which $F(SC)$ vs. SC follows the power law (equation 6), SC had to be linearly proportional to the concentration of ThT bound to the endospores.

Statistical tests for the presence of autocorrelation features, not depicted by the data fits, provide the means for examining the appropriateness of the fitting models for the examined data sets. An ideal model for data analysis results in random distribution of the data points around the regressed values for the data-fit function: i.e., the data-fit residuals should not exhibit autocorrelation. Durbin-Watson statistics, DW , provides the means for examining the presence of autocorrelation. DW can assume values between 0

and 4, and DW close to 2 signifies no autocorrelation, DW close to 0 indicates for positive autocorrelation (15, 28, 72, 99). In particular, DW smaller than a critical value with statistical significance α , $dwL_{\alpha,k}$, indicates for positive autocorrelation in the residuals from data fits with k regression parameters in addition to the intercept. Concurrently, $DW > dwU_{\alpha,k}$ indicates for no autocorrelation. For linear fits, $k = 1$, and for the nonlinear power-function fits (equation 6), $k = 2$. (The critical values $dwL_{\alpha,k}$ and $dwU_{\alpha,k}$ are obtained from Durbin-Watson significance tables for α , k , and for the number of examined data points n .) For the data set presented on Figure 13b and 13c, for $\alpha = 0.01$ and $n = 9$, $dwL_{0.01,1} = 0.554$ and $dwU_{0.01,1} = 0.998$ (for linear fits); and $dwL_{0.01,2} = 0.408$ and $dwU_{0.01,2} = 1.39$. For linear analysis, $DW = 0.841$ (Figure 13b) and $dwL_{0.01,1} < DW < dwU_{0.01,1}$ indicated for inconclusiveness in rejecting the presence of autocorrelation. Employing nonlinear analysis yielded $DW = 1.75$ (Figure 13c) and $DW > dwU_{0.01,2}$ proving a lack of autocorrelation with at least 99% certainty. This statistical analysis demonstrated the preferred feasibility for using a power law (equation 6), instead of linear relation, for correlating emission intensity with chromophore concentration.

2.2.3 Binding affinity of ThT for endospores

In addition to the increase in the emission quantum yield of ThT upon binding to the endospores, accumulation of the dye in the endospores will also enhance the imaging

contrast of the fluorescently stained species. The distribution coefficient, K_D , for ThT allowed us to quantify the propensity of the dye to accumulate in bacterial endospores:

$$K_D = \frac{C_{SPORE}}{C_{SOLUTION}} \quad \text{Equation (7)}$$

where C_{SPORE} and $C_{SOLUTION}$ are the concentrations of ThT in the endospore and in the aqueous solution, respectively, after completion of the staining process.

We incubated known amounts of endospores in ThT solutions with preset concentrations and absorbance (Figure 14a). After the incubation, we centrifuged the endospores and collected the supernatant. From the absorbance of the supernatant we determined $C_{SOLUTION}$ (Figure 14a). The difference between the absorbance of the ThT prior to the endospore treatment and the absorbance of the supernatant allowed us to estimate the amount of dye that was bound to the endospores and removed from the solution via the centrifugation step. Therefore, $C_{SPORE} = n_{ThT} / n_S V_S$, where n_{ThT} is the moles of ThT bound to the endospores, n_S is the number of endospores, and V_S is the volume of a single endospore estimated from their images.

For *B. globigii*, we obtained $K_D = 4 \times 10^3$ (Figure 14a). For *G. stearothermophilus*, a similar approach of incubating 2×10^7 spore ml^{-1} in 17 μM solution of ThT, yielded $K_D = 7 \times 10^3$. The measured distribution coefficient, K_D , however, exhibited dependence on the spore count, SC , and on the initial dye contrition, $C_{ThT}^{(0)}$, in which the spores were incubated. An increase in $C_{ThT}^{(0)}$ and a decrease in SC caused an increase in K_D (Figure 14b). This concentration dependence of K_D reflected the fact that a two-state/two-phase model (equation 7) did not encompass the complexity of the dye uptake by the

endospores. Nevertheless, the estimated K_D ranged between about 10^3 and 10^4 , and these values were indicative for the high propensity of ThT to accumulate in bacterial endospores: i.e., the average concentration of ThT in the endospores exceeded three orders of magnitude the dye concentration in the surrounding aqueous media. Therefore, the fluorescence staining of endospores with ThT is driven not only by the increase in the emission quantum yield of ThT upon uptake, but also by the high propensity of endospores to uptake ThT.

2.2.4 Imaging in the presence of contaminants

Despite the established pronounced affinity of ThT for β -amyloid types of protein structures prevalent in endospores and in the cell walls of vegetative bacteria (19, 22, 43, 62-65), binding of the positively charged chromophore (Scheme 3) to a range of other macromolecular structures presents a limitation for employing ThT as a stain for biomedical and environmental samples.

We examined the capability of ThT to selectively stain bacterial endospores in the presence of: a biochemical contaminant, deoxyribonucleic acid, DNA; a biomedical contaminant, fetal bovine serum, FBS; and an environmental contaminant, humic acid, HA (42, 83). The presence of these contaminants, indeed, increased the level of background fluorescence as reflected by the decrease in the DC component of the signal-to-noise ratios (equation 5a): i.e., S/N_{DC} was $5.5 \text{ dB} \pm 1.7 \text{ dB}$, $2.5 \text{ dB} \pm 0.2 \text{ dB}$ and $1.8 \text{ dB} \pm 1.5 \text{ dB}$ for the endospore containing DNA, FBS and HA samples, respectively.

While phase-contrast and differential interference contrast (DIC) microscopy provide means for visualization of bacterial endospores in relatively pure samples (12, 47, 105), they fail when substantial amounts of particulate contaminants are present. Fluorescence microscopy of ThT-stained samples, on the other hand, appears to have the potential for visualizing bacterial endospores even when such contaminants are prevalently present.

2.2.5 Signal to noise of staining bacterial endospores with ThT

The dynamic range of the recording equipment, however, placed limits on the S/N improvement resultant from extending the exposure time. Indeed, an increase in the exposure time increased not only the signal, but also the noise. For spore samples treated with 100 μM ThT, exposure time of 5 to 7 s allowed for attaining the maximum signal intensity, i.e., $\langle h \rangle \approx 6 \times 10^4$ (Figure 18a). Extending the exposure time beyond 7 s, thus, did not increase the signal (i.e., led to saturation), but increased the noise, i.e., A_{AC} and RMS (Figure 18a). As a result, extended exposure times (beyond ~ 7 s) resulted in deterioration of the measured S/N (Figure 18b).

The ThT concentration, C_{ThT} , had a less pronounced effect on the S/N , especially for short exposure times (Figure 18c). For 0.5-s exposure time, varying C_{ThT} over six orders of magnitude affected neither S/N_{AC} , nor S/N_{DC} (Figure 18c). For relatively short exposure times (e.g., 1 s), the baseline RMS manifested no dependence on C_{ThT} ; and the background fluorescence from the free ThT, ΔI , contributed less than $\sim 15\%$ to the

intensity of A_{DC} (Figure 10c, e). Conversely, for exposure times extending to 5 s, the optimal S/N_{AC} and S/N_{DC} appeared optimal for C_{ThT} in the range of tens and hundred mM (Figure 18c).

In aqueous media, ThT aggregates as its concentration increases above about 1 μ M (44, 75). An improved staining ability of aggregated ThT (44), therefore, can be an underlying plausible reason for the observed improvement in the image S/N as C_{ThT} is raised above 10^{-6} M.

It should be emphasized that it is S/N_{AC} that presents limitation for improving the quality of images. For processing of small signals on large DC background, inherent for numerous spectroscopic and imaging techniques (39, 96-98), analogue or digital subtraction of A_{DC} from the whole data set immensely improves S/N_{DC} , and the overall S/N . Improving S/N_{AC} , however, is considerably more challenging and less straightforward than the relatively facile optimization of S/N_{DC} . The images of the fluorescently stained endospores manifested $A_{DC} \gg RMS$, and hence $S/N_{AC} > S/N_{DC}$, revealing the promising potentials of ThT for facile visualization of dormant bacterial cells.

2.2.6 Signal to noise analysis of staining with contaminants

Despite the relatively low S/N_{DC} values, the stained spores were still visualizable in the presence of the contaminants (Figure 15e, j, o). Although the presence of the contaminants also compromised the AC component of S/N (equation 6b), the S/N_{AC} values were still large enough to allow for visualization of the endospores. The signals

from the stained endospores noticeably surpassed the background baseline noise, and S/N_{AC} was $40 \text{ dB} \pm 4 \text{ dB}$, $35 \text{ dB} \pm 1 \text{ dB}$ and $28 \text{ dB} \pm 10 \text{ dB}$ for the endospore containing DNS, FBA and HA samples, respectively.

The presence of DNA had no detectable effect on S/N_{AC} and a mildly noticeable effect on S/N_{DC} . Among the three contaminants, however, HA proved to be the most challenging for the ThT staining and the fluorescence imaging of the endospores. Nevertheless, S/N_{AC} exceeding 10 dB (even for the HA samples) still provide means for achieving acceptable image contrasts for visualizing the endospores.

2.3 Discussion

In this study, we hypothesized that an amyloid histology stain, thioflavin T (Scheme 3), allows for visualization of bacterial endospores via the means of fluorescence imaging. To test this hypothesis, we employed a range optical spectroscopy and microscopy techniques. The experimental evidence not only allows for accepting the hypothesis, but also elucidates some of the governing processes leading to the desirable staining outcomes.

What is the relationship between the observations from the three different techniques: i.e., between spectroscopy, imaging, and dye-distribution analysis? To answer this question, it is essential to consider the heterogeneous nature of the samples. The fluorescence spectra present the intensity of the light emitted from all dye molecules that are in the sample volume illuminated by the excitation beam. Therefore, the spectra

encompass the emission from the weakly fluorescent ThT free in solution and from the strongly fluorescent ThT taken up by the endospores. By far, most of the dye molecules in the stained samples are free in solution (Figure 14). As previously reported, an increase in the medium viscosity causes an increase in the ThT fluorescence quantum yield (86), which is expected for the properties of chromophores, such as ThT, classified as molecular rotors (4, 85). A viscosity-induced increase in the emission quantum yield of ThT amounts to about three orders of magnitude (86). Therefore, due to the relatively viscous microenvironment of the dye molecules bound within the endospores, the free ThT in the aqueous media has emission quantum yield that is about two-to-three orders of magnitude smaller than the quantum yield of ThT taken up by the spores.

For example, in samples containing 10^6 spore ml^{-1} , the total volume of the spores is about 10^6 times smaller than the volume of the aqueous media (i.e., a volume of a single spores is in the order of a femtoliter). Considering the measured distribution coefficients, K_D , of about $10^3 - 10^4$, indicates that in such samples, only about 0.1% to 1% of the dye is taken up by the endospores. While the number of dye molecules in the aqueous media is about 100 to 1,000 larger than the number of dye molecules in the endospores, the dye in the aqueous media has about two to three orders of magnitude smaller fluorescence quantum yield than the endospore-bound dye, which explains the relatively “moderate” emission enhancement (less than an order of magnitude for 10^6 spore ml^{-1}) spectroscopically observed (Figure 12 and 13). For this discussion, we considered negligible perturbation in the absorption of ThT when binding to the spores (as evident from the absorption and excitation spectra, Figure 12a and 13a, respectively),

and we did not include the multiple pathways of excitation and emitted light due to the scattering nature of the samples.

Because an increase in $C_{\text{ThT}}^{(0)}$ (within the μM range) tends to decrease K_D (Figure 14b), the endospore-induced enhancement of the emission of ThT is more pronounced for lower dye concentrations (Figure 12b vs. Figure 13b and 13c). Diluting the ThT staining solutions, thus, tends to improve the extent of the endospore-induced emission enhancement essential for spectroscopic and imaging applications. The sensitivity of the measurements, the background emission from endogenous fluorophores (no matter how weak it might be), the photostability of ThT, and the upper limit of K_D , imposes limitations on how low $C_{\text{ThT}}^{(0)}$ feasibly can be.

We readily imaged endospores using 1 nM ThT, and we demonstrated excellent correlations between spore count and emission intensity for 1 μM ThT (Figure 13b). These findings demonstrate an immensely wide working range of ThT concentrations, providing the basis for a broad applicability and optimization for each specific application that might resort to ThT-aided spectrofluorometry and imaging.

How do the images relate to the spectroscopic findings? The imaged contrast ratio between the fluorescence intensity corresponding to ThT-stained endospores, $\langle\Delta h\rangle + \Delta I$, and the emission intensity from the free ThT in the background, ΔI (Figure 10), considerably exceeds one and even two orders of magnitude. The endospores, however, occupy only a few percent (or less) of the total area of the images. As a result, the cumulative intensity from the pixels corresponding to the stained endospores does not exceed with more than about an order of magnitude the cumulative intensity from the

pixels corresponding to the image background. This trend is, indeed, in agreement with the fluorescence enhancement observed from the spectral data.

Overall, the accumulation of ThT in the bacterial endospores increases the dye local concentration, followed by increasing the amount of light absorbed and emitted by the stained endospores. The increase in the ThT emission quantum yield upon endospore uptake further enhances the intensity of the emitted light from the stained regions. The synergy between these two effects is essential for attaining images with sufficiently large signal-to-noise ratios, essential for visualization of bacterial endospores with acceptable contrast, especially when the background autofluorescence signal is prevalent.

Where does ThT bind? This study does not provide a direct answer. Indeed, ThT targets β -amyloid-type proteins with high affinity (44), and bacterial endospores are coated with β -strand proteins as evident, for example, from high-resolution AFM images (62-65). These facts provide the rationale of this study for staining endospores with ThT targeting their endogenous protein structures. The images of the ThT-stained endospores showed bright contours outlining their elliptical shapes, which is consistent with the dye collecting in the surface regions of the spores (where the high β -protein content has been demonstrated) (62-65), and with an increase in the fluorescence quantum yield of the dye bound to these endospores surface regions. Our evidence does not rule out the possibility for weakly fluorescent ThT present in the interior of the stained endospores. The pronounced density of intact endospores, however, suppresses facile diffusion of molecular species with the size of ThT into their interior (contributing to the resilience of these dormant species). Furthermore, unless endogenous fluorescence quenchers are

present, the rigid interior of endospores entrapping the dye and restraining its molecular motions will cause an increase in its fluorescence quantum yield (86). Therefore, most probably, the emission intensity of the ThT-stained endospore images represents the dye distribution throughout these dormant bacterial entities.

Furthermore, ThT staining did not noticeably affect the viability of bacterial endospores. Cell-culture tests of endospores incubated in various concentrations of ThT revealed that staining with this dye does not compromise their ability to germinate. Thus, ThT either does not bind to key components of the endospores essential for their germination, or does not negatively affect these molecular components even if it binds to them.

What are the practical implications of the endospore-induced ThT fluorescence enhancement? The tight correlations between the emission enhancement and the spore count (Figure 12b and 13b) illustrate the applicability for the use of ThT for spectroscopic quantification of endospore suspensions. Such spectroscopic measurements, however, are immensely susceptible to the presence of contaminants, such as vegetative bacterial cells, environmental polymers and blood proteins. Such restrictions, therefore, limits the ThT spectrofluorometry to pure endospore samples.

For imaging, on the other hand, ThT presents unexplored potentials. Based on their morphology, ThT-stained bacterial endospores are readily discernible in the presence of vegetative bacterial cells (Figure 11). Debris of lysed cells (due to sporulation, for example) does not compromise the visualization of the endospores (Figure 11d). Furthermore, staining with ThT provides means for facile visualization of bacterial

endospores in the presence of macromolecular contaminants that not only enhance the ThT fluorescence, but also contribute to the autofluorescence of the sample.

Speed and ease are the principal advantages of using amyloid stains for imaging bacterial endospores. The mild ambient conditions, under which the administration of such stains is conducted along with the absence of any pre- and/or post-treatment steps, provide important and unique capabilities for this approach of fluorescence imaging. Therefore, while classical methods for staining endospores, such as Schaeffer-Fulton stain, require a series of thermal fixing and wash steps, mixing of ThT solution with the bacterial sample without any further treatment allowed for simultaneous staining of vegetative bacteria and endospores during sporulation, for example (Figure 10d). ThT amyloid stain, indeed, provides an unprecedented facility for fluorescence imaging of bacterial endospores.

2.4 Conclusions

The ability of ThT to stain bacterial endospores was driven by its binding affinity, and by the increase in its fluorescence quantum yield upon uptake. While a variety of stains for vegetative cells are available for bioanalytical assays and imaging with minimal treatment, the choice of methodologies for enhancement and visualization of bacterial endospores is somewhat limited. Our demonstration of fluorescence imaging with an amyloid histology stain exhibits venues for utilization of alternative endogenous markers

(such as β -folded protein aggregates) for facile and expedient bioanalytical applications targeting bacterial endospores, known for their resilience and structural stability.

2.5 Experimental

2.5.1 Materials

The reagents and solvents (spectroscopic grade) were purchased from VWR, Fisher Scientific and Sigma-Aldrich. Bacterial endospore cultures: (i) *B. subtilis*, *B. globigii* and *Bacillus thuringiensis* (*B. thuringiensis*) were obtained from U.S. Army Research Laboratory facility, as desiccated powders. (ii) *B. atrophaeus* and *G. stearothermophilus* were purchased from SPS Medical (Rush, NY) in the form of 20% ethanol aqueous suspension. Vegetative bacteria cultures: *Escherichia coli* (*E. coli*), TOP 10 cells, were obtained from Invitrogen (Carlsbad, CA); and wild type *B. subtilis* were obtained from ATCC (Manassas, VA). (Details on sample preparation and handling are detailed in the experimental material section.)

For the imaging studies, thioflavin T (>95 % purity, Aldrich, Milwaukee, WI) was used as received. For some of the spectroscopy studies, the dye was further purified via recrystallization as a hexafluorophosphate salt. Comparison of the UV/visible absorption and emission spectra of the purified and the commercial-purity dye, however, did not reveal any detectable differences.

All other reagents, spectroscopic grade, were purchased from VWR and from Fisher Scientific. The buffered aqueous solutions were prepared using Millipore H₂O and filtered through 0.2 μ m polycarbonate syringe filters (Corning) before use. The notion of lowered protein binding affinity of ThT in acidic media (23), along with improved protein-induced fluorescence enhancement in slightly alkaline media (16), governed our choice of buffering the samples for this study at pH between 8.5 and 9. The absorption and excitation spectra showed that under the employed conditions, the used weakly alkaline media does not induce hydroxylation of ThT, as previously reported for elevated temperatures and prolonged exposure to aqueous media (18).

For contaminant studies, the deoxyribonucleic acid (DNA) was extracted from and purified using QIAGEN Plasmid Maxi Kit (purchased from QIAGEN Inc., Valencia, CA), humic acid and fetal bovine serum were purchased from Fischer Scientific.

2.5.2 Optical imaging

Bright field and fluorescence images were acquired using a Nikon Ti-U inverted microscope (Nikon Inc., Melville, NY), equipped with a 100 $\times$ Nikon oil immersion objective (N.A. = 1.49, WD = 120 μ m) and a Hamamatsu EM-CCD digital camera (model C9100-13, Hamamatsu Corp., Bridgewater, NJ). A 100-watt Xenon arc lamp (LUDL Electronic products, Hawthorne, NY), in combination with a FITC filter set (λ_{ex} = 482, λ_{em} = 536 nm, band widths = 25 nm, Chroma Technology, Bellows Falls, VT), was used for fluorescence imaging. The images were recorded with a Hamamatsu EM-CCD

digital camera (model C9100-13, Hamamatsu Corp., Bridgewater, NJ) controlled by HCImage application software; and saved in a 16-bit gray-scale format: i.e., $2^{16} = 65,536$ hues of gray for each pixel, where 0 corresponds to black (no signal) and 65,535 corresponds to white (maximum light intensity / saturation). Image analysis of the gray-scale distribution was performed using Wavemetrics Igor Pro software v.6.02A installed on Windows and Mac OS workstations as we have previously demonstrated (11, 29, 56, 88, 89).

2.5.3 Spectroscopy

Absorption spectra were recorded using DU-640B Beckman spectrophotometer and JASCO V-670 UV/Vis/NIR spectrophotometer. Steady-state emission spectra were recorded using Felix/Timemaster spectrofluorometer (Photon Technology International, Inc.), using slit width 10 nm; and Fluorolog-3-22 spectrofluorometer (Horiba Jobin Yvon) using slit widths between 2 and 5 nm (3, 4, 30, 52, 93, 99). All measurements were performed at ambient temperature, in polystyrene 2-sided and 4-sided cuvettes, with 1 cm optical paths (unless stated otherwise), after 10 – 15 min. pre-incubation in dark. Calibration and purity confirmation measurements of thioflavin T were reproduced in quartz cuvettes. The thioflavin T concentrations for all samples were estimated from its apparent molar absorption coefficient at peak wavelength 412 nm ($36,000 \pm 500 \text{ M}^{-1} \text{ cm}^{-1}$). (23)

Microplate reader fluorescence measurements with thioflavin T were performed in triplicates in black 96-well plates (Greiner), using SpectraMax Gemini XS fluorescence plate reader (Molecular Devices), at excitation wavelengths 430 nm and emission wavelength 495 nm, with filter cutoff 475 nm, after 2 min. shaking and 15 min. pre-incubation in dark. The pH of the aqueous solutions was monitored using an Orion 420A pH meter. Data analysis and calculations were performed using Igor Pro software v.6.02A (Wavemetrics) installed on Windows and Mac OS workstations (11, 29, 34-36, 40, 41, 56).

2.5.4 Bacterial samples

Bacterial endospore cultures: (i) *B. subtilis*, *B. globigii* and *Bacillus thuringiensis* (*B. thuringiensis*) were obtained from U.S. Army Research Laboratory facility, as desiccated powders. (ii) *B. atrophaeus* and *G. stearothermophilus* were purchased from SPS Medical (Rush, NY) in the form of 20% ethanol aqueous suspension. They were stored at 2°C suspended in deionized double distilled water (DDW) or in 40 % ethanol – DDW.

Before assays, all suspended cultures were washed with ice-cold DDW at least 5 times. They were then kept at 2°C or on ice until use. Lyophilized endospores were resuspended and repeatedly washed with ice-cold deionized double distilled water. Prior to measurements, endospores or particles were transferred to the corresponding buffered dye-free solution, resuspended, sonicated 2-3 times (15 sec. per pulse) and kept on ice.

After mixing with the dye stock solutions (in the same buffer or in water), the samples were sonicated 1-2 times. In several comparative experiments, Tween® 20 or Tween® 40 (91) (0.1 % w/v) or alcohol (20 – 40 % v/v) was used to facilitate handling (resuspension washing and centrifugation) of several endospore samples; no essential difference was observed in the thioflavin T reactivity of the samples thus treated, as compared with the control.

Endospores were counted under a phase contrast microscope (Nikon, magnification $\times 400$), using a Petroff-Hausser chamber (VWR). Concentrated endospore stocks were diluted toward final count $5 \times 10^8 - 3 \times 10^9$ per ml and stored up to one week at 2°C in the dark until use. Standard count error was 10 – 20 %. Phase-contrast microscopic appearance of the water-washed endospore samples before purification confirmed them as essentially pure (90 – 97 % endospores). Large (about 4 - 10 μm) endospore aggregates and other organic particles of uncertain origin (apparently resembling large particulates reported in previously examined endospore specimens)(102) occurred in the lyophilized endospore samples in minor quantities. No vegetative cells were observed in endospore cultures studied.

Vegetative bacteria cultures: *Escherichia coli* (*E. coli*), TOP 10 cells, were obtained from Invitrogen (Carlsbad, CA); and wild type *B. subtilis* were obtained from ATCC (Manassas, VA). Primary *Bacillus* cultures (18 hrs) of vegetative bacteria were outgrown from endospores in 2 % LB medium (Sigma) at 30°C. The cultures were then washed with plenty of cold DDW. They appeared under microscope as viable colonies of average size 2-10 cells, characteristic of *Bacillus*. These bacteria were then treated,

counted, and directly assayed. For sporulation, the *Bacillus* vegetative cells were plated and grown on thin layers of LB Agar for 10 days at 37 °C, and the growth was swabbed to collect the formed endospores (36).

2.5.5 Contaminant samples

Large quantities of plasmid DNA were extracted from *E. coli* cells strain DH5 α (transformed with NSP1 gene; Clone Id: YJL041W) using a standard QIAGEN maxi prep kit, and methods described in the Quick-Start protocol handbook. Buffer volumes used were adjusted based on the size of the cell pellet. Concentration of the purified plasmid DNA was obtained spectroscopically via pre-calibrated standard curve. Plasmid DNA contaminant was used at a final concentration of 1 mg ml⁻¹ in the presence of ThT and/or spores.

Humic acid contaminants were prepared at a final concentration of 1 mg ml⁻¹, without additional processing, in the presence of ThT and/or spores. Using stock fetal bovine serum, a 1:1 v/v dilution was made with 2 mM Tris buffer, pH 8.5, containing ThT and/or spores.

2.5.6 Signal-to-noise ratios of images

The signal-to-noise ratios, S/N , were calculated from the horizontal or vertical gray-scale traces across each of the imaged spores (Figure 10). While S/N is defined as a

decimal logarithm of the ratio between the power of the signal and the power of the noise (that for the same image can be estimated from the square of the ratio between the amplitude of the signal and the amplitude of the noise), in the fluorescence microscope images the noise has direct current, DC, and alternative current, AC, components. The amplitude of the DC noise, A_{DC} , is extracted from the dark background of the image (Figure 10b); and the amplitude of the AC noise, A_{AC} , is estimated from the root-mean-square of the fluctuations of the background (Figure 10c):

$$A_{AC} = RMS = \sqrt{\frac{1}{n} \sum_{i=1}^n (I_i - A_{DC})^2} \quad \text{Equation (8a)}$$

$$A_{DC} = \frac{1}{n} \sum_{i=1}^n I_i \quad \text{Equation (8b)}$$

where n is the number of “background” pixels analyzed and I_i is the intensity of pixel i .

The AC and DC contributions to S/N were estimated separately using the noise amplitudes and the average heights, $\langle h \rangle$, of the peaks on the traces crossing over imaged spores (Figure 10b, c):

$$S/N_{DC}[\text{dB}] = 20 \lg \left(\frac{\langle h \rangle}{A_{DC}} \right) \quad \text{Equation (9a)}$$

$$S/N_{AC}[\text{dB}] = 20 \lg \left(\frac{\langle \Delta h \rangle}{RMS} \right) \quad \text{Equation (9b)}$$

where the multiple of 10 in front of the logarithms converts the units to dB; and $\langle \Delta h \rangle = \langle h \rangle - A_{DC}$.

For each set of imaging conditions, we obtained the S/N values as an average of the gray-scale trace analysis (Figure 10) of at least ten imaged spores from multiple images, located in different quadrants of the images.

2.5.7 Dye distribution

The distribution coefficients, K_D , are defined as the concentration ratios between the dye taken up by the spores and the dye remained in solution:

$$K_D = \frac{C_{SPORE}}{C_{SOLUTION}} \quad \text{Equation (10)}$$

where C_{SPORE} and $C_{SOLUTION}$ are the concentrations of ThT in the endospore and in the aqueous solution, respectively, after completion of the staining process. The values of C_{SPORE} and $C_{SOLUTION}$ were extracted from spectroscopic data.

The samples were prepared by incubating a 1 ml solution of varying concentrations of thioflavin T (ThT) with known count of bacterial endospores for 10 minutes under constant stirring. (The 10-minute period ensured completion of the staining processes, which we determined using kinetic measurements as we previously demonstrated(90, 107).) The ThT-endospore mixture was centrifuged for two minutes at 12,000 rpm, using Eppendorf Centrifuge 5415 with a rotational radius of 7.5 cm, and the supernatant was collected. (The spectra were averaged from at least three different experiments for the same species and spore counts.) Using the molar extinction coefficient values, ThT concentrations were estimated from the absorption spectra. For

these studies, we selected dye concentrations and cuvette thicknesses that yielded absorbance values in the range between 0.10 and 1.0. For $A < 0.1$, less than 20 % of the light is absorbed by the sample; and for $A > 1$, the sample absorbs more than 90% of the light permitting analysis based on changes only within less than 10% of the incident light intensity.

Approximating the shape of the endospores to an ellipsoid (i.e., prolate spheroid), allowed us to estimate the volume of a single endospore, $V_S = (4/3)\pi a^2 b$, where a and b are the short and long elliptic radii, respectively, estimated from the images of the endospores. The decrease in the absorbance at 412 nm, after the incubation and centrifugation steps, provided information about the amount (moles) of dye taken up by the endospores. Dividing the amount of taken up dye by the endospore volume, yielded the average ThT concentration in the endospores, C_{SPORE} (equation 5). $C_{SOLUTION}$ needed for calculating the distribution coefficient (equation 5) was obtained from the absorption of the supernatant.

Acknowledgements. Funding for this research was provided by the U. S. Army Research Laboratory (for G.J.), by the National Science Foundation (CBET 0935995, for V.I.V.), by the U. C. Regents Faculty Development Award (for V.I.V.) and by the National Institute of Health (2R01-DC02775-06, for B.A.). We also extend our gratitude to Dr. B. Hyle Park for the discussions on image analysis.

2.6 References

1. **Ankolekar, C., T. Rahmati, and R. G. Labbe.** 2009. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in US rice. *International Journal of Food Microbiology* **128**:460-466.
2. **Arnon, S. S., R. Schechter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow, K. Tonat, and W. G. C. Biodefense.** 2001. Botulinum toxin as a biological weapon - Medical and public health management. *Journal of the American Medical Association* **285**:1059-1070.
3. **Bao, D., B. Millare, W. Xia, B. G. Steyer, A. A. Gerasimenko, A. Ferreira, A. Contreras, and V. I. Vullev.** 2009. Electrochemical Oxidation of Ferrocene: A Strong Dependence on the Concentration of the Supporting Electrolyte for Nonpolar Solvents. *Journal of Physical Chemistry A* **113**:1259-1267.
4. **Bao, D., S. Ramu, A. Contreras, S. Upadhyayula, J. M. Vasquez, G. Beran, and V. I. Vullev.** 2010. Electrochemical Reduction of Quinones: Interfacing Experiment and Theory for Defining Effective Radii of Redox Moieties. *Journal of Physical Chemistry B* **114**:14467-14479.
5. **Belgrader, P., W. Benett, D. Hadley, J. Richards, P. Stratton, R. Mariella, Jr., and F. Milanovich.** 1999. PCR detection of bacteria in seven minutes. *Science* **284**:449-450.
6. **Beverly, M. B., K. J. Voorhees, T. L. Hadfield, and R. B. Cody.** 2000. Electron monochromator mass spectrometry for the analysis of whole bacteria and bacterial spores. *Analytical Chemistry* **72**:2428-2432.
7. **Biancalana, M., and S. Koide.** 2010. Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochimica Et Biophysica Acta-Proteins and Proteomics* **1804**:1405-1412.
8. **Bisset, K. A.** 1950. Evolution in Bacteria and the Significance of the Bacterial Spore. *Nature* **166**:431-432.
9. **Cable, M. L., J. P. Kirby, D. J. Levine, M. J. Manary, H. B. Gray, and A. Ponce.** 2009. Detection of Bacterial Spores with Lanthanide-Macrocyclic Binary Complexes. *Journal of the American Chemical Society* **131**:9562-9570.
10. **Cable, M. L., J. P. Kirby, K. Sorasane, H. B. Gray, and A. Ponce.** 2007. Bacterial Spore Detection by [Tb³⁺(macrocyclic)(dipicolinate)] Luminescence. *Journal of the American Chemical Society* **129**:1474-1475.

11. **Chau, K., B. Millare, A. Lin, S. Upadhyayula, V. Nuñez, H. Xu, and V. I. Vullev.** 2011. Dependence of the quality of adhesion between poly(dimethylsiloxane) and glass surfaces on the composition of the oxidizing plasma. *Microfluidics and Nanofluidics* **10**:907-917.
12. **Chen, D., S. S. Huang, and Y. Q. Li.** 2006. Real-time detection of kinetic germination and heterogeneity of single *Bacillus* spores by laser tweezers Raman spectroscopy. *Analytical Chemistry* **78**:6936-6941.
13. **Dobson, C. M.** 2003. Protein folding and misfolding. *Nature* **426**:884-890.
14. **Driks, A.** 2003. The dynamic spore. *Proceedings of the National Academy of Sciences of the United States of America* **100**:3007-3009.
15. **Eaton, D. F.** 1990. Recommended methods for fluorescence decay analysis. *Pure and Applied Chemistry* **62**:1631-1648.
16. **Eisert, R., L. Felau, and L. R. Brown.** 2006. Methods for enhancing the accuracy and reproducibility of Congo red and thioflavin T assays. *Analytical Biochemistry* **353**:144-146.
17. **Fichtel, J., J. Koester, J. Rullkoetter, and H. Sass.** 2007. Spore dipicolinic acid contents used for estimating the number of endospores in sediments. *FEMS Microbiology Ecology* **61**:522-532.
18. **Fodera, V., M. Groenning, V. Vetri, F. Librizzi, S. Spagnolo, C. Cornett, L. Olsen, M. van de Weert, and M. Leone.** 2008. Thioflavin T Hydroxylation at Basic pH and Its Effect on Amyloid Fibril Detection. *Journal of Physical Chemistry B* **112**:15174-15181.
19. **Fowler, D. M., A. V. Koulov, W. E. Balch, and J. W. Kelly.** 2007. Functional amyloid - from bacteria to humans. *Trends in Biochemical Sciences* **32**:217-224.
20. **Franz, D. R., P. B. Jahrling, A. M. Friedlander, D. J. McClain, D. L. Hoover, W. R. Bryne, J. A. Pavlin, C. W. Christopher, and E. M. Eitzen.** 1997. Clinical recognition and management of patients exposed to biological warfare agents. *Journal of the American Medical Association* **278**:399-411.
21. **Fykse, E. M., B. Langseth, J. S. Olsen, G. Skogan, and J. M. Blatny.** 2008. Detection of bioterror agents in air samples using real-time PCR. *Journal of Applied Microbiology* **105**:351-358.
22. **Gebbink, M. F. B. G., D. Claessen, B. Bouma, L. Dijkhuizen, and H. A. B. Wosten.** 2005. Amyloids - A functional coat for microorganisms. *Nature Reviews Microbiology* **3**:333-341.

23. **Groenning, M., M. Norrman, J. M. Flink, M. van de Weert, J. T. Bukrinsky, G. Schluckebier, and S. Frokjaer.** 2007. Binding mode of Thioflavin T in insulin amyloid fibrils. *Journal of Structural Biology* **159**:483-497.
24. **Gueltekin, A., A. Ersoez, N. Y. Sarioezlue, A. Denizli, and R. Say.** 2010. Nanosensors having dipicolinic acid imprinted nanoshell for *Bacillus cereus* spores detection. *Journal of Nanoparticle Research* **12**:2069-2079.
25. **Hammer, N. D., X. Wang, B. A. McGuffie, and M. R. Chapman.** 2008. Amyloids: Friend or Foe? *Journal of Alzheimer's Disease* **13**:407-419.
26. **Harel, M., L. K. Sonoda, I. Silman, J. L. Sussman, and T. L. Rosenberry.** 2008. Crystal structure of thioflavin T bound to the peripheral site of Torpedo californica acetylcholinesterase reveals how thioflavin T acts as a sensitive fluorescent reporter of ligand binding to the acylation site. *Journal of the American Chemical Society* **130**:7856-7861.
27. **Hindle, A. A., and E. A. H. Hall.** 1999. Dipicolinic acid (DPA) assay revisited and appraised for spore detection. *Analyst* **124**:1599-1604.
28. **Hisamatsu, H., and K. Maekawa.** 1994. The Distribution of the Durbin-Watson Statistic in Integrated and near-Integrated Models. *Journal of Econometrics* **61**:367-382.
29. **Hong, C., D. Bao, M. S. Thomas, J. M. Clift, and V. I. Vullev.** 2008. Print-and-Peel Fabrication of Microelectrodes. *Langmuir* **24**:8439-8442.
30. **Hu, J., B. Xia, D. Bao, A. Ferreira, J. Wan, G. Jones, and V. I. Vullev.** 2009. Long-Lived Photogenerated States of α -Oligothiophene-Acrinium Dyads Have Triplet Character. *Journal of Physical Chemistry A* **113**:3096-3107.
31. **Inglesby, T. V., T. O'Toole, A. Henderson Donald, G. Bartlett John, S. Ascher Michael, E. Eitzen, M. Friedlander Arthur, J. Gerberding, J. Hauer, J. Hughes, J. McDade, T. Osterholm Michael, G. Parker, M. Perl Trish, K. Russell Philip, and K. Tonat.** 2002. Anthrax as a biological weapon, 2002: updated recommendations for management. *Journal of the American Medical Association* **287**:2236-2252.
32. **Iqbal, S. S., M. W. Mayo, J. G. Bruno, B. V. Bronk, C. A. Batt, and J. P. Chambers.** 2000. A review of molecular recognition technologies for detection of biological threat agents. *Biosensors & Bioelectronics* **15**:549-578.
33. **Jakob-Roetne, R., and H. Jacobsen.** 2009. Alzheimer's Disease: From Pathology to Therapeutic Approaches. *Angewandte Chemie-International Edition* **48**:3030-3059.

34. **Jones, G., II, V. Vullev, E. H. Braswell, and D. Zhu.** 2000. Multistep Photoinduced Electron Transfer in a de Novo Helix Bundle: Multimer Self-Assembly of Peptide Chains Including a Chromophore Special Pair. *Journal of the American Chemical Society* **122**:388-389.
35. **Jones, G., II, and V. I. Vullev.** 2001. Contribution of a Pyrene Fluorescence Probe to the Aggregation Propensity of Polypeptides. *Organic Letters* **3**:2457-2460.
36. **Jones, G., II, and V. I. Vullev.** 2001. Ground- and Excited-State Aggregation Properties of a Pyrene Derivative in Aqueous Media. *Journal of Physical Chemistry A* **105**:6402-6406.
37. **Jones, G., II, and V. I. Vullev.** 2002. Medium effects on the photophysical properties of terbium(III) complexes with pyridine-2,6-dicarboxylate. *Photochemical & Photobiological Sciences* **1**:925-933.
38. **Jones, G., II, and V. I. Vullev.** 2002. Medium Effects on the Stability of Terbium(III) Complexes with Pyridine-2,6-dicarboxylate. *Journal of Physical Chemistry A* **106**:8213-8222.
39. **Jones, G., II, and V. I. Vullev.** 2002. Photoinduced Electron Transfer between Non-Native Donor-Acceptor Moieties Incorporated in Synthetic Polypeptide Aggregates. *Organic Letters* **4**:4001-4004.
40. **Jones, G., II, D. Yan, J. Hu, J. Wan, B. Xia, and V. I. Vullev.** 2007. Photoinduced Electron Transfer in Arylacridinium Conjugates in a Solid Glass Matrix. *Journal of Physical Chemistry B* **111**:6921-6929.
41. **Jones, G., II, X. Zhou, and V. I. Vullev.** 2003. Photoinduced electron transfer in alpha-helical polypeptides: dependence on conformation and electron donor-acceptor distance. *Photochemical & Photobiological Sciences* **2**:1080-1087.
42. **Jones, G., and G. L. Indig.** 1996. Spectroscopic and chemical binding properties of humic acids in water. *New Journal of Chemistry* **20**:221-232.
43. **Jordal, P. B., M. S. Dueholm, P. Larsen, S. V. Petersen, J. J. Enghild, G. Christiansen, P. Hojrup, P. H. Nielsen, and D. E. Otzen.** 2009. Widespread Abundance of Functional Bacterial Amyloid in Mycolata and Other Gram-Positive Bacteria. *Applied and Environmental Microbiology* **75**:4101-4110.
44. **Khurana, R., C. Coleman, C. Ionescu-Zanetti, S. A. Carter, V. Krishna, R. K. Grover, R. Roy, and S. Singh.** 2005. Mechanism of thioflavin T binding to amyloid fibrils. *Journal of Structural Biology* **151**:229-238.
45. **Kim, K., J. Seo, K. Wheeler, C. Park, D. Kim, S. Park, W. Kim, S. I. Chung, and T. Leighton.** 2005. Rapid genotypic detection of *Bacillus anthracis* and the

- Bacillus cereus group by multiplex real-time PCR melting curve analysis. *FEMS Immunology and Medical Microbiology* **43**:301-310.
46. **Klunk, W. E., Y. M. Wang, G. F. Huang, M. L. Debnath, D. P. Holt, and C. A. Mathis.** 2001. Uncharged thioflavin-T derivatives bind to amyloid-beta protein with high affinity and readily enter the brain. *Life Sciences* **69**:1471-1484.
 47. **Kong, L. B., P. F. Zhang, P. Setlow, and Y. Q. Li.** 2010. Characterization of Bacterial Spore Germination Using Integrated Phase Contrast Microscopy, Raman Spectroscopy, and Optical Tweezers. *Analytical Chemistry* **82**:3840-3847.
 48. **Kricka, L. J., and P. Fortina.** 2009. Analytical ancestry: "firsts" in fluorescent labeling of nucleosides, nucleotides, and nucleic acids. *Clinical Chemistry* **55**:670-683.
 49. **Kukreja, R. V., S. K. Sharma, and B. R. Singh.** 2010. Molecular Basis of Activation of Endopeptidase Activity of Botulinum Neurotoxin Type E. *Biochemistry* **49**:2510-2519.
 50. **LeVine, H., 3rd.** 1993. Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Science* **2**:404-410.
 51. **Ljosa, V., and A. E. Carpenter.** 2008. High-throughput screens for fluorescent dye discovery. *Trends in Biotechnology* **26**:527-530.
 52. **Lu, H., D. Bao, M. Penchev, M. Ghazinejad, V. I. Vullev, C. S. Ozkan, and M. Ozkan.** 2010. Pyridine-coated lead sulfide quantum dots for polymer hybrid photovoltaic devices. *Advanced Science Letters* **3**:101-109.
 53. **Maji, S. K., M. H. Perrin, M. R. Sawaya, S. Jessberger, K. Vadodaria, R. A. Rissman, P. S. Singru, K. P. R. Nilsson, R. Simon, D. Schubert, D. Eisenberg, J. Rivier, P. Sawchenko, W. Vale, and R. Riek.** 2009. Functional amyloids as natural storage of peptide hormones in pituitary secretory granules. *Science* **325**:328-332.
 54. **Makino, S., and H. Cheun.** 2003. Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores. *Journal of Microbiological Methods* **53**:141-147.
 55. **Maskevich, A. A., V. I. Stsiapura, V. A. Kuzmitsky, I. M. Kuznetsova, O. I. Povarova, V. N. Uversky, and K. K. Turoverov.** 2007. Spectral Properties of Thioflavin T in Solvents with Different Dielectric Properties and in a Fibril-Incorporated Form. *Journal of Proteome Research* **6**:1392-1401.
 56. **Millare, B., M. Thomas, A. Ferreira, H. Xu, M. Holesinger, and V. I. Vullev.** 2008. Dependence of the quality of adhesion between polydimethyl siloxane and

glass surfaces on the conditions of treatment with oxygen plasma. *Langmuir* **24**:13218-13224.

57. **Montecucco, C., and J. Molgo.** 2005. Botulinal neurotoxins: revival of an old killer. *Current Opinion in Pharmacology* **5**:274-279.
58. **Naiki, H., K. Higuchi, M. Hosokawa, and T. Takeda.** 1989. Fluorometric-Determination of Amyloid Fibrils Invitro Using the Fluorescent Dye, Thioflavine-T. *Analytical Biochemistry* **177**:244-249.
59. **Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow.** 2000. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews* **64**:548.
60. **Ohhashi, Y., M. Kihara, H. Naiki, and Y. Goto.** 2005. Ultrasonication-induced amyloid fibril formation of beta(2)-microglobulin. *Journal of Biological Chemistry* **280**:32843-32848.
61. **Pellegrino, P. M., N. F. Fell, Jr., D. L. Rosen, and J. B. Gillespie.** 1998. Bacterial Endospore Detection Using Terbium Dipicolinate Photoluminescence in the Presence of Chemical and Biological Materials. *Analytical Chemistry* **70**:1755-1760.
62. **Plomp, M., T. J. Leighton, K. E. Wheeler, H. D. Hill, and A. J. Malkin.** 2007. In vitro high-resolution structural dynamics of single germinating bacterial spores. *Proceedings of the National Academy of Sciences of the United States of America* **104**:9644-9649.
63. **Plomp, M., T. J. Leighton, K. E. Wheeler, and A. J. Malkin.** 2005. Architecture and High-Resolution Structure of Bacillus thuringiensis and Bacillus cereus Spore Coat Surfaces. *Langmuir* **21**:7892-7898.
64. **Plomp, M., T. J. Leighton, K. E. Wheeler, M. E. Pitesky, and A. J. Malkin.** 2005. Bacillus atrophaeus outer spore coat assembly and ultrastructure. *Langmuir* **21**:10710-10716.
65. **Plomp, M., and A. J. Malkin.** 2009. Mapping of Proteomic Composition on the Surfaces of Bacillus Spores by Atomic Force Microscopy-Based Immunolabeling. *Langmuir* **25**:403-409.
66. **Premasiri, W. R., D. T. Moir, M. S. Klempner, N. Krieger, G. Jones, and L. D. Ziegler.** 2005. Characterization of the Surface Enhanced Raman Scattering (SERS) of bacteria. *Journal of Physical Chemistry B* **109**:312-320.
67. **Rao, S. S., K. V. K. Mohan, and C. D. Atreya.** 2010. Detection technologies for Bacillus anthracis: Prospects and challenges. *Journal of Microbiological Methods* **82**:1-10.

68. **Rosen, D. L.** 1999. Bacterial endospore detection using photoluminescence from terbium dipicolinate. *Reviews in Analytical Chemistry* **18**:1-21.
69. **Rosen, D. L.** 1999. Bacterial endospore detection using photoluminescence from terbium dipicolinate. *Reviews in Analytical Chemistry* **18**:1-21.
70. **Rosen, D. L., C. Sharpless, and L. B. McGown.** 1997. Bacterial Spore Detection and Determination by Use of Terbium Dipicolinate Photoluminescence. *Analytical Chemistry* **69**:1082-1085.
71. **Rueckert, A., R. S. Ronimus, and H. W. Morgan.** 2005. Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders. *Journal of Microbiological Methods* **60**:155-167.
72. **Rutledge, D. N., and A. S. Barros.** 2002. Durbin-Watson statistic as a morphological estimator of information content. *Analytica Chimica Acta* **454**:277-295.
73. **Sadik, O. A., W. H. Land, and J. Wang.** 2003. Targeting chemical and biological warfare agents at the molecular level. *Electroanalysis* **15**:1149-1159.
74. **Schaeffer, A., and M. D. Fulton.** 1933. A simplified method of staining endospores. *Science* **77**:194.
75. **Schirra, R.** 1985. Dye Aggregation in Freezing Aqueous-Solutions. *Chemical Physics Letters* **119**:463-466.
76. **Singh, P. K., M. Kumbhakar, H. Pal, and S. Nath.** 2010. Ultrafast Bond Twisting Dynamics in Amyloid Fibril Sensor. *Journal of Physical Chemistry B* **114**:2541-2546.
77. **Singh, P. K., M. Kumbhakar, H. Pal, and S. Nath.** 2009. Ultrafast Torsional Dynamics of Protein Binding Dye Thioflavin-T in Nanoconfined Water Pool. *Journal of Physical Chemistry B* **113**:8532-8538.
78. **Singh, P. K., M. Kumbhakar, H. Pal, and S. Nath.** 2010. Viscosity Effect on the Ultrafast Bond Twisting Dynamics in an Amyloid Fibril Sensor: Thioflavin-T. *Journal of Physical Chemistry B* **114**:5920-5927.
79. **Slieman, T. A., and W. L. Nicholson.** 2001. Role of dipicolinic acid in survival of *Bacillus subtilis* spores exposed to artificial and solar UV radiation. *Applied and Environmental Microbiology* **67**:1274-1279.
80. **Snyder, A. P., A. Tripathi, J. P. Dworzanski, W. M. Maswadeh, and C. H. Wick.** 2005. Characterization of microorganisms by thermogravimetric analysis-mass spectrometry. *Analytica Chimica Acta* **536**:283-293.

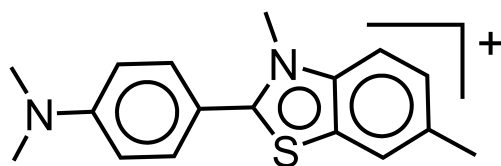
81. **Song, J. M., M. Culha, P. A. Kasili, G. D. Griffin, and T. Vo-Dinh.** 2005. A compact CMOS biochip immunosensor towards the detection of a single bacteria. *Biosensors & Bioelectronics* **20**:2203-2209.
82. **Steele, P. T., A. Srivastava, M. E. Pitesky, D. P. Fergenson, H. J. Tobias, E. E. Gard, and M. Frank.** 2005. Desorption/ionization fluence thresholds and improved mass spectral consistency measured using a flattop laser profile in the bioaerosol mass spectrometry of single *Bacillus* endospores. *Analytical Chemistry* **77**:7448-7454.
83. **Steinberg, C. E. W., T. Meinelt, M. A. Timofeyev, M. Bittner, and R. Menzel.** 2008. Humic substances. *Environmental Science and Pollution Research* **15**:128-135.
84. **Stratis-Cullum, D. N., G. D. Griffin, J. Mobley, A. A. Vass, and T. Vo-Dinh.** 2003. A miniature biochip system for detection of aerosolized *Bacillus globigii* spores. *Analytical Chemistry* **75**:275-280.
85. **Stsiapura, V. I., A. A. Maskevich, V. A. Kuzmitsky, V. N. Uversky, I. M. Kuznetsova, and K. K. Turoverov.** 2008. Thioflavin T as a Molecular Rotor: Fluorescent Properties of Thioflavin T in Solvents with Different Viscosity. *Journal of Physical Chemistry B* **112**:15893-15902.
86. **Sulatskaya, A. I., A. Maskevich Alexander, M. Kuznetsova Irina, N. Uversky Vladimir, and K. Turoverov Konstantin.** 2010. Fluorescence quantum yield of thioflavin T in rigid isotropic solution and incorporated into the amyloid fibrils. *PloS one* **5**:e15385.
87. **Taylor, K. M. L., and W. Lin.** 2009. Hybrid silica nanoparticles for luminescent spore detection. *Journal of Materials Chemistry* **19**:6418-6422.
88. **Thomas, M. S., J. M. Clift, B. Millare, and V. I. Vullev.** 2010. Print-and-Peel Fabricated Passive Micromixers. *Langmuir* **26**:2951-2957.
89. **Thomas, M. S., B. Millare, J. M. Clift, D. Bao, C. Hong, and V. I. Vullev.** 2010. Print-and-peel fabrication for microfluidics: what's in it for biomedical applications? *Annals of biomedical engineering* **38**:21-32.
90. **Thomas, M. S., V. Nuñez, S. Upadhyayula, E. R. Zielins, D. Bao, J. M. Vasquez, B. Bahmani, and V. I. Vullev.** 2010. Kinetics of Bacterial Fluorescence Staining with 3,3'-Diethylthiacyanine. *Langmuir* **26**:9756-9765.
91. **Turnbough, C. L.** 2003. Discovery of phage display peptide ligands for species-specific detection of *Bacillus* spores. *Journal of Microbiological Methods* **53**:263-271.

92. **Uithoven, K. A., J. C. Schmidt, and M. E. Ballman.** 2000. Rapid identification of biological warfare agents using an instrument employing a light addressable potentiometric sensor and a flow-through immunofiltration-enzyme assay system. *Biosensors & Bioelectronics* **14**:761-770.
93. **Vasquez, J. M., A. Vu, J. S. Schultz, and V. I. Vullev.** 2009. Fluorescence enhancement of warfarin induced by interaction with beta -cyclodextrin. *Biotechnology Progress* **25**:906-914.
94. **Vetri, V., C. Canale, A. Relini, F. Librizzi, V. Militello, A. Gliozzi, and M. Leone.** 2007. Amyloid fibrils formation and amorphous aggregation in concanavalin A. *Biophysical Chemistry* **125**:184-190.
95. **Voropai, E. S., M. P. Samtsov, K. N. Kaplevskii, A. A. Maskevich, V. I. Stepuro, O. I. Povarova, I. M. Kuznetsova, K. K. Turoverov, A. L. Fink, and V. N. Uverskii.** 2003. Spectral Properties of Thioflavin T and Its Complexes with Amyloid Fibrils. *Journal of Applied Spectroscopy (Translation of Zhurnal Prikladnoi Spektroskopii)* **70**:868-874.
96. **Vullev, V. I., and G. Jones.** 2002. Photoinduced electron transfer in alkanoylpyrene aggregates in conjugated polypeptides. *Tetrahedron Letters* **43**:8611-8615.
97. **Vullev, V. I., and G. Jones, II.** 2005. Nanosecond laser flash photolysis: Dealing with dynamic-range and response-time limitations of the detection system. *Journal of Applied Sciences* **5**:517-526.
98. **Vullev, V. I., J. Wan, V. Heinrich, P. Landsman, P. E. Bower, B. Xia, B. Millare, and G. Jones, II.** 2006. Nonlithographic Fabrication of Microfluidic Devices. *Journal of the American Chemical Society* **128**:16062-16072.
99. **Wan, J., A. Ferreira, W. Xia, C. H. Chow, K. Takechi, P. V. Kamat, G. Jones, and V. I. Vullev.** 2008. Solvent dependence of the charge-transfer properties of a quaterthiophene-anthraquinone dyad. *Journal of Photochemistry and Photobiology, A: Chemistry* **197**:364-374.
100. **Wan, J., M. S. Thomas, S. Guthrie, and V. I. Vullev.** 2009. Surface-bound proteins with preserved functionality. *Annals of biomedical engineering* **37**:1190-1205.
101. **Wictome, M., K. Newton, K. Jameson, B. Hallis, P. Dunnigan, E. Mackay, S. Clarke, R. Taylor, J. Gaze, K. Foster, and C. Shone.** 1999. Development of an in vitro bioassay for Clostridium botulinum type B neurotoxin in foods that is more sensitive than the mouse bioassay. *Applied and Environmental Microbiology* **65**:3787-3792.

102. **Williams, D. D., and C. L. Turnbough, Jr.** 2004. Surface layer protein EA1 is not a component of *Bacillus anthracis* spores but is a persistent contaminant in spore preparations. *Journal of Bacteriology* **186**:566-569.
103. **Xing, Y., and K. Higuchi.** 2002. Amyloid fibril proteins. *Mechanisms of Ageing and Development* **123**:1625-1636.
104. **Yang, W.-W., and A. Ponce.** 2009. Rapid endospore viability assay of *Clostridium sporogenes* spores. *International Journal of Food Microbiology* **133**:213-216.
105. **Zhang, P. F., L. B. Kong, G. W. Wang, P. Setlow, and Y. Q. Li.** 2010. Combination of Raman tweezers and quantitative differential interference contrast microscopy for measurement of dynamics and heterogeneity during the germination of individual bacterial spores. *Journal of Biomedical Optics* **15**:.
106. **Zhang, X. Y., M. A. Young, O. Lyandres, and R. P. Van Duyne.** 2005. Rapid detection of an anthrax biomarker by surface-enhanced Raman spectroscopy. *Journal of the American Chemical Society* **127**:4484-4489.
107. **Zielins, E., M. Thomas, and V. I. Vullev.** 2008. Bacterium-Induced Fluorescence-Enhancement Kinetics: Breaking 100-Year Old Traditions of Staining Bioanalyses. *UCR Undergraduate Research Journal* **2**:75-82.

2.7 SCHEMES

Scheme 3. Thioflavin T (ThT).



2.8 FIGURES

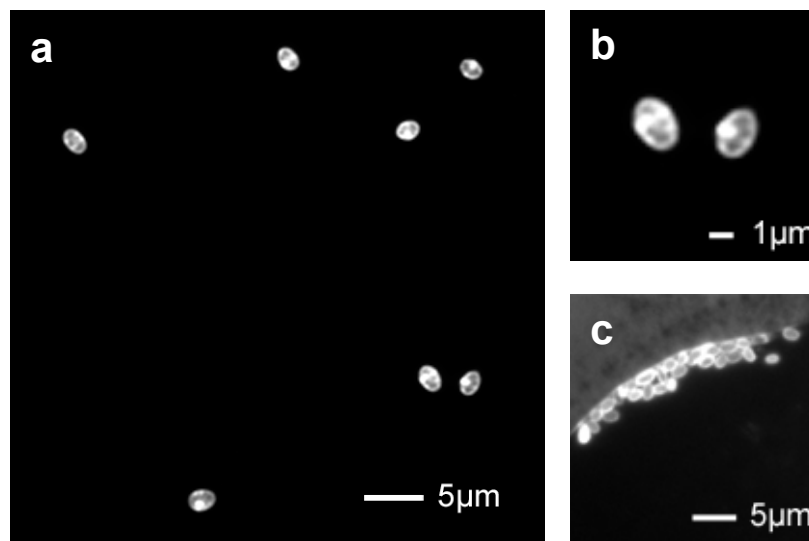


Figure 9. Epifluorescence microscopy images of bacterial endospores, (a, b) *B. atrophaeus* and (c) *G. stearothermophilus*. All endospore samples were imaged while suspended in 150 μM ThT aqueous solution (2 mM Tris buffer, pH 8.5, *exposure time* = 5 s). The spores were incubated in the ThT solution for 10 min prior to imaging.

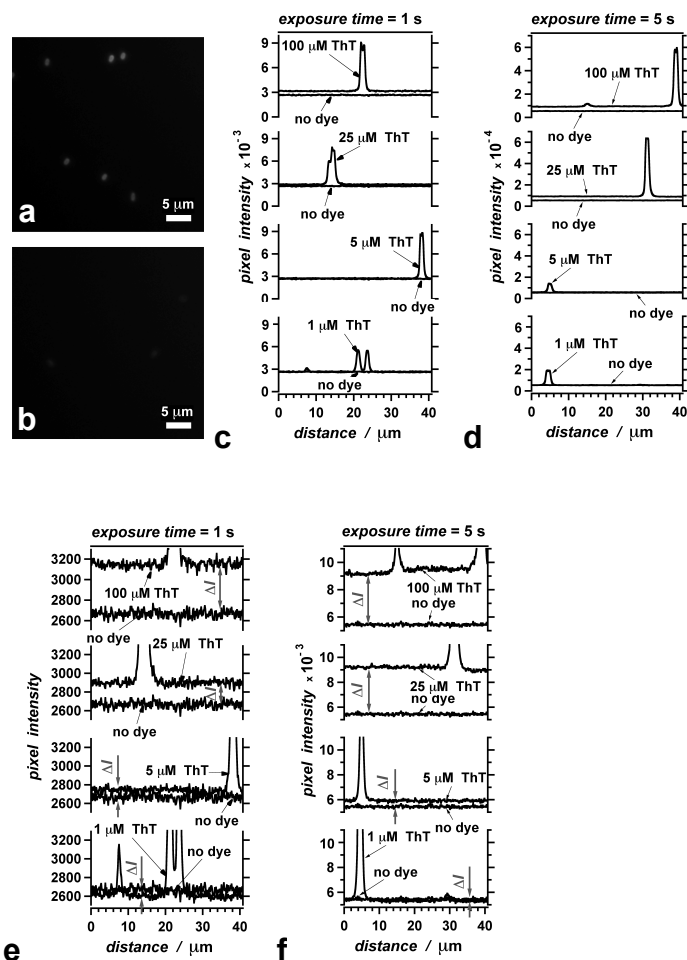


Figure 10. Gray-scale analysis (88) of epifluorescence microscopy images of *B. atrophaeus* endospores suspended in solutions with various concentrations of ThT (2 mM Tris buffer, pH 8.5). **(a, b)** Fluorescence images (*exposure time* = 5 s): **(a)** in the presence of 1 μM ThT and **(b)** with no dye present. **(c, d)** Traces from images of spores suspended in different concentrations of ThT recorded at *exposure time* = **(c)** 1 s, and **(d)** 5 s. **(e, f)** The same traces plotted against expanded ordinates, demonstrating the differences between the baselines of the images with and without dye, ΔI . ΔI represents the contribution of the fluorescence from the free ThT to the backgrounds of the microscope images, A_{DC} . ($\Delta I = 490, 240, 60$ and <20 for 100 μM, 25 μM, 5 μM and 1 μM ThT at *exposure time* = 1 s; and $\Delta I = 4,100, 3,300, 460$ and <50 for 100 μM, 25 μM, 5 μM and 1 μM ThT at *exposure time* = 5 s.) The peak signals corresponding to the spores labeled with ThT (i.e., to the dye taken up by the spores) exceed the background fluorescence, ΔI , by more than an order of magnitude: i.e., $(\langle \Delta h \rangle + \Delta I) / \Delta I > 10$. For 1-s *exposure time*, the baseline *RMS* = 35, 24, 32, 25 and 35 for 0 μM, 1 μM, 5 μM, 25 μM and 100 μM ThT;

and for 5-s exposure time, $RMS = 75, 65, 92, 96$ and 120 for $0\ \mu\text{M}$, $1\ \mu\text{M}$, $5\ \mu\text{M}$, $25\ \mu\text{M}$ and $100\ \mu\text{M}$ ThT.

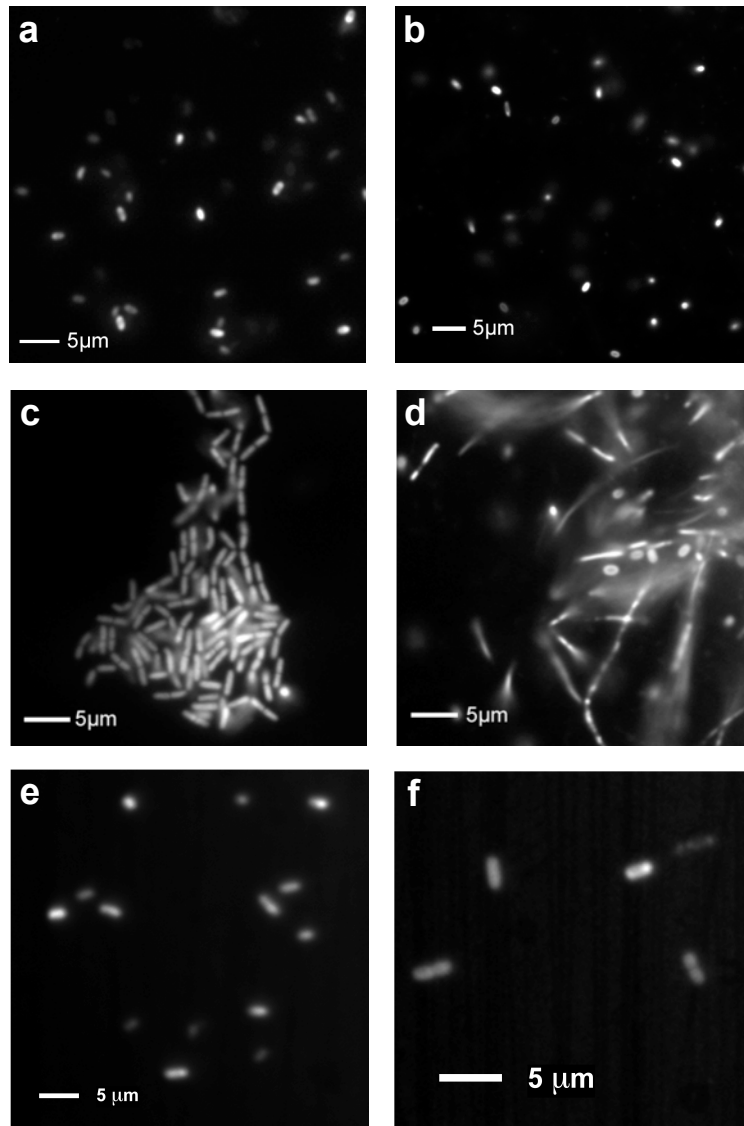


Figure 11. Epifluorescence microscopy images of: **(a)** *B. globigii* endospores; **(b)** *B. subtilis* endospores; **(c)** *B. subtilis* vegetative cells; **(d)** *B. subtilis* during sporulation; and **(e, f)** *E. coli* cells. All bacterial samples were imaged while suspended in 150 μ M ThT aqueous solution (2 mM Tris buffer, pH 8.5). The bacteria were incubated in the ThT solution for 10 min prior to imaging.

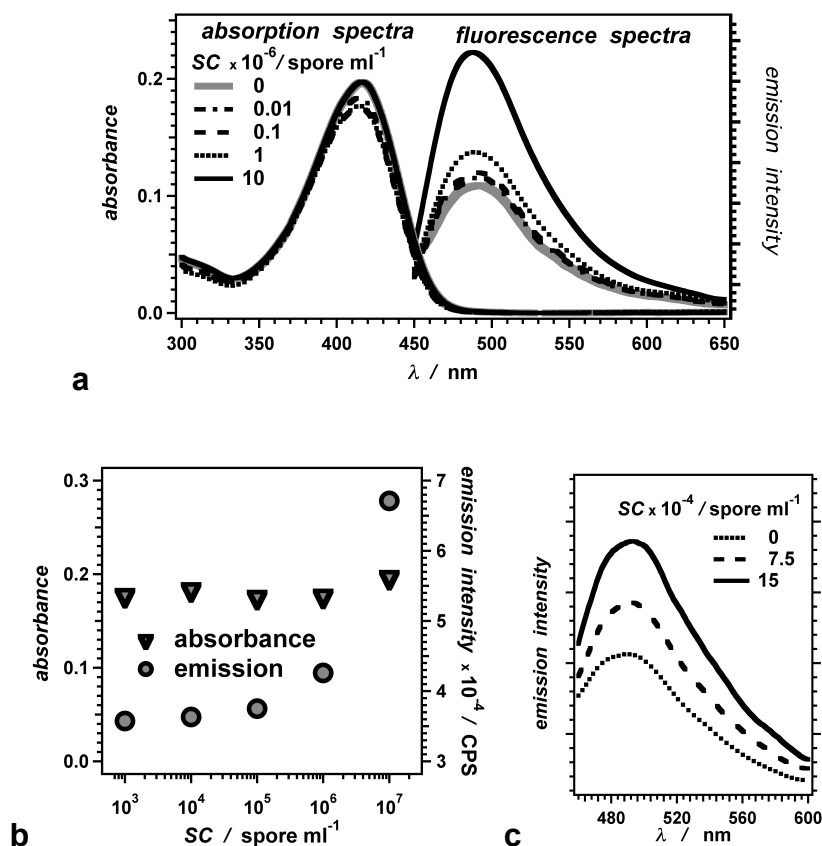


Figure 12. Absorption and fluorescence properties of ThT (11 μM) dissolved in aqueous solutions (2 mM Tris buffer, pH 8.5, absorption and excitation optical path length = 0.5 cm) in the absence and the presence of different amounts bacterial endospores (SC = spore count): **(a)** absorption and emission ($\lambda_{\text{ex}} = 430$ nm) spectra of ThT samples containing *B. atrophaeus* with different SC (for SC exceeding 10^7 spore ml^{-1} the baseline of the absorption spectra in the blue and UV region shifts due to scattering); **(b)** dependence of the absorbance ($\lambda_{\text{abs}} = 415$ nm) and emission intensity ($\lambda_{\text{ex}} = 415$ nm; $\lambda_{\text{em}} = 490$ nm) on the spore count; and **(c)** emission spectra of ThT in the presence and absence of *B. globigii* endospores ($\lambda_{\text{ex}} = 430$ nm).

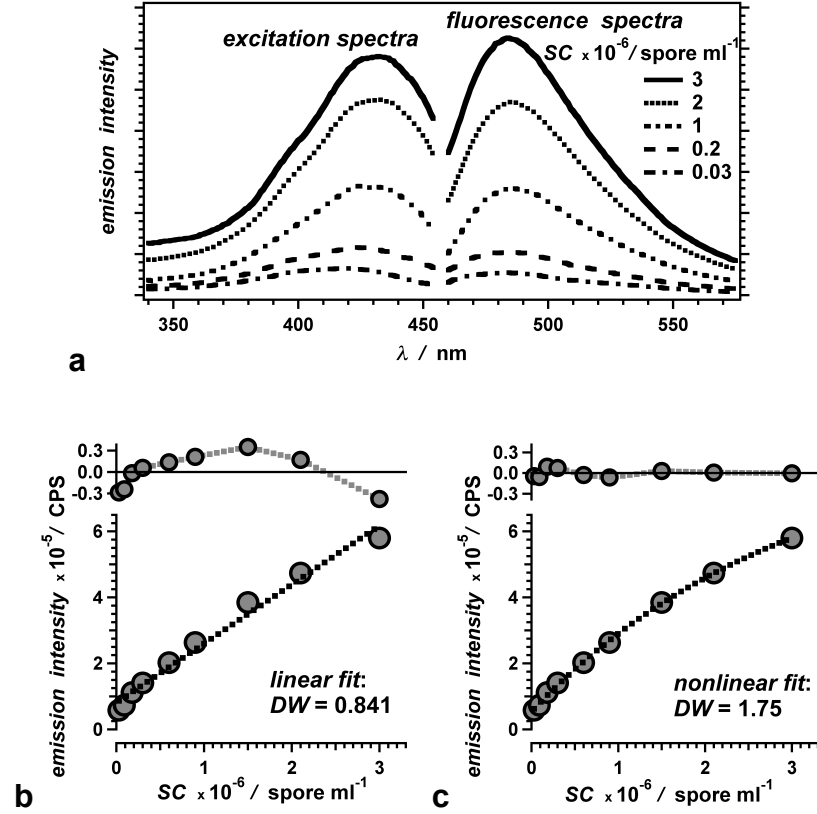


Figure 13. Fluorescence properties of ThT (1 μ M) dissolved in aqueous solution (4 mM glycine buffer, pH 9.0) in the presence of different amounts of *B. subtilis* endospores: **(a)** excitation ($\lambda_{\text{em}} = 480$ nm) and emission ($\lambda_{\text{ex}} = 430$ nm) spectra; and **(b, c)** dependence of the emission intensity ($\lambda_{\text{ex}} = 430$ nm) on the spore count (SC). The circles represent the emission intensity at 480 nm, and the dotted lines are data fits. The residuals from the data fits are plotted against a separate ordinate above the data. **(b)** Linear analysis of the fluorescence dependence on the spore count: $F(SC) = F_0 + \Delta F SC$. **(c)** Nonlinear analysis employing a power-law function (equation 6) to fit the fluorescence dependence on SC. The appropriateness of the function used for data fits was examined using the Durbin-

Watson test statistics: $DW = \frac{\sum_{i=2}^n (\delta_i - \delta_{i-1})^2}{\sum_{i=1}^n \delta_i^2}$, where δ_i are the residual values at data points i , and n is the total number of data points.

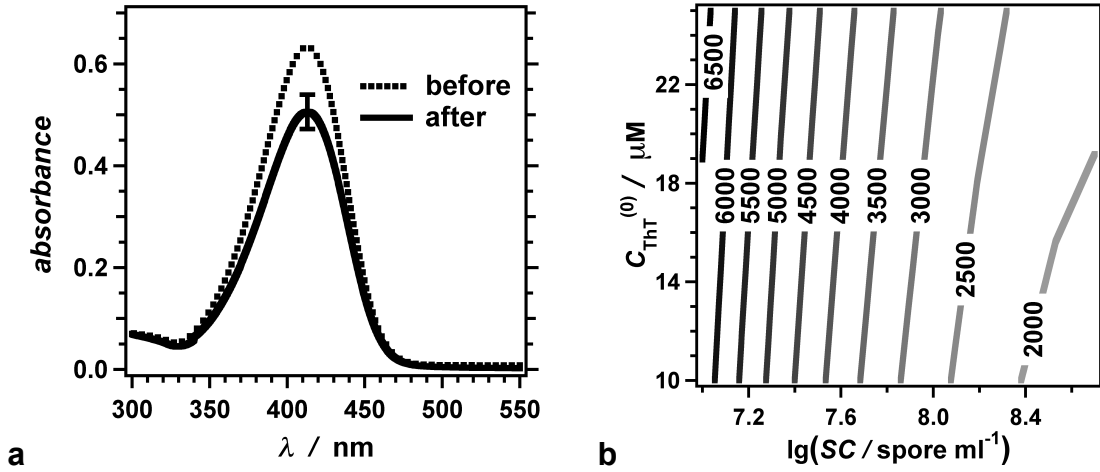


Figure 14. Distribution coefficient, K_D (equation 7), of ThT for endospores of *B. globigii* and *B. thuringiensis*. **(a)** Absorption spectra of ThT dissolved in aqueous media (2 mM Tris buffer, pH 8.5, optical path length = 0.5 cm). The dotted line represents the spectrum of ThT recorded **before** any treatment ($C_{ThT}^{(0)} = 35\ \mu M$). The solid line corresponds to a spectrum of the supernatant **after** the dye solution was incubated with *B. globigii* endospores (8×10^7 spores ml^{-1}) for 10 min and the spores were removed using centrifugation. The decrease in the absorbance corresponds to an 8- μM decrease in the ThT concentration: i.e., the concentration of ThT, which remained free in the solution after the spore uptake, was $28 \pm 2\ \mu M$. (The error bar represents $\pm$ one single standard deviation.) Considering that the volume of a single *B. globigii* spore is about 8×10^{-13} ml, the average ThT concentration in the stained spores was 0.11 M, yielding distribution coefficient $K_D = 3.9 \times 10^3 \pm 0.4 \times 10^3$. **(b)** Contour plot depicting the dependence of ThT K_D for *B. thuringiensis* endospores on the initial dye concentration, $C_{ThT}^{(0)}$, and on the spore count, SC . The lines represent $K_D(C_{ThT}^{(0)}, SC)$ with the identical values as designated by the corresponding numbers tagged to the lines.

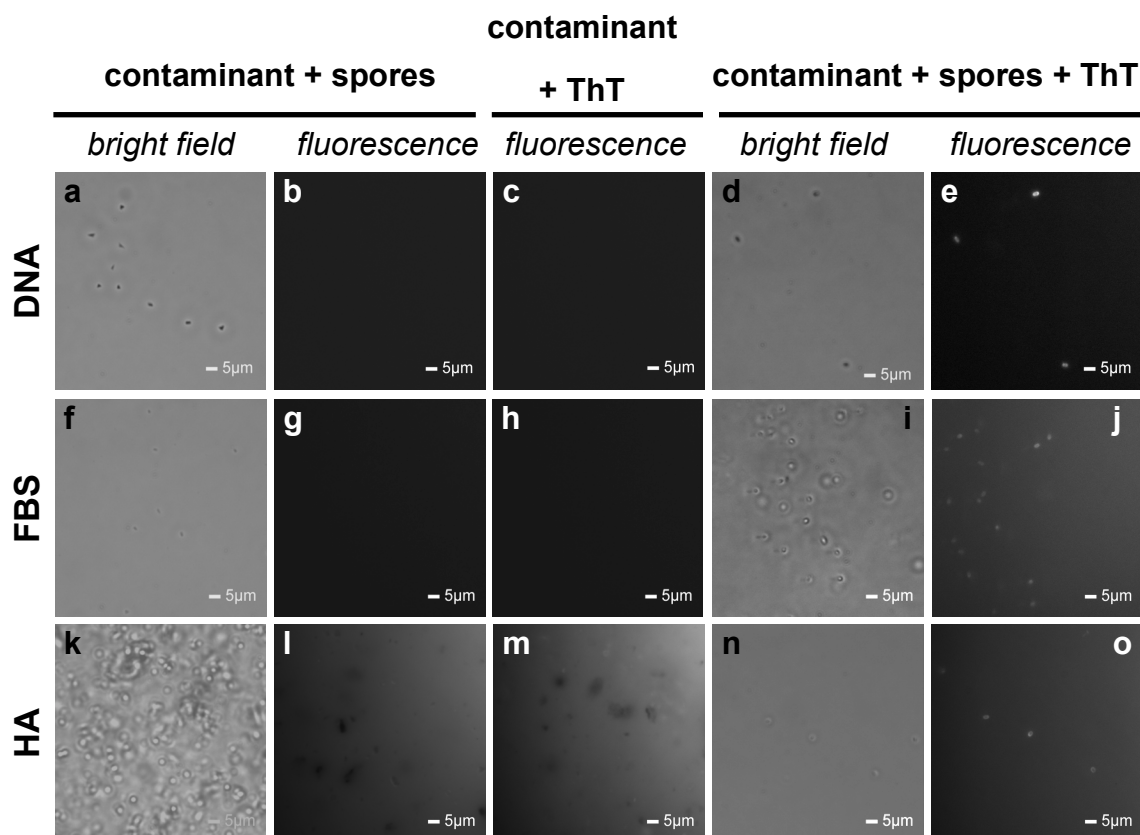


Figure 15. Imaging of endospores of *B. atrophaeus* suspended in solution of biological and environmental contaminants: **(a-e)** 1 mg ml⁻¹ DNA extract from *E. coli*; **(f-j)** 50% v/v fetal bovine serum (FBS); and **(k-o)** 1 mg ml⁻¹ humic acid (HA); dissolved in aqueous buffer (2 mM Tris buffer, pH 8.5; *exposure time* = 0.5 s). The ThT concentration for the stained samples **(c-e, h-j, and m-o)** was 150 µM. The heterogeneity and the pronounced fluorescence of HA (overlapping with the ThT fluorescence range) presented challenges for imaging of the added endospores. We do not have a proof if any of the structures depicted on the bright-field images **(k)** belong to the suspended spores. Staining the sample with ThT, however, allowed for visualization of the spores **(o)** and focusing the objective on them, provided means for observing them even in a bright-field mode **(n)**. Such fluorescence structures were not present in the HA samples without endospores **(m)**.

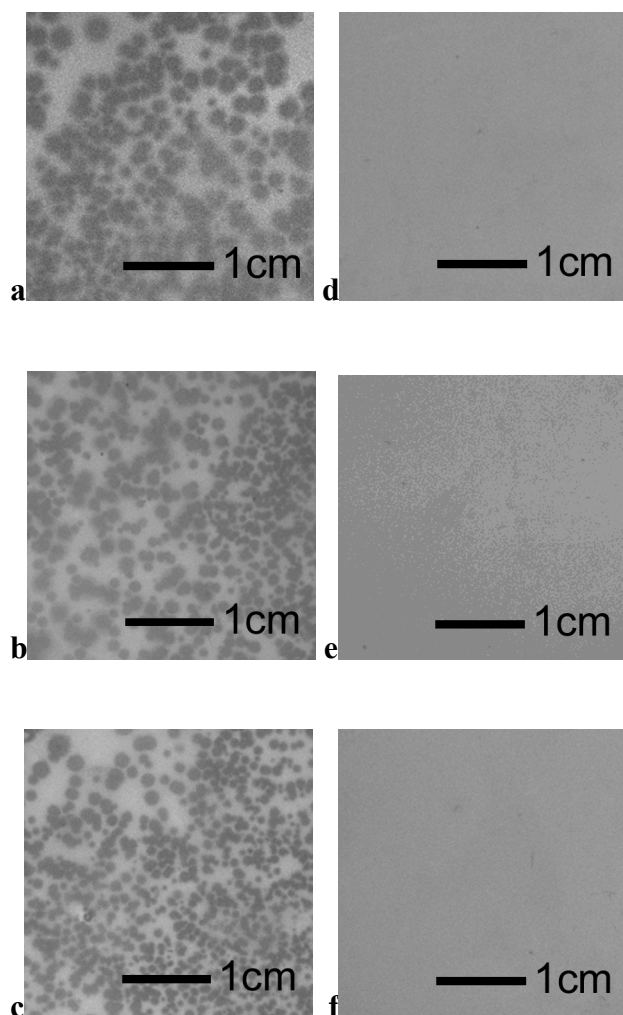


Figure 16. Solid cultures of *B. atrophaeus* plated from endospore suspensions (10^6 spore ml^{-1} , 2 mM Tris buffer, pH 8.5) in 7.5-cm Petri dishes. The endospores were suspended in a solution of (a) 0 μM , (b) 100 μM and (c) 3 mM ThT for > 10 min prior to plating. Similar plating with endospore-free buffer solutions containing: (d) no ThT (e) 100 μM and (f) 3 mM, did not produce detectable colonies.

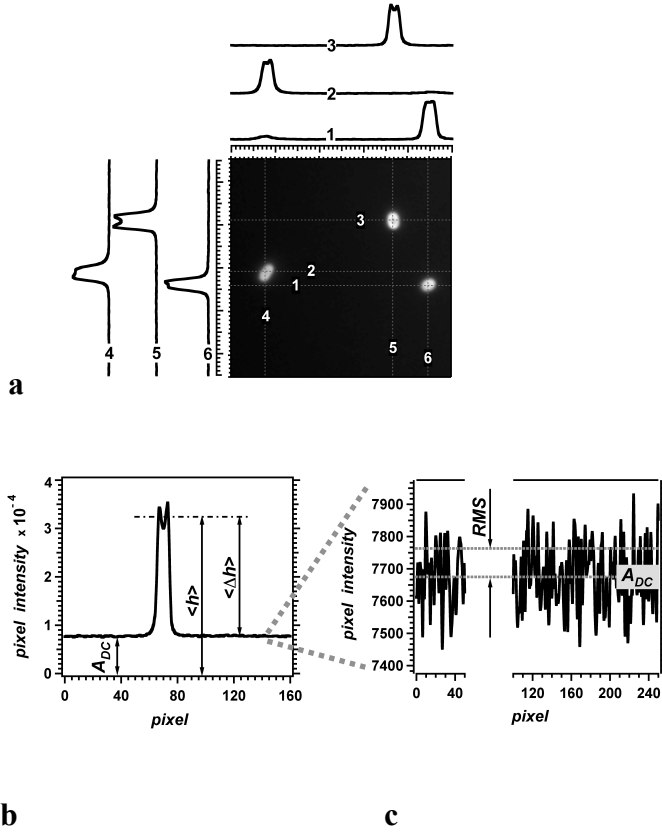


Figure 17. Signal-to-noise analysis of epifluorescence microscopy image of *B. atrophaeus* endospores suspended in a 100 μ M solution of ThT (2 mM Tris buffer, pH 8.5). **(a)** Microscopy image with corresponding horizontal and vertical traces crossing through the fluorescent spores (*exposure time* = 4 s). **(b)** Designation of trace parameters: $A_{DC} = 7.7 \times 10^3$; $\langle h \rangle = 3.2 \times 10^4$; $\langle \Delta h \rangle = 2.4 \times 10^4$. **(c)** The trace baseline with average value of A_{DC} and $RMS = 90$. The data for the trace on **(b)** and **(c)** yield $S/N_{AC} = 69$ dB, and $S/N_{DC} = 12$ dB (equation 6).

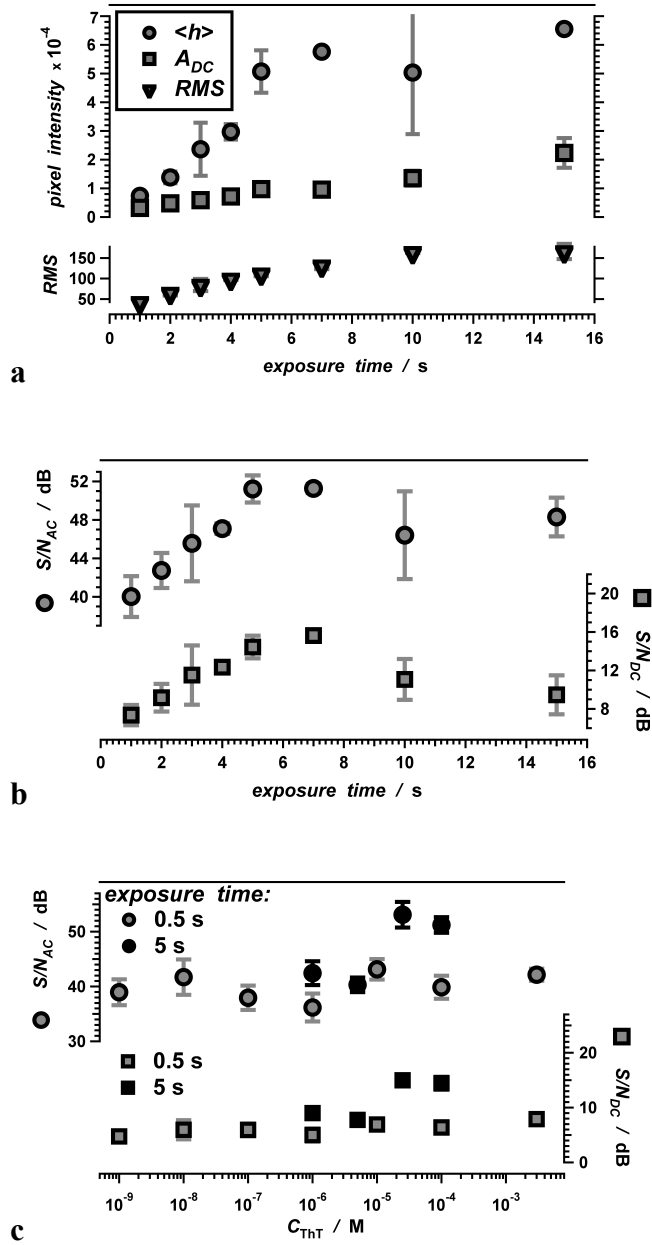


Figure 18. Dependence of signal-to-noise ratios, S/N , on exposure time and on dye concentration, C_{ThT} , for epifluorescence images of *B. atrophaeus* suspended in ThT solution (2 mM Tris buffer, pH 8.5). **(a)** Dependence of the amplitude of the signal, $\langle h \rangle$, and the amplitudes of the DC and AC noise, A_{DC} and RMS , respectively, on the exposure time for $C_{ThT} = 100 \mu\text{M}$. **(b)** Dependence of the AC and DC noise on the exposure time for $C_{ThT} = 100 \mu\text{M}$. **(c)** Dependence of the AC and DC noise on the ThT concentration for $exposure\ time = 0.5\ s$ and $5\ s$.

Chapter 3

Dynamic Staining of *Bacillus* Endospores with Thioflavin T

ABSTRACT

This study examines the discernibility between kinetics of fluorescence staining of endospores of *Bacillus atrophaeus* and *Bacillus thuringiensis*. This study demonstrates avenues for expanding the applicability of dynamic staining to intact endospores, and thus, at furthering the field beyond the previously demonstrated examples with vegetative bacteria. Dynamic staining utilized the time-course of the increase in the fluorescence intensity of an amyloid-staining dye, Thioflavin T (ThT), after mixing with intact endospores. The time constants, τ , for *Bacillus atrophaeus* and *Bacillus thuringiensis* clustered around 7 s and 3 s respectively for the different dye and endospore concentrations. The rates of staining of intact endospores with ThT were statistically different for the two *Bacilli*. Rapid detection and identification of endospores presents a range of complex challenges, and our results from dynamic staining demonstrate a venue for attaining information about the sporulated bacterial species without lysing, germinating or other pretreatment steps.

3.1 Introduction

This report demonstrates the use of thioflavin T (ThT) for dynamic staining of intact endospores of *Bacillus atrophaeus* (*B. atrophaeus*) and *Bacillus thuringiensis* (*B. thuringiensis*). While the fluorescence images of the ThT-stained endospores of the two species had similar appearances (Figure 19a, 19b), the kinetics of the staining process were different for the two *Bacilli* (Figure 19c).

Endospores are packed and resilient entities, and spore-forming bacteria are responsible for the production of some of the most puissant toxins. Despite numerous advances in biomedical technology, the rapid detection and identification of bacterial endospores remains challenging. Assays for bacterial endospores require a range of sample pretreatment steps, such as lysing to extract their content for analysis or allowing them to germinate and analyzing the resulting cultures of vegetative bacteria (4).

The endospore coats contain a wealth of information about the bacterial species (1, 3, 5, 6, 9). Therefore, analytical techniques, targeting components of exosporium, provide the means for rapid endospore biosensing with certain species-specificity.

We previously demonstrated the use of ThT, an amyloid fluorescent stain, for the rapid visualization of endospores (10). ThT binds with a high affinity to proteins with amyloid-like β -sheet motifs—like those that constitute the exosporium of the endospores. Furthermore, upon binding to endospores, the fluorescence quantum yield of ThT increases, resulting in the ability to image endospores in the presence of the leftover

unbound dye. This emission enhancement effectively eliminates the necessity for pre- or post-treatment steps for the visualization of endospores (10).

Recently, we demonstrated that the kinetics of fluorescence enhancement of cyanine stains, when applied to different vegetative bacteria, are characteristic of the species, regardless of whether the bacteria are Gram positive or Gram negative (7). The timed progression of staining, monitored via the enhancement of the fluorescence of the staining dye, differed for different species, which, in turn, provided venues for statistically significant discernibility among microbial organisms (7).

3.2 Results and Discussion

Herein, we proceed to demonstrate the application of this dynamic approach to intact bacterial endospores by utilizing the emission enhancement of ThT. We observed that the rates of fluorescence staining of *B. thuringiensis* were faster than the rates of staining of *B. atrophaeus*, which can be representative of the composition of the endospore coats of the two species.

The experimental data was fit to a monoexponential rise function showing the increase in the emission intensity with time, $F(t)$. This analysis allowed us to determine the characteristic time constants, τ , for the quantification of the dynamics of the fluorescence staining (Figure 19c):(7)

$$F(t)_{t \geq t_0} = F(\infty) - \Delta F \exp\left(-\frac{t-t_0}{\tau}\right) \quad \text{Equation (11)}$$

where $F(\infty)$ is the fluorescence intensity upon completion of the staining, ΔF is the maximum change in the fluorescence intensity, and t_0 is the starting time of endospore-stain interaction initiated by rapid mixing of the sample with the dye.

The intensity change, ΔF , varies with the dye concentration, C_{ThT} , and with the endospore density, represented as spore count, SC (Figure 19d). Doubling the density from 7.5×10^4 to 15×10^4 spores ml^{-1} caused an increase in ΔF that exceeded twofold. Concurrently, a fivefold increase in C_{ThT} from 1 to 5 $\mu\text{mol l}^{-1}$ caused an increase in ΔF that was less than a factor of two. A further increase in C_{ThT} from 5 to 10 $\mu\text{mol l}^{-1}$ marginally affected ΔF (Figure 19d). These trends were similar for the endospores of both species (Figure 19d), indicating that static measurements of fluorescence intensity of the stained samples (i.e., $F(\infty)$ or ΔF) could not provide sufficient information for discerning between the two *Bacilli*.

Conversely, the dynamic characteristic of the straining processes, τ , manifested distinctly different trends in comparison with the static characteristic, ΔF (Figure 19e). For endospore densities varying around 10^5 spores ml^{-1} , the time constants for each of the two *Bacilli* did not manifest a statistically significant dependence on the dye concentration in the range between 1 and 10 $\mu\text{mol l}^{-1}$. (For two-factor analysis of variance (ANOVA2), the two null hypotheses were: $H_{0,1}$: the values of τ do not depend on C_{ThT} ; and $H_{0,2}$: the values of τ do not depend on the spore count, SC. For *B. atrophaeus*,

ANOVA2 produced $p_{SC} = 0.97$, $p_{ThT} = 0.75$, and $p_{SC,ThT} = 0.38$, i.e., neither $H_{0,1}$, nor $H_{0,2}$, can be rejected. For *B. thuringiensis*, ANOVA2 produced $p_{SC} = 0.36$, $p_{ThT} = 0.29$, and $p_{SC,ThT} = 0.53$, i.e., neither of the null hypotheses can be rejected.) Such a lack of statistically significant concentration dependence of the time constants agreed with the kinetic trends we have observed for vegetative bacterial cells (7).

For *B. atrophaeus* endospores, the τ values for staining with ThT clustered around 7 s, while *B. thuringiensis* τ values clustered around 3 s for the same dye. This difference constitutes a statistically significant discernibility in the respective τ values of the *Bacilli*. (Comparing all values of τ measured for *B. atrophaeus* with all values of τ for *B. thuringiensis*, using one-factor ANOVA (H_0 : the values of τ do not depend on the endospore species), produced $p = 1.8 \times 10^{-14}$. Therefore, H_0 can be rejected at any reasonable level of confidence.) The faster rate of staining of *B. thuringiensis*, in comparison with *B. atrophaeus*, was attributed to the reduced amount of proteins on *B. thuringiensis*' surface to be stained, as reflected by its genotype (6).

The absolute values of fluorescence intensities, representing static characteristics such as $F(\infty)$, which is the sum of the initial fluorescence intensity and ΔF , depend on: (1) sample properties, such as the partition coefficient of ThT between the aqueous media and the endospores, the fluorescence quantum yield of the free and the bound dye, and the scattering properties of the endospore suspension; and (2) instrumental settings, such as the manners of sample photoexcitation and emission measurement. Therefore, ΔF manifests dependence on SC and C_{ThT} , which is not necessarily linear (Figure 19d).

Conversely, the dynamic characteristics of staining, such as the time constants, τ , depend solely on the internal sample properties. Because τ characterizes the rates of fluorescence changes, the emission intensities prior to staining (unbound ThT) and after the completion of the staining process served as an internal standard, allowing for compensating for potential changes in the instrumental settings.

Based on a diffusion model of staining, τ should exhibit negligible concentration dependence and, concurrently, a strong dependence on the cell or endospore media that takes up the staining dye (2, 7). This simplified view appears to be in accord with the staining dynamic of species and stains that we currently report and previously investigated (7).

3.3 Conclusions

These findings show the species discernibility between intact bacterial endospores produced by the dynamic staining approach. Moreover, the observed trends demonstrate the advantages of applying dynamic, instead of static, approaches to bioanalysis. We believe that dynamic staining provides an avenue of unprecedented simplicity and facility for endospore assays.

3.4 Experimental

Following the methods we previously reported (8, 10), we inoculated the *Bacilli*, sporulated the cells, and purified the endospores. Bright field and epifluorescence microscopy allowed us to examine the samples (Figure 19a, b) (8, 10). We recorded the kinetics of fluorescence staining with ThT using protocols developed in our lab (7).

Acknowledgements. Funding for this research was provided by the U. S. National Science Foundation (CBET 0935995), by the UC Regents Faculty Development Award.

3.5 References

1. **Chada, V. G., E. A. Sanstad, R. Wang, and A. Driks.** 2003. Morphogenesis of bacillus spore surfaces. *Journal of Bacteriology* **185**:6255-6261.
2. **Crank, J.** 1975. *The Mathematics of Diffusion*, 2nd ed. Clarendon Press: Oxford, U.K.
3. **Kim, H., M. Hahn, P. Grabowski, D. C. McPherson, M. M. Otte, R. Wang, C. C. Ferguson, P. Eichenberger, and A. Driks.** 2006. The *Bacillus subtilis* spore coat protein interaction network. *Molecular Microbiology* **59**:487-502.
4. **Knecht, L. D., P. Pasini, and S. Daunert.** 2011. Bacterial spores as platforms for bioanalytical and biomedical applications. *Analytical and Bioanalytical Chemistry* **400**:977-989.
5. **Plomp, M., T. J. Leighton, K. E. Wheeler, H. D. Hill, and A. J. Malkin.** 2007. In vitro high-resolution structural dynamics of single germinating bacterial spores. *Proceedings of the National Academy of Sciences of the United States of America* **104**:9644-9649.
6. **Plomp, M., T. J. Leighton, K. E. Wheeler, and A. J. Malkin.** 2005. Architecture and High-Resolution Structure of *Bacillus thuringiensis* and *Bacillus cereus* Spore Coat Surfaces. *Langmuir* **21**:7892-7898.
7. **Thomas, M. S., V. Nunez, S. Upadhyayula, E. R. Zielins, D. Bao, J. M. Vasquez, B. Bahmani, and V. I. Vullev.** 2010. Kinetics of Bacterial Fluorescence Staining with 3,3'-Diethylthiacyanine. *Langmuir* **26**:9756-9765.
8. **Vullev, V. I., J. Wan, V. Heinrich, P. Landsman, P. E. Bower, B. Xia, B. Millare, and G. Jones, II.** 2006. Nonlithographic Fabrication of Microfluidic Devices. *Journal of the American Chemical Society* **128**:16062-16072.
9. **Wang, R., S. N. Krishnamurthy, J. S. Jeong, A. Driks, M. Mehta, and B. A. Gingras.** 2007. Fingerprinting species and strains of *Bacilli* spores by distinctive coat surface morphology. *Langmuir* **23**:10230-10234.
10. **Xia, B., S. Upadhyayula, V. Nuñez, P. Landsman, S. Lam, H. Malik, S. Gupta, M. Sarshar, J. Hu, B. Anvari, G. Jones, and I. Vullev Valentine.** 2011. Amyloid histology stain for rapid bacterial endospore imaging. *Journal of clinical microbiology* **49**:2966-2975.

3.6 FIGURES

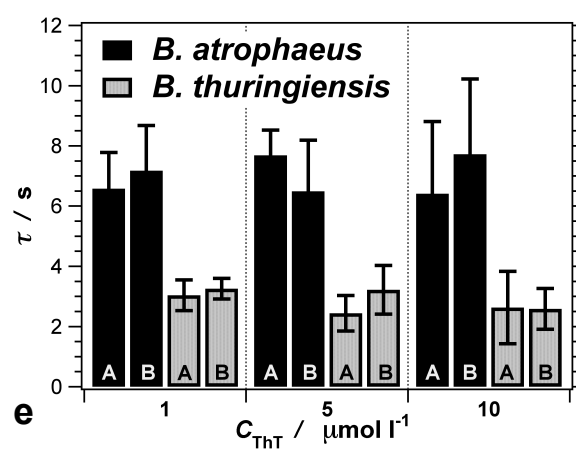
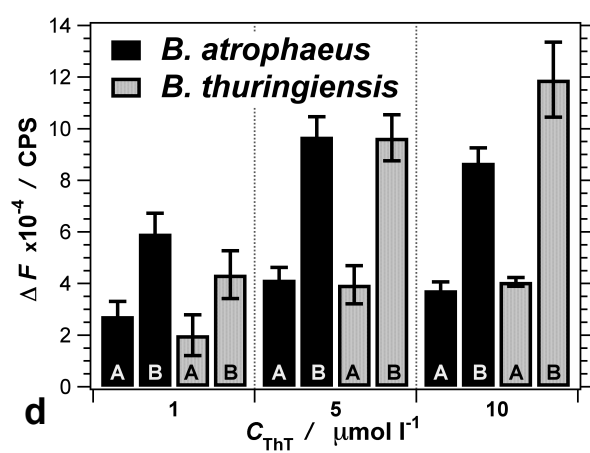
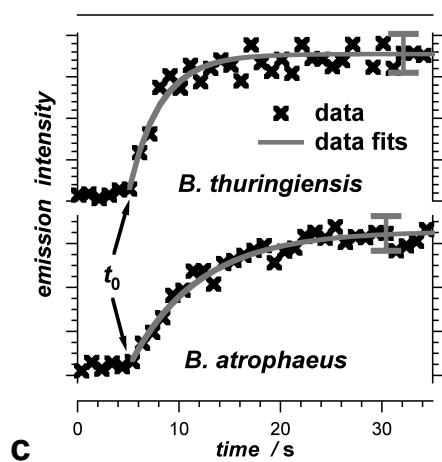
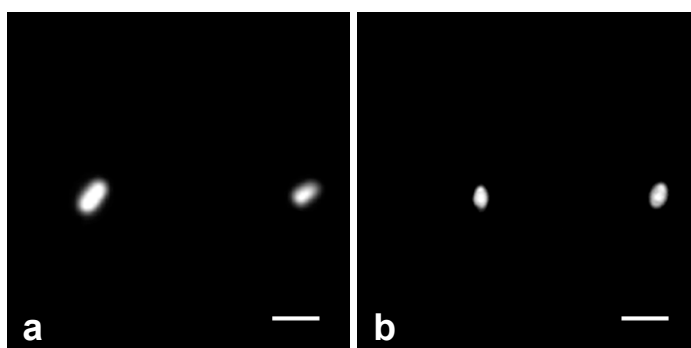


Figure 19. Fluorescence staining of endospores of *B. atrophaeus* and *B. thuringiensis* with ThT. (a, b) Epifluorescence microscope images of endospores of (a) *B. atrophaeus* and (b) *B. thuringiensis* stained by mixing with a ThT aqueous solution ($C_{\text{ThT}} = 1 \mu\text{mol l}^{-1}$; 2 mM Tris buffer, pH 8.5, with 1 mM TWEEN[®] 40; $\lambda_{\text{ex}} 482 \text{ nm}$, $\lambda_{\text{em}} 536 \text{ nm}$, the band widths of the imaging filters were 25 nm; scale bar corresponds to 3 μm). (c) Kinetics of the enhancement of the emission of $1 \mu\text{mol l}^{-1}$ ThT upon mixing with endospores, $1.5 \times 10^5 \text{ spores ml}^{-1}$, at time t_0 , with the monoexponential data fits (2 mM Tris buffer, pH 8.5, with 1 mM TWEEN[®] 40; $\lambda_{\text{ex}} 430 \text{ nm}$, $\lambda_{\text{em}} 480 \text{ nm}$). Category plots of: (d) static, ΔF , and (e) dynamic, τ , characteristics of staining of *B. atrophaeus* and *B. thuringiensis* with ThT. The time constants, τ , and the change in the fluorescence intensity, ΔF , were extracted from least-square data fits of kinetic emission data using Equation 11 for different dye contraptions, C_{ThT} , and endospore densities of: A = $7.5 \times 10^4 \text{ spores ml}^{-1}$, and B = $15 \times 10^4 \text{ spores ml}^{-1}$. (d, e) The average values and standard deviations of τ and ΔF shown in the category plots were obtained from four repeats. ΔF values represent the maximum difference between the average final emission intensity ($\sim 120 \text{ s}$ after the addition of endospores) and the average initial emission intensity (before the addition of endospores).

Chapter 4

Dynamic Staining of Bacteria at a Single-Cell Level

ABSTRACT

Bacterial infectious diseases remain one of the major health hazards nation- and worldwide. The expedience of detection and identification of bacterial pathogens determines how early the diagnosis is, and hence, what the treatment and the outcome of the illness would be. As we have previously reported, the dynamics of fluorescence staining provides venues for the development of expedient assays for detection and identification of bacterial species (19). We measured the kinetics of bacterial staining with cyanine and thioflavin dyes and investigated their photophysical properties. We demonstrated that the pseudo first-order kinetic constants of the fluorescence staining processes have species specificity without contrition dependence. Combining the dynamics of staining with real-time fluorescence microscopy we characterized the fluorescence staining process at the single-cell level with improved sensitivity and contrast.

4.1 Introduction

This chapter describes the investigation of the dynamic staining (DS) of bacteria with fluorescent dyes: 3,3'-diethylthiacyanine (THIA) and thioflavin T (ThT) (Scheme & 3 respectively)(19, 24). We recorded the kinetics of staining of suspensions of vegetative bacterial cells and analyzed the uniqueness of the extracted time constants. Utilizing imaging allowed us to record DS at a single-cell level.

A quarter of the human deaths worldwide are caused by bacterial infections (1, 2, 4, 8, 11). Although bacterial infections are not necessarily lethal and they can generally be successfully treated, they can strike in an opportunistic manner and put the lives of immune-compromised populations at risk of further complications. Additionally, the identification of a pathogen most often requires culturing which can range between 12-72 hours or even up to weeks in the case of the notorious *Mycobacterium tuberculosis*. During this period the treatment method unavoidably becomes employing broad-spectrum antibiotics, which yields idle results for the patient, and may also contribute to the progression of multi-drug resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) (9, 11, 15, 16). Recent advances in medical training of military health-care personnel, improved protective equipment for soldiers and advanced surgery procedures have improved the survival rates of warfare casualties (10, 14). As a result, mortality rates of wounded soldiers is increasingly attributed to bacterial infections such as sepsis (13).

While speed is crucial for the diagnosis of bacterial infections, conventional cell-staining methods, which offer expedience and relative simplicity, inherently provide

Boolean outcomes (based only on the final appearance of the tested samples). Ever since the development of Gram staining more than a century ago, such “yes-no” information has been the traditional outcome, revealing if the sample belongs to one of two groups of bacterial species (e.g., Gram-positive or Gram-negative) (5-7). Staining assays with improved specificity, such as immunoassays, also yield Boolean information. They separate the results into groups, either the species are the ones targeted by the antibodies used in the test, or everything else. Inherently, such assays provide information only about the species that are targeted by each specific test. Therefore, pathogens present in the sample, which are not targeted by the conducted tests, remain undetected.

Time-consuming amplification steps are essential for improving the sensitivity of bioassays, and the most common way to amplify the number of bacterial cells is by culturing them in the appropriate growth conditions. Other amplification procedures such as the enzyme-linked immunosorbent assay (ELISA) enhance the signal from the immune-based assay (3), however, prior knowledge of the species in question and the availability of the targeting immunoglobins is required. Additionally, PCR-based assays (12, 17) amplify the amount of genetic material extracted from the bacteria. This approach, however, requires a number of pretreatment steps, such as cell lysing, as well as the prior knowledge of the targeted DNA sequence and the availability of the specific primers. Such sample pretreatment tends to take most of the time and thus compromises the expedience of the analysis. Therefore, an expedient bioanalysis method for identification of bacteria without pretreatment, i.e. single-cell analysis is urgently needed.

The utilization of the DS of bacteria with fluorescent dyes provides a promising alternative to traditional staining- and amplification-based methodologies. As we previously demonstrated, DS has the potential for rapid bacteria identification assays that provide results beyond the “yes-no” Boolean outcomes of the traditional methodologies (Figure 20) (19, 25). By investigating the DS of a range of microorganisms, we demonstrated that a fluorogenic dye manifests an increase in its fluorescence quantum yield in the presence of bacterial cells. This fluorescence enhancement results in a significant contrast, allowing for investigating the fluorescence signal from the stained cells without the removal of the staining dye that remains free in the surrounding suspension media. Due to the relatively weak background emission of the free dye, however, a limit to the sensitivity for characterizing the time-dependant staining arises when this ensemble average approach is applied to diluted bacterial suspensions. Increased interfering background fluorescence of free dye in the solution is observed as cell density in the suspension drops below 10^5 cell / ml. Furthermore, the feasibility of a bacteria detection assay that requires cell concentrations above 10^5 cell / ml is compromised especially if the sample of interest is from the blood of a patient.

Herein we describe the principles of DS and demonstrate kinetics-based discernibility of an infectious species, *Pseudomonas aeruginosa* (*P. aeruginosa*). Combining fluorescence microscopy and the dynamic staining approach, allowed us to bring DS to a single-cell level. We obtained sequential images (i.e., fluorescence microscopy movie) of individual cells during the DS process with excellent signal-to-

noise ratios. The single-cell DS revealed kinetic features that were not apparent in the ensemble-average measurements we carried with the bacterial suspensions.

4.2 Results

4.2.1 DS of *E. coli* with 3,3'-diethylthiacyanine

Kinetic analysis of the emission enhancement allows for extracting time constant, τ , which is characteristic of the staining dye and of the bacterial species. Unlike-steady state intensity, a dynamic quantity, such as τ , does not require references and standards and is not as prone to experimental errors. We recorded fluorescence kinetics curves for various bacteria species and utilized a mono-exponential fit of the curves to extract the time constant for the fluorescence enhancement (Figure 20 & Equation 1).

$$F(t) = F_{\infty} \left(1 - e^{-\frac{t}{\tau}} \right) + F_0 \quad \text{Equation (12)}$$

We observed that the time constants of staining did not depend on the dye concentration and on the cell density (Table 4).

This species/dye specificity of τ , along with its lack of concentration dependence, is the principal characteristic of dynamic staining essential for becoming a viable bioanalytical technique for identification of bacterial pathogens.

4.2.2 DS of *P. aeruginosa* with 3,3'-diethylthiacyanine

We investigated the DS of *P. aeruginosa* with THIA based on the prevalence of multi-drug resistant (MDR) *P. aeruginosa* (14) infections reported among military personnel exposed to active combat operations. Figure 21a shows the enhancement of THIA fluorescence in the presence of *P. aeruginosa*. After mono-exponential fitting, the average DS of *P. aeruginosa*, shown in Figure 21b, yielded a time constant (τ) for this species of 7.54 ± 0.3 s.

4.2.3 Single-cell dynamic staining of *E. coli*

Despite the pronounced bacterium-induced enhancement of the fluorescent dye emission, the lower detection limit we observed is in the order of approximately 10^5 cell/ml. This sensitivity limit is posed by the background fluorescence of the non-bound dye in the solution media. Concurrently, we readily imaged individual ThT-stained bacterial cells with excellent contrast and signal-to-noise ratios. Therefore, employing imaging for dynamic staining (DS) of entrapped bacterial cells allows for bringing the sensitivity of DS to a single-cell level.

At the single-cell level, we observed that the DS with ThT does not follow a mono-exponential rise, as we previously reported with THIA using an ensemble approach containing at least 10^5 cells/ml (19).

In addition to the immense sensitivity improvement, single-cell DS provides means for analysis of samples containing mixtures of bacterial species. The ability to visualize bacterial cells in the presence of environmental contaminants (such as humic acid) or in biological samples (such as in blood serum), provides important means for DS analysis of samples that do not require pre or post treatment (such as purification or cell lysis) (24).

Based on the rise of the fluorescence intensity for a series of single cells upon the introduction of ThT dye (as seen in figure 22), the time constant consistently clustered around 9.8 ± 2.3 seconds (figure 23).

4.3 Conclusions

The kinetics of fluorescence staining can serve as a robust platform for the detection and identification of bacterial agents, including endospores and vegetative cells. This unprecedented approach utilizes the DS, i.e., the time course of fluorescence-enhancement signals, as a signature for identification of microorganisms.

Acknowledgements. This research was supported by the National Science Foundation (CBET 0935995 and DBI 0731660) and by the U.C. Regents Faculty Development Award. We would like to extend our gratitude to Prof. Bahman Anvari and Dr. Sharad Gupta for assisting with the fluorescence imaging; and to Prof. Jiayu Liao for providing the samples of *E. coli*.

4.4 Experimental

4.4.1 Preparation of ThT and THIA solutions

The thioflavin T (ThT) dye solution was prepared using by taking the appropriate amount of dye powder and solubilizing it in 40% methanol, 10% ethanol and 50% Milli-Q water. Then the ThT solution was filtered through a 0.22 μm BD Visitec™ high flow filter. The concentration of the dye solution was characterized by taking its absorbance and using the molar extinction coefficient of 36,000 $\text{mol} / \text{l} \cdot \text{cm}$. The stock ThT solution was then diluted to a concentration of 100 μM with 2 mM tris(hydroxymethyl)aminomethane (TRIS) buffer solution and was then used for staining of the cells.

A 60 μM 3,3'-diethylthiacyanine (THIA) dye stock solution was prepared by adding the appropriate amount of THIA dye powder to a 70% ethanol and 30% Milli-Q water solution. The stock solution was diluted ten times into 2 mM TRIS buffer solution and was then used for staining of the cells.

4.4.2 Cell culture and preparation procedures

Cultures of *B. subtilis*, *B. sphaericus* and *P. aeruginosa* were purchased from ATCC (Manassas, VA). The *E. coli* TOP 10 cells were obtained from Invitrogen (Carlsbad, CA) by Prof. Jiayu Liao. The bacterial cultures were grown at 37° C in sterilized LB broth liquid media, under aerobic conditions and constant shaking (at 200

rpm on Forma Orbital Shaker, Thermo). For sample preparation, the bacterial cultures were subjected to a sequence of centrifugation/wash steps (using Eppendorf 5415D centrifuge) and finally suspended in 2 mM Tris buffer, pH = 8.5. If satiated, the aqueous Tris buffer solution contained 0.02 mM TWEEN® 40. Cell counting was performed using an improved Neubauer counting chamber (Paul Marienfeld GmbH & Co. KG) and a MicroMaster microscope (Fisher Scientific) equipped with a 40× (0.40 NA) objective. To ensure that the bacterial cultures, which we used for the sample preparation, were in their exponential growth phase, we constructed growth curves for the investigated bacterial species. The curves revealed that the investigated bacteria, cultured for 18-24 hours, are still in their active growth phase.

4.4.3 Imaging DS

Using surface chemistry methods developed in our lab (23), we aminated clean glass surfaces in order to immobilize bacterial cells for imaging. Subsequent to immobilizing cells on surfaces, we introduced 100µM ThT for florescent staining. We captured the DS images using Nikon Ti-U microscope (Nikon Inc., Melville, NY) along with a Hamamatsu EM-CCD digital camera (model C9100-13, Hamamatsu Corp., Bridgewater, NJ). The exposure time was manually set to 0.1 s, which corresponds to a capture rate of 10 frames / s. Excitation of ThT was facilitated using a 100-watt Xenon arc lamp (LUDL Electronic products, Hawthorne, NY), coupled with a fluorescein isothiocyanate (FITC) optical filter (λ_{ex} 482 nm, λ_{em} 536 nm with 25 nm bandwidth).

4.4.4 Image analysis

Captured images were recorded and saved as TIFF stacks for analysis using Imaris 7.0 (Bitplane Inc., Saint Paul, MN). Imaris Spot recognition algorithm parameters were optimized for recognizing and tracking single bacterial cells for almost the entirety of the DS process.

Limitation in extracting kinetics based on fluorescent intensity signals from single cells arose from: (1) exposure time required for detecting fluorescent cell intensity, and (2) threshold of signal-to-noise ratios necessary to distinguish intensity signals from background versus stained cells. To overcome these limitations, we employed 0.1 s as the exposure time, allowing us to study kinetics in the seconds timescale. Furthermore, we started DS analysis of captured image sequences on Imaris 1 s after the introduction of ThT dye. The recognized single-cell spot intensities were exported for further analysis using Igor Pro software v.6.02A (Wavemetrics) installed on Windows and Mac OS workstations as we have previously demonstrated (18, 20-25).

We approximated the time constant, τ , for the *E. coli* cells stained with 100 μ M ThT using equation 2 (eq. 2), where τ is the time when the fluorescence intensity signal is F_τ . F_0 and F_∞ refer to the initial and final fluorescence intensities respectively. For all time constant calculations, F_0 was approximated after the background dye intensity reached a steady state level.

$$F_\tau = \left(1 - \frac{1}{e}\right)(F_\infty - F_0) \quad \text{Equation (13)}$$

4.5 References

1. **Anderson, J.** report 2007. Basic Research on Bacteria. The Essential Frontier. American Society for Microbiology and National Institute of Health.
2. **Beaglehole, R., A. Irwin, and T. Prentice** 2004, posting date. The World Health Report 2004.
3. **Beutner, E. H., E. J. Holborow, and G. D. Johnson.** 1965. A New Fluorescent Antibody Method - Mixed Antiglobulin Immunofluorescence or Labelled Antigen Indirect Immunofluorescence Staining. *Nature* **208**:353.
4. **Cossart, P., and P. J. Sansonetti.** 2004. Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens. *Science* **304**:242-248.
5. **Friedländer, C.** 1883. Die Mikrokokken der Pneumonie. *Fortschritte der Medizin* **1**:715-733.
6. **Gram, H. C.** 1884. Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten. *Fortschritte der Medizin* **2**:185–189.
7. **Green, L. H.** 2009. *Practical Handbook of Microbiology*, 2nd ed. CRC Press, New York.
8. **Kumble, K. D.** 2006. Anti-bacterial drug discovery using systems biology. *Mini-Reviews in Medicinal Chemistry* **6**:1275-1283.
9. **Maloy, S., and M. Schaechter.** 2006. The era of microbiology: A golden phoenix. *International Microbiology* **9**:1-7.
10. **Mazzuchi, J., R. Claypool, K. Hyams, and D. Trump.** 2000. Protecting the health of US military forces: a national obligation. oai.dtic.mil.
11. **Millar, B. C., J. Xu, and J. E. Moore.** 2007. Molecular diagnostics of medically important bacterial infections. *Current Issues in Molecular Biology* **9**:21-40.
12. **Mothershed, E. A., and A. M. Whitney.** 2006. Nucleic acid-based methods for the detection of bacterial pathogens: Present and future considerations for the clinical laboratory. *Clinica Chimica Acta* **363**:206-220.
13. **Murray, C. K.** 2008. Epidemiology of infections associated with combat-related injuries in Iraq and Afghanistan. *Journal of Trauma-Injury Infection and Critical Care* **64**:S232-S238.
14. **Murray, C. K.** 2008. Infectious disease complications of combat-related injuries. *Critical Care Medicine* **36**:S358-S364.

15. **Overbye, K. M., and J. F. Barrett.** 2005. Antibiotics: where did we go wrong? *Drug discovery today* **10**:45-52.
16. **Owens, S.** 2001. Killing the right bug. *Embo Reports* **2**:757-760.
17. **Saikaly, P. E., M. A. Barlaz, and F. L. de Los Reyes, 3rd.** 2007. Development of quantitative real-time PCR assays for detection and quantification of surrogate biological warfare agents in building debris and leachate. *Applied and Environmental Microbiology* **73**:6557-6565.
18. **Thomas, M., J. Wan, and V. Vullev.** 2009. Immobilization of Proteins on a Glass Surface at High Density. *AIP Conference Proceedings*.
19. **Thomas, M. S., V. Nunez, S. Upadhyayula, E. R. Zielins, D. Bao, J. M. Vasquez, B. Bahmani, and V. I. Vullev.** 2010. Kinetics of Bacterial Fluorescence Staining with 3,3'-Diethylthiacyanine. *Langmuir* **26**:9756-9765.
20. **Upadhyayula, S., D. D. Bao, B. Millare, S. S. Sylvia, K. M. M. Habib, K. Ashraf, A. Ferreira, S. Bishop, R. Bonderer, S. Baqai, X. Y. Jing, M. Penchev, M. Ozkan, C. S. Ozkan, R. K. Lake, and V. I. Vullev.** 2011. Permanent Electric Dipole Moments of Carboxyamides in Condensed Media: What Are the Limitations of Theory and Experiment? *Journal of Physical Chemistry B* **115**:9473-9490.
21. **Upadhyayula, S., T. Quinata, S. Bishop, S. Gupta, N. R. Johnson, B. Bahmani, K. Bozhilov, J. Stubbs, P. Jreij, P. Nallagatla, and V. I. Vullev.** 2012. Coatings of Polyethylene Glycol for Suppressing Adhesion between Solid Microspheres and Flat Surfaces. *Langmuir* **28**:5059-5069.
22. **Vullev, V., J. Wan, V. Heinrich, and P. Landsman.** 2006. Nonlithographic fabrication of microfluidic devices. *Journal of American Chemical Society* **50**:16062-16072.
23. **Wan, J., M. Thomas, S. Guthrie, and V. Vullev.** 2009. Surface-Bound Proteins with Preserved Functionality. *Annals of biomedical engineering* **37**: 1190-1205.
24. **Xia, B., S. Upadhyayula, V. Nuñez, P. Landsman, S. Lam, H. Malik, S. Gupta, M. Sarshar, J. Hu, B. Anvari, G. Jones, and I. Vullev Valentine.** 2011. Amyloid histology stain for rapid bacterial endospore imaging. *Journal of clinical microbiology* **49**:2966-2975.
25. **Zielins, E., M. Thomas, and V. I. Vullev.** 2008. Bacterium-Induced Fluorescence-Enhancement Kinetics: Breaking 100-Year Old Traditions of Staining Bioanalyses. *UCR Undergraduate Research Journal* **2**:75-82.

4.6 TABLES

Table 4. Ensemble measurements of time constants for the fluorescence enhancement of THIA upon staining of *E. coli* cells at varying dye and cell concentrations.

	<i>E. coli</i>		
	5×10^6 cell / ml	5×10^7 cell / ml	5×10^8 cell / ml
0.6 μ M	34 ± 4	33 ± 6	22 ± 6
6 μ M	27 ± 2	27 ± 6	29 ± 4
60 μ M	31 ± 6	36 ± 5	26 ± 1

Table 5. Time constants for *P. aeruginosa*, *B. sphaericus*, *B. subtilis* and *E. coli*. Cell concentrations are 2×10^7 cell / ml for *P. aeruginosa* and 5×10^7 cells / ml for the other species.

	<i>E. coli</i>	<i>B. sphaericus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>
6 μ M	27 ± 2 s	52 ± 8 s	3.9 ± 1.6 s	7.54 ± 0.3 s

4.7 FIGURES

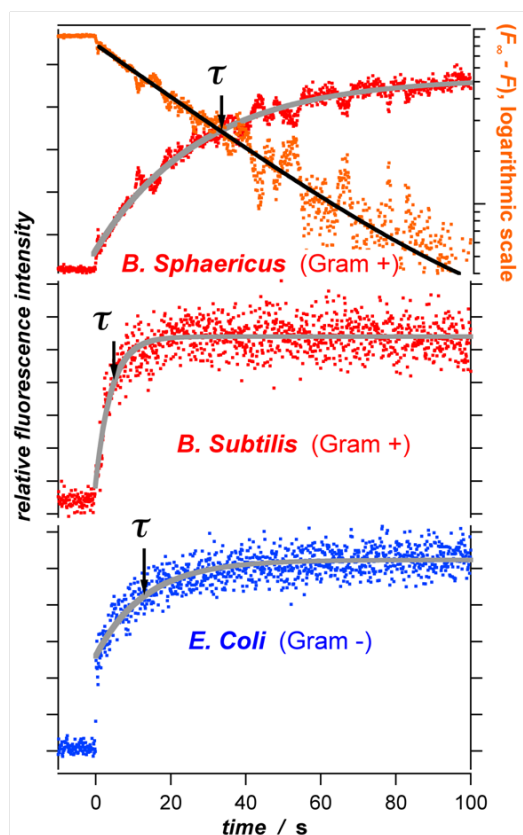


Figure 20. Fluorescence-enhancement kinetic curves ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 475$ nm) for THIA (6 μ M in 2 mM TRIS buffer, pH = 8.5) recorded for suspensions of three different bacterial vegetative cells. The solid lines represent the monoexponential data fits.

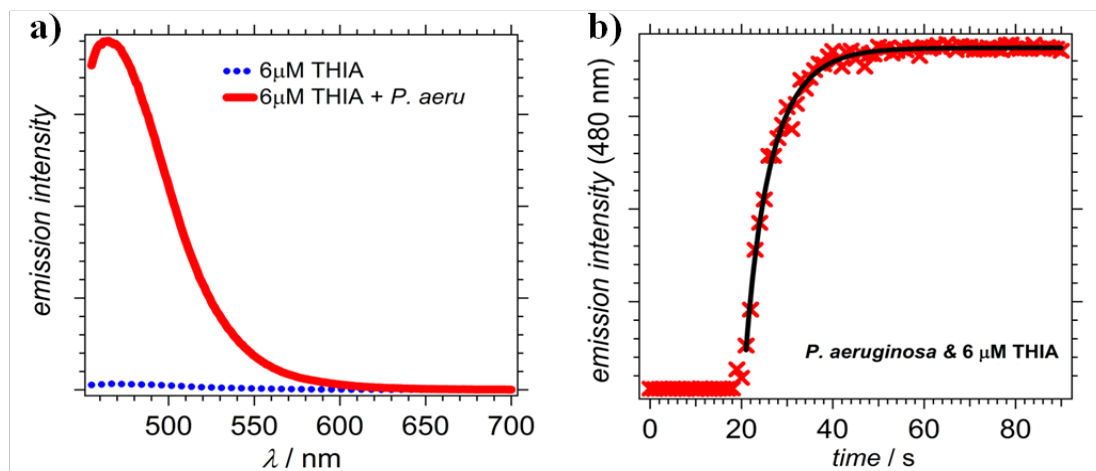


Figure 21. a) Fluorescence spectra of 6 μM THIA versus 6 μM THIA with 2×10^7 *P. aeruginosa* cells. b) DS of *P. aeruginosa* cells with 6 μM THIA.

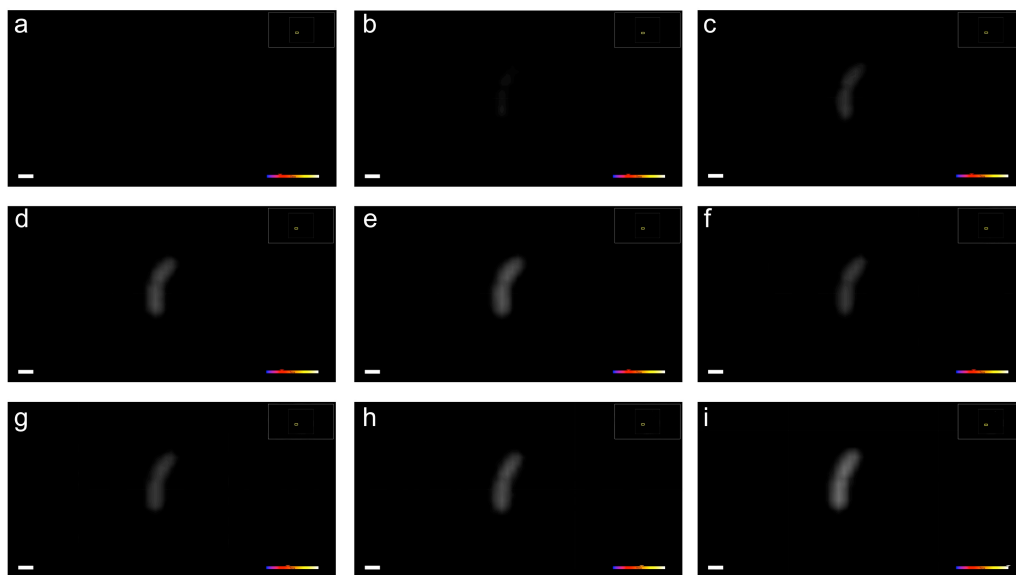


Figure 22. Sequential images showing the DS of single *E. coli* cells trapped on aminated surfaces, and stained with 100 μ M ThT. a) no dye present; b) imaged 1.7 s after 100 μ M ThT introduced; c) imaged 1.8 s after ThT addition; start of DS; d-i) imaged after ThT addition 2.0, 2.1, 3.7, 7.1, 13.3, 33.2 s respectively. The scale bar on the bottom left corresponds to 2 μ m.

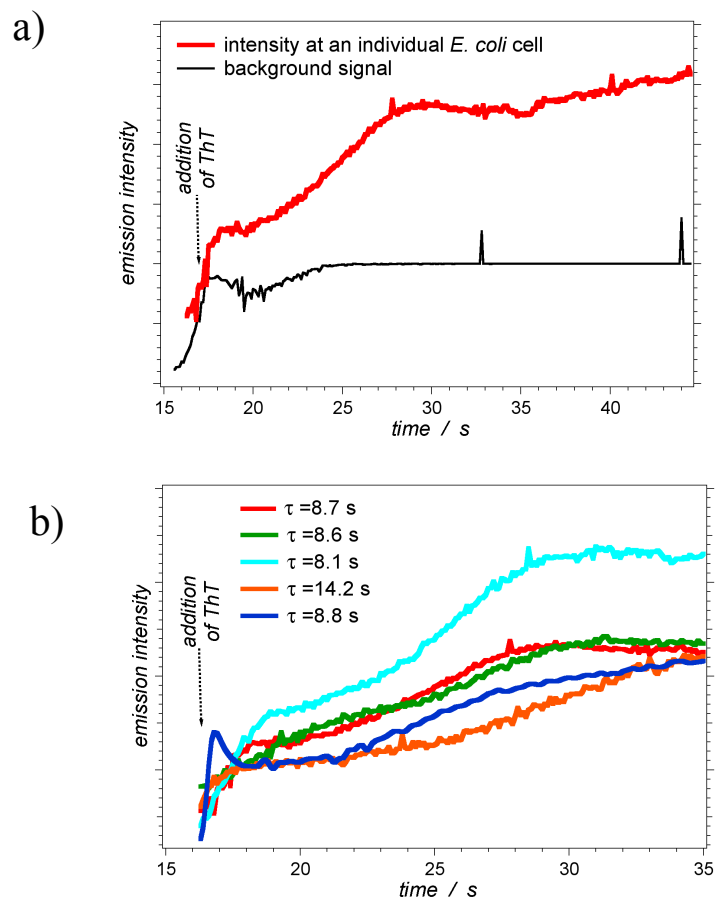


Figure 23. Dynamic staining of individual *E. coli* cells based on the fluorescence intensity signal. a) comparison between intensity kinetics of background and single *E. coli* cell after the addition of 100 μ M ThT extracted from image sequence shown in figure 22. b) time constants derived for multiple single *E. coli* cells after addition of 100 μ M ThT; data plotted from multiple experiment image sets.

Srigokul Upadhyayula

Ph.D. Candidate; *Department of Biochemistry and Molecular Biology*
M.Sc. Candidate; *Department of Bioengineering*
University of California, Riverside, Material Science & Engineering 233
(951) 732 8424; supad003@ucr.edu; www.vullevgroup.org

Employment:

University of California, Riverside, California (Oct. 2007 – present)
Graduate student researcher, Department of Bioengineering, June 2008 - present
Graduate student researcher, Department of Plant Pathology, Oct 2007 – June 2008
Teaching assistant, Fall 2008 – present

Education:

University of California, Riverside, California
July 2008 – Present Ph.D. Candidate, Biochemistry and Molecular Biology
Jan 2012 – Sept 2012 M.Sc. Candidate, Bioengineering (Awarded Fall 2012)
Sept. 2007 – June 2008 M.Sc., Biochemistry and Molecular Biology (Awarded March 2009)
University of California, Irvine, California
Sept. 2005 – June 2007 B.S., Biological Sciences (Awarded June 2007)
Riverside Community College, Riverside, California (2003 – 2005)

Awards and Recognitions:

2012 **Earle C. Anthony Research Travel Award** presented by the Academic Senate Faculty Committee, UCR to present research at a top tier National/International professional conference
2012 **Demonstration of Energy-Efficient Developments** awarded by the American Public Power Association for research contributing to energy innovation: improving efficiencies and lowering costs
2012 **Dissertation Year Fellowship** awarded by the Graduate Division, UCR, competitively selected based on academic and research merit
2012 **James and Margaret Lesley Annual Prize** awarded by the Committee on Honors and Scholarships, College of Natural and Agricultural Sciences, UCR, for original research in microfluidic biosensing
2012, 2011 **Randolph T. and Mary K. Wedding Travel Award** awarded by the Department of Biochemistry, UCR
2012, 2011 **Outstanding Teaching Assistant** awarded by the Department of Biochemistry and Graduate Division, UCR, for excellence in teaching
2011 **Walton B. Sinclair Award** presented by the Department of Biochemistry, UCR, for excellence in teaching
2011 **Randolph T. and Mary K. Wedding Prize** awarded by the Department of Biochemistry, UCR, for significant contribution to the peer reviewed scientific literature in biochemistry and molecular biology
2011 **University Teaching Certificate** awarded by the Graduate Dean and Teaching Assistant Developmental Program, UCR
2011 **Graduate Dean's Dissertation Research Grant** awarded by the Graduate Council's Fellowship Committee, UCR, for a competitive dissertation proposal
2007 **Excellence in Research** awarded by the University of California, Irvine, for outstanding undergraduate research in Neuropharmacology
2005 **Dean's List** awarded by the Dean of Biological sciences, UC Irvine, for academic excellence

Research Experience:

Single molecule surface chemistry, Photoinduced charge transfer (2008–present)

University of California Riverside, Department of Bioengineering, Riverside, CA

Using surface chemistry methodologies and *in vitro* solid phase peptide synthesis (SPPS) we study the biophysical dissociation properties of Leucine zippers on a single molecule level. In parallel, using the SPPS along with nontraditional amino acids we produce oligomers with enhanced charge transfer properties for use in photovoltaic devices and characterize them using ultrafast spectroscopic techniques (such as femtosecond TAS).

Microfluidic Biosensing (2009–present)

University of California Riverside, Department of Bioengineering, Riverside, CA

Developing utilitarian tools by incorporating microfluidics for magnetic biosensing applications that are practical for laboratory use as well as immensely valuable for field deployment. Moreover, optimize the tools to decrease the ambiguity with which medical professionals currently treat infections by emphasizing simplicity, expedience and versatility.

Viral coat protein-replicase interaction and packaging studies (2007 – 2008)

University of California Riverside, Department of Plant Pathology, Riverside, CA

Using bimolecular fluorescence studies with enhanced yellow fluorescent protein genes fused to Bromo Mosaic Virus (BMV) coat protein genes and BMV replicase in various conformations, we examined the essential interactions between coat proteins and viral RNA packaging by the replicase.

***In situ* hybridization of GPCR and ligand mRNA during development** (2005 – 2007)

University of California Irvine, Department of Neuropharmacology, Irvine, CA

Demonstrated the localization of GPR7 and its ligands, neuropeptide B (NPB) and neuropeptide W (NPW) during development. Sections were incubated with radiolabeled cRNA probes for GPR7, NPB precursor, or NPW precursor during the *in situ* hybridization process and exposed to MR-1 Kodak films.

Teaching Experience:

University of California, Riverside, California (Sept. 2008 – present)

Teaching assistant Fall 2008 - Present

Elementary Biochemistry (BCH 100), **Fall 2008, Winter 2010 & Winter 2011**

Microbiology Laboratory (MCBL 121L), **Winter 2009, Spring 2009, Spring 2010, Spring 2011, Winter 2012 & Spring '12**

Biochemistry (BCH 110A), **Fall 2009, Fall 2010 & Fall 2011**

Bioengineering Senior Design (BIEN 175 A&B), **Winter 2010 & Spring 2010**

BRITE Mentorship, **Summer 2009**

MY BEST Mentorship, **Summer 2009 - Present**

Computer Experience:

Molecular Modeling: SwissPDB, RasTop, MolMol, Chimera

Animation and 3D Design: SolidWorks, Maya 3D, Google Sketchup

Microsoft Office products: Word, Excel, PowerPoint, Publisher, Access, Outlook

Adobe products: Photoshop, Illustrator, Acrobat, Flash, Dreamweaver, Indesign, After Effects

Web design: created and manage research group website: www.vullevgroup.org

Interactive learning: Animated educational video production and editing for *Journal of Physical Chemistry Letters*

13. Lock, J. Y.; Dragonov, M.; Whall, A.; Dhillon, S.; **Upadhyayula, S.**; Vullev, V.; Liu, H. "Antimicrobial Properties of Biodegradable Magnesium for Next Generation Ureteral Stent Applications," *Proc. IEEE-EMBS* **2012**, Paper WeC20.5.
12. **Upadhyayula, S.**; Lam, S.; Ha, A.; Malik-Chaudhry, H. K.; Vullev, V. "Dynamic Staining of Bacilli Endospores with Thioflavin T," *Proc. IEEE-EMBS* **2012**, Paper WeB06.1.
11. **Upadhyayula, S.**; Quinata, T.; Bishop, S.; Gupta, S.; Johnson, N.; Bahmani, B.; Bozhilov, K.; Stubbs, J.; Jreij, P.; Nallagatla, P.; Vullev, V. "Coatings of Polyethylene Glycol for Suppressing Non-specific Interactions between Solid Microspheres and Flat Surfaces," *Langmuir* **2012**, 28 (11), 5059-5069.
10. **Upadhyayula, S.**; Bao, D.; Millare, B.; Sylvia, S.; Habib, K. M. M.; Ashraf, M.; Ferreira, A.; Bishop, S.; Bonderer, R.; Baqai, S.; Jing, X.; Penchev, M.; Ozkan, M.; Ozkan, C.; Lake, R.; Vullev, V. "Permanent Electric Dipole Moments of Carboxyamides in Condensed Media: What Are the Limitations of Theory and Experiment?," *J. Phys. Chem. B* **2011**, 115(30), 9473-9490.
8. Xia, B.; **Upadhyayula, S.**; Nunez, V.; Landsman, P.; Lam, S.; Malik, H.; Gupta, S.; Sarshar, M.; Hu, J.; Anvari, B.; Jones, G.; Vullev, V. I. "Amyloid Histology Stain for Rapid Bacterial Endospore Imaging," *Journal of Clinical Microbiology* **2011**, 49(8), 2966-2975.
8. Nuñez, V.; **Upadhyayula, S.**; Lin, A.; Chau, K.; Vullev, V. "Dynamic staining of bacteria at a single-cell level," *Proc. SPIE* 8018, 80180A **2011**; doi:10.1117/12.884186
7. Bahmani, B.; Gupta, S.; **Upadhyayula, S.**; Vullev, V.; Anvari, B. "Effect of Polyethylene Glycol Coatings on Uptake of Indocyanine Green Loaded Nanocapsules by Human Spleen Macrophages In-Vitro," *Journal of Biomedical Optics* **2011**, 16(5) 051303.
6. Chau, K.; Millare, B.; Lin, A.; **Upadhyayula, S.**; Nunez, V.; Xu, H.; Vullev, V. I. "Dependence of the Quality of Adhesion between Poly(dimethylsiloxane) and Glass Surfaces on the Composition of the Oxidizing Plasma," *Microfluidics and Nanofluidics* **2011**, 10(4), 907-917.
5. Bao, D.; Ramu, S.; Contreras, A.; **Upadhyayula, S.**; Vasquez, J.M.; Beran, G. and Vullev, V. I. "Electrochemical Reduction of Quinones: Interfacing Experiment and Theory for Defining Effective Radii of Redox Moieties," *J. Phys. Chem. B* **2010**, 114, 14467-14479.
4. Thomas, M. S.; Nunez, V.; **Upadhyayula, S.**; Zielins, E.; Bao, D.; Vasquez, J.M.; Bahmani, B. and Vullev, V. I. "Kinetics of Bacterial Fluorescence Staining with 3,3'-Diethylthiacyanine," *Langmuir* **2010**, 26 (12), 9756-9765.
3. **Upadhyayula, S.**; Nuñez, V.; Wan, J.; Vullev, V.; "Bacterial-Spore Assay Based on Fluorescence-Enhancement Kinetics as a Platform for Lab-on-a-Chip Setting," *Proc. of the 11th Annual UC Bioengineering Meeting* **2010** UCBioE-19-21
2. Nuñez, V.; Thomas, M.; Zielins, E.; **Upadhyayula, S.**; Vullev, V.; "Rapid Bacterial Identification Using Kinetics of Fluorescence Staining," *Proc. of the 11th Annual UC Bioengineering Meeting* **2010** UCBioE-3-5

1. **Upadhyayula, S.**, Jackson, V., Civelli, O. "Differential expression of GPR7 receptor, Neuropeptide B, and Neuropeptide W, in the brains of postnatal Sprague-Dawley rats during development," *Journal of Undergraduate Research in the Biological Sciences, University of California, Irvine, Volume 37* **2007**