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Gregory L. Rorrer

A label-free, photoluminescence (PL) based biosensor for 2,4,6-trinitrotoluene (TNT) detection was developed by functionalizing diatom biosiilica with a TNT-specific single-chain variable fragment (scFv). The scFv loading was estimated to be 0.040±0.003 (μg scFv/μg biosilica). In saturated concentration, TNT binding to scFv-biosilica quenched about 13% of its PL. Dose response follows Langmuir isotherm with half saturated concentration at $3.66\times10^{-8}$ M. The method detection limit (MDL) was estimated to be $3.87\times10^{-8}$ M. No interference was observed in the presence of non-complementary explosives. Limitations like small signal intensity and high background signal can be address by optimizing combination of transducer and bio-receptor. In addition, the biosensor system has the potential to be self-assembled by genetically modified diatom, eliminating the whole chemical functionalization steps. This biosensor shows potential for low-cost, high-sensitivity, on-site and selective TNT detection.

# 2,4,6-trinitrotoluene Detection using Photoluminescence Response of Diatom Biosilica Functionalized with Single-chain Variable Fragment

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Le Zhen, Author

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# 2,4,6-trinitrotoluene Detection using Photoluminescence Response of Diatom Biosilica Functionalized with Single-chain Variable Fragment

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**Abstract**

A label-free, photoluminescence (PL) based biosensor for 2,4,6-trinitrotoluene (TNT) detection was developed by functionalizing diatom biosiilica with a TNT-specific single-chain variable fragment (scFv). The scFv loading was estimated to be 0.040±0.003 (μg scFv/μg biosilica). In saturated concentration, TNT binding to scFv-biosilica quenched about 13% of its PL. Dose response follows Langmuir isotherm with half saturated concentration at $3.66\times10^{-8}$ M. The method detection limit (MDL) was estimated to be $3.87\times10^{-8}$ M. No interference was observed in the presence of non-complementary explosives. Limitations like small signal intensity and high background signal can be address by optimizing combination of transducer and bio-receptor. In addition, the biosensor system has the potential to be self-assembled by genetically modified diatom, eliminating the whole chemical functionalization steps. This biosensor shows potential for low-cost, high-sensitivity, on-site and selective TNT detection.

**Key words:** diatom biosilica, photoluminescence, scFv, TNT detection, label-free

## 1. Introduction

The common application of TNT in military and industry leads to widespread soil and water pollution (Rieger and Knackmuss 1995). TNT detection is the prerequisite for remediation. Standard detection method (EPA method 8330) is invasive, requires specific instrument (HPLC) and has low sensitivity. High price (Dasary et al. 2009), complexity in manufacture (Medintz et al. 2004) and requirement of specific instruments (Yang et al. 2014) often render newly

developed detection methods inapplicable. Cheap, on-site, quantitative and selective method for TNT detection is needed.

Diatoms, single-celled algae almost ubiquitous to natural water environment, fabricate nano-porous siliceous exoskeleton, called frustules. The photoluminescence (PL) response of frustule biosilica was discovered to be quenched by electrophiles and enhanced by nucleophiles (Stefano et al. 2005). As a transducer, biosilica was first used in non-specific gas sensing (Stefano et al. 2005), and subsequently harnessed in specific immunoassays for proteins in solution (Gale et al. 2009; Stefanoa et al. 2009). TNB2 is a single-chain fragment of antibody variable (scFv) engineered to complementarily bind TNT (Goldman et al. 2003), which has been incorporate into several competitive immunoassays for TNT detection (Anderson et al. 2006; Goldman et al. 2005a; Goldman et al. 2005b; Liu et al. 2013; Medintz et al. 2004). Since basilica immunosensor does not require a competitive antigen as transducer, integrating biosilica and scFv provides possibility to engineer cheap and selective biosensor for TNT.

In this study, scFv was immobilized on basilica via three steps of chemical reactions. The immobilization was verified by epifluorescence images and quantified by Bradford test. The resulted scFv-biosilica was challenged with TNT, the PL response of which was elicited through proper control and normalizations. TNT dose response data were fitted to Langmuir Isotherm. Method detection limit (MDL) was quantitatively measured. The selectivity of the sensor was tested by competitive antigens challenge.

## 2. Material and Methods

### 2.1 Biosilica generation and harvest.

Marine diatoms Pinnularia sp. obtained from the UTEX Culture Collection of Algae (UTEX#B679) were cultivated in 100 ml of autoclaved Harrison's artificial seawater medium enriched with 0.5 mM $Na_2SiO_3$ in 500 ml glass flasks. Cultures were incubated in 22 °C under 150 $\mu E\ m^{-2}\ s^{-1}$ incident light intensity on a 14h/10h light/dark cycle for 21 days. To facilitate $CO_2$ transport, each flask was rigorously shaken for 10 s every day. Biosilica frustules were harvested using the hydrogen peroxide treatment reported previously (Gale et al. 2009). Briefly, two flasks of 21-day-old diatoms were combined, centrifuged down at 2,000 rpm, and washed twice with deionized water. The cell pellets were transferred into 100 ml 30% hydrogen peroxide solution mixed with 200 µL 35% hydrochloride acid. The hydrogen peroxide treatment was run in 80 °C and 100 rpm shaking for 24 hours. The resulting white biosilica frustules were washed twice with deionized, then twice with 100% ethanol, and stored in ethanol.

**2.2 Amination of biosilica.**

Amine groups were introduced onto biosilica surface using previously reported method (Gale et al. 2009). In short, an aliquot of 5 mg biosilica, 2 ml anhydrous ethanol and 5µL 3-aminopropyltrimethoxysilane (APTMS, Sigma-Aldrich Lot#BCBF9895V, 179.29 g/mol) were added to an amber glass vial and allowed to react under constant mixing in 80 °C for 2 hours. The reaction continued for another 22 hours without heating. The aminated biosilica frustules were centrifuged down at 2,000 rpm for 10 min, and washed with anhydrous ethanol for 3 times. The final biosilica frustules were stored in ethanol.

**2.3 ScFv crosslinking on biosilica film.**

For subsequent functionalization and PL measurement, the aminated biosilica frustules were first made into a film on a 22mm×22mm square cover glass according to method reported recently

(Gale et al. 2009). Briefly, aminated biosilica was first washed with deionized water twice and adjusted to 1 mg/ml in deionized water. 10 μL of the resulted frustule suspension was pipetted on the center of a cover glass and allowed to dry in room temperature. After dried, the frustules formed the first layer of a 5-mm-diameter round film. The suspension pipetting and drying were repeated on top of the first layer for another 3 times to form a film containing 40 μg biosilica. The film making steps were performed in a laminar flow hood to prevent dust contamination. The film was incubated in 90 °C for 1 h and aged in room temperature and dark for at least one day and used before three days for further functionalization or PL measurement.

For scFv immobilization, 1 mg disuccinimydylsuberate (DSS, Pierce Biotechnology, Prod#21655, 368.35 g/mol) was dissolved in 1ml anhydrous acetonitrile (ACN, EMD$^{TM}$, HPLC grade, Lot#47295, dried with 0.4 nm molecular sieves for 2 days). 10 μL of the DSS solution was immediately pipetted on each of the aminated biosilica films and allowed to react for 10 min. The DSS functionalized biosilica films was dip-rinsed the in phosphate-buffered saline (PBS, pH=7.4). The excess PBS left on the films was removed with a kimwipe. 10 μL of scFv solution (MW=30,000, 0.6 mg/ml) was immediately pipetted on each of the DSS functionalized biosilica films. The film was then transfer into a Petri dish (60×15mm) humidified by laying a wet kimwipe on the bottom. The crosslinking reaction was run under 100 rpm shaking for 2 h. After the reaction, the biosilica films were rinsed thoroughly in PBS for 3 times to remove excess scFv. Excess PBS on the films was removed with a kimwipe. The scFv-biosilica films were dried in stagnant air and room temperature for exactly 1 hour before PL measurement. Up to 9scFv functionalized biosilica films fora round of PL measurement were made at the same time with one DSS solution.

**2.4 Complementary binding of TNT on scFv-biosilica.**

The scFv-biosilica film was challenged with TNT PBS solution immediately after PL measurement. First, 2,4,6-trinitrotoluene (Chemical Service, Lot# 439200, min 30% water, purity 99.1%) was dissolved in ACN to make 1 mg/ml stock solution. The stock solution was diluted into a series of concentrations with ACN, and 5 μL of the series dilutions were added to 45 mL of PBS to make TNT PBS solutions with initial concentrations ranging from $4.8\times10^{-9}$ M to $4.8\times10^{-7}$ M. The TNT solution was equally separated into three 15 ml solutions in60×15mm Petri dishes. Then, the scFv functionalized biosilica films was placed upside down on the surface of the TNT solutions for 2 hours under 50 rpm shaking in room temperature. As controls, scFv-biosilca films made at the same time were also soaked in PBS with same amount of ACN for 2 hours. After the binding, the biosilica films were rinsed in PBS for 3 times. The excess PBS was removed with a kimwipe. The films were dried in stagnant air and room temperature for exactly 1 h before PL measurement.

**2.5 Competitive antigen challenge.**

To test the specificity of scFv functionalized biosilica, 1,3,5-trinitrobenzen (TNB, SUBELCO, Lot#LB91318, 1000 μg/ml in Acetonitrile, Mw=213.10), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX, Cerilliant, Lot#ER12012-01, 1000 μg/ml in Acetonitrile, Mw=222.12), 2,6-dinitrotoluene (2,6-DNT, Cerilliant, Lot#FN092811-01, 1000 μg/ml in Acetonitrile, Mw=182.13) were added to PBS, as well as $4.8\times10^{-7}$ M TNT PBS solution, to make $4.8\times10^{-7}$ M solutions which were used to challenge scFv functionalized biosilica using the same method described Section 2.4. To test the strength of the binding,the PL responses of challenged scFv-biosilica films after 1 rinse in PBS and 3 rinses were recorded separately.

**2.6 Scanning Electron Microscopy.**

To see the surface morphology of the frustules, $H_2O_2$-treated biosilica film made with the method described in Section 2.4 was sputter-coated with 60/40 gold/platinum for 30 s, and imaged using an FEI Quanta 600 FEG scanning electron microscope at 10 kV.

**2.7 Epifluorescence Microscopy.**

To verified the immobilization of scFv on biosilica surface, a DSS-biosilica film (as control) and scFv-biosilica film were soaked in Tris-buffer (pH=7.4) for 3 days, then challenged with 2 ml of 5.6 μg/L Alex Fluor 555 labeled TNB (AFTNB) PBS solution using similar method with TNT challenge. After rinsing for 3 times in PBS and dried for one day, the white light images and their corresponding fluorescence images of the AFTNB challenged biosilica films were taken with a Leica DM inverted light microscope equipped with CY3 filter (excitation 530/50 nm, emission 610/75 nm) with 40 × magnification and 2 s integration time. Imaging and analysis were done using the software Image-Pro Plus 5.0. For visual comparison, images were taking on the edge of the films. For quantitative comparison, 5 fluorescence images in the multilayer region of each film were taken. These images were converted into "Gray Scale 8" format whose pixel information was sampled every 20 pixels using the Bitmap Analysis function. The sum of the sampled pixel counts quantitatively represented the fluorescence intensity of each image. The average intensities obtained were normalized by that of the DSS-biosilica film.

**2.8 Bradford test.**

To determine the amount of scFv immobilized on biosilica, 20 uL of scFv solution before and after scFv functionalization was collected. The concentrations of both solutions were determined by Bradford test using scFv solution with known concentrations as standards. The amount of scFv immobilized was calculated by material balance.

**2.9. Photoluminescence spectroscopy.**

PL measurement was performed using the system adapted from the one reported recently. Laser was emitted from a 337-nm $N_2$-gass-laser source (Spectra-Physics VSL, maximum power output: 2.0mW, peak output power: 40 kW, pulse length: 3 ns, pulse energy: 120μJ, wavelength: 337 nm, repetition rate: 10 Hz, beam dimension: 3mm×8mm). Before hitting the sample, the laser beam was first filtered by a 337 nm band pass filter and then shaped by a 1mm×3mm slit. The sample was positioned at a 45 ° angle to the incident laser on a sample holder capable of locking the sample in all three dimensions. The emission light on a 45 ° angle to the sample surface was focused by an optical lens, filtered by a 360 nm cut-off filter, and detected by an Acton SP2150 spectra detector (Priceton Instrument,1mm slit, 600 groves/mm grating blazed for 300 nm) equipped with a PIXIS 100 CCD detector (20s integration time). The whole light pass was enclosed in a 26mm×23mm optical box. Background signal was measured and subtracted from each measurement. Each scFv-biosilica film was measured in exactly the same position before and after antigen challenge.

**2.10 Data analysis.**

The change in PL peak intensity of a biosilica film was packaged in to a first-order normalized value Q:

$$Q = \frac{A_0 - A_f}{A_0} \qquad (1)$$

Where Q is the proportion of decrease in integrated intensity, $A_0$ is the integrated intensity between 400 and 450 nm ofthe spectra of scFv-biosilica film, $A_f$is the same integrated intensity of the scFv-biosilica film after challenge.

A second-order normalization was represented by the equation:

$$Q^0 = Q - Q_{PBS} \qquad (2)$$

Where $Q^0$ is the second-order normalized proportion of quenching, Q is the average first-order normalized proportion of quenching of challenged scFv-biosilica films, $Q_{PBS}$ is the first-order normalized proportion of signal change of PBS soaked scFv-biosilica films.

The dose response curve was modeled by Langmuir Isotherm:

$$Q^0 = \frac{Q_{max} \times {C_{TNT}}^n}{K_d + {C_{TNT}}^n} \qquad (3)$$

Where the $Q_{max}$ is the maximum second-order normalized response, $K_d$ is the apparent dissociation constant, $C_{TNT}$ is the concentration of TNT, n is the apparent number of TNT molecules one antibody binds. $Q_{max}$, $K_d$ and n were estimated using the Matlab code shown in Figure S1.

The method detection limit (MDL) as well as the upper (UCL) and lower (LCL) bounds of its 95% confidence limits were estimated using the procedure described in Glaser et al. (1981). Briefly, N (N=7) scFv-biosilica films were challenged with $3.5\times10^{-8}$ M TNT PBS solution. The PL responses obtained were substituted into equation (3) to get measured concentrations using Matlab code shown in Figure S2. The standard deviation (s) of the seven measured concentrations was used for estimating MDL using the following equation:

$$MDL = t_{(6\ df, 1-\alpha=0.99, one\ tail)} \times s = 3.14s \qquad (4)$$

where t is the student's t value for a one tail test at the 99% confidence level with N-1 degrees of freedom.

The 95% confidence limits for the MDL were computed using the equations:

$$UCL = 2.20\ MDL \quad (5)$$

$$LCL = 0.64\ MDL$$

All quantitative data are presented with descriptive statistics (mean ± standard deviation). The significance of all comparisons with two data points were tested using student's t test. Multiple comparisons in competitive antigens experiment were tested using analysis of variance (ANOVA) method described in Wallenstein et al. (1980).

## 3. Results and discussion

### 3.1 Fine structures of the biosilica frustule film.

The biosilica film consists of multi-layers of randomly stacked half-ellipsoid-shape valves and belt-shape girdle bands (Figure 1A, 1B). A periodical array of wells covers the surface of valves and girdle bands (Figure 1B). The diameter of each well is about 200 nm. Four or five pores of the diameters between 40 nm and 100 nm lies on the bottom of each well (Figure 1C). In addition, previous study (Qin et al. 2008) observed 3-5 nm nanoparticles lining the base of pores not properly formed, indicating lower scale of nanostructure than the ones observed here. Although the origin of the biosilica PL is still under debate, all the putative origins are closely related the nanostructure of frustule: quantum confinement (Glinka et al. 2001; Liu et al. 2005) is a direct result of the scale of the structure; the enlarged surface area from the porous structure favors both localized surface states (Setaro et al. 2007; Xie et al. 1992) and surface-confined molecular emitters (Gole et al. 1997; Qin et al. 2008).

### 3.2 ScFv immobilization.

The anti-TNT scFv was immobilized on the biosilica surface using a three-step method (Figure 2), of which the chemistry was described in previous research (Gale et al. 2009). To verify the scFv immobilization on biosilica surface, the DSS-biosilica and the scFv-biosilica were challenged with AF-TNB, a fluorescently labeled TNT surrogate (Anderson et al. 2006). White light images of the AF-TNB challenged DSS-biosilica (Figure 3A) and scFv-biosilica (Figure 3C) shows a similar distribution of frustules, while epifluorescence image of the same spot of DSS-bioslica (Figure 1B) is much darker than that of the scFv-biosilica(Figure 3D).Fluorescence intensity increased significantly ($p=3.10 \times 10^{-5}$)by about 4 times from DSS-biosilica to scFv-biosilica. These results indicate the success of scFv immobilization. The amount of scFv immobilized on biosilica was estimated to be 0.040±0.003 (μg scFv/μg biosilica) using Bradford test. This number can be converted into surface site density of about 50,000 scFv molecules $\mu m^{-2}$, which is an order of magnitude higher than previous study on functionalizing Cyclotella sp. frustule with IgG (Gale et al. 2009). This result is expected considering scFv is much smaller than IgG.

**3.3 Maximum PL Response to TNT.**

The decrease of biosilica PL upon TNT binding to scFv is the basis of our biosensor (Figure 2). However, the PL of scFv-biosilica increases after soaking in PBS solution (Figure 4A), which can neutralize the decrease cause by TNT binding (Figure 4B). Also, both effects of PBS soaking and TNT binding are relatively small. In addition, both effects are most recognizable at the wavelengths from 400 nm to 450 nm (Figure 4A, B). To quantitatively represent the PL changes, the PL spectra of individual scFv-biosilica film before and after challenge were integrated from 400 nm to 450 nm, the decrease of which was normalized, according to equation (1), by the value before challenge. Because of the structure of the first-order normalized

quantity, a positive value represents a decrease in PL, vice versa. Thus the negative value for PBS-soaked scFv-biosilica and positive value for TNT-challenged scFv-biosilica (Figure 4C) depicts the changes directly observed in spectra (Figure 4A, B). However, because the scFv-biosilica films were challenged in TNT-PBS solution, their direct observed PL changes are the combined effect of both TNT binding and PBS soaking. To exclude the PBS effect, the first-order normalized PL change of PBS-soaked scFv-biosilica was subtracted from that of the TNT-challenged scFv-biosilica. The resulted second-order normalized PL change represents the sole effect of TNT-binding (Figure 4D), which shows that TNT quenches about 13% of the PL signal. Statistically the same saturated PL responses can be reproducibly measured (Figure 6B). These two results demonstrate that, with proper control and data processing procedure, the scFv-biosilica biosensor can detect TNT.

TNT is a well-known PL quencher (Content et al. 2000; Gao et al. 2008; Yang and Swager 1998). Study on non-specific gas adsorption to diatom biosilica indicates that its PL is quenched by electrophiles such as $NO_2$ and enhanced by nucleophiles such as Pyridine (Stefano et al. 2005). Given the electrophilic nature of TNT and its similarity to $NO_2$, TNT is expected to quench biosilica PL by attracting electron from biosilica suface. The studies on $NO_2$'s quenching effect show that it cannot quench the biosilica PL to zero (Bismuto et al. 2008), instead, certain limits exist depending on the frustule surface morphologies of different diatom species (Setaro et al. 2007). In one study, a rugged, irregular and more porous surface shows a quenching limit of 31% and a smooth, periodical, less porous surface only 14% (Lettieri et al. 2008). The latter surface is very similar to that of our biosilica. Thus the surface morphology may post an intrinsic limit to the signal intensity of the scFv-biosilica. It's possible to improve signal intensity by selecting diatom species with more porous biosilica surface.

TNT is also a small molecule (Mw=227.13) with three nitrite groups which attracts electron from biosilica surface. Proteins, on the other hand, are macromolecules with molecule weights hundreds of times larger than that of TNT. Studies have explored methods for specific detection of proteins using antibody-functionalized biosilica (Gale et al. 2009; Stefanoa et al. 2009). In our previous work on detection of anti-rabbit IgG, PL peak intensity tripled upon immunocomplex formation (Gale et al. 2009). The PL enhancement was attributed to the nucleophilic free amine groups which one anti-rabbit IgG has more than a hundred. In contrast, small molecule like TNT is expected to trigger less response. In addition, scFv (Mw=30,000) is more than a hundred times bigger than TNT. Even every scFv on the biosilica surface binds to TNT, there aren't much more TNT to quench PL then anti-rabbit IgG to enhance it. The size factor posts an extrinsic limit to the signal intensity. One way to resolve this problem is to use smaller bioreceptors like dodecapetide (Goldman et al. 2002) which can bind more TNT to biosilica surface.

ScFv-biosilica PL response to PBS soaking is almost identical to that of the DSS-biosilica (Figure S3). This background signal is thus speculated to origin from the reaction between residual DSS and water. The magnitude of this background signal is negligible to the PL response to anti-rabbit IgG, thus wasn't discovered in our previous study (Gale et al. 2009). However, as the background signal is in the same magnitude of the PL response to TNT, it increase the complexity of TNT detection. Recent studies have shown that diatom can be genetically modified to express protein on its frustule (Marshall et al. 2012; Poulsen et al. 2007). The background signal in our sensor system could be eliminated by applying genetical functionalization.

**3.4 Quantitative TNT Detection.**

The observed PL responses of scFv-biosilica to different concentrations of TNT are best fitted to Langmuir Isotherm with a bivalence model (Figure 5). The apparent bivalence behavior does not necessarily translate into one scFv binding two TNT, since the weakened driving force of diffusion caused by decrease in concentration may intertwine with the ideal binding mechanism, which results in an apparent bivalence behavior. The actual binding mechanism is out of the scope of this research. Quantitative detection of TNT demands a model that fits the data, thus a bivalence model is used. The 50% saturated TNT concentration derived from the modeled dose response curve is $3.66\times10^{-8}$ M. MDL estimated with the standard method described in Section 2.10 is $3.87\times10^{-8}$ M. The UCL and LCL of the MDL are $8.51\times10^{-8}$ M and $2.48\times10^{-8}$ M.

Since first engineered in 2003, the scFv used in this research has been incorporated into several competitive immunoassays for TNT detection (Anderson et al. 2006; Goldman et al. 2003; Goldman et al. 2005a; Goldman et al. 2005b; Liu et al. 2013; Medintz et al. 2004). In these studies, fluorescently-labeled TNT surrogates first bound to scFv, and the displacement of these surrogates by TNT triggered fluorescence decrease as response. The half saturated TNT concentrations range from $1.6\times10^{-8}$ M to $1.57\times10^{-5}$ M, and the MDLs are between $4.4\times10^{-9}$ M and $4.4\times10^{-6}$ M. Our study achieves the same level of sensitivity with label-free method.

**3.5 Specificity of TNT detection.**

Bovine serum albumin (BSA) was immobilized on biosilica using the same method of scFv functionalization. The BSA-bisilica shows a significantly ($p=3.37\times10^{-3}$) lower PL response to $4.8\times10^{-7}$ M TNT than that of the scFv-biosilica (Figure 6A).In addition, the BSA-biosilica response is not significantly different than zero (p=0.42).As the porous structure of biosilica could adsorb TNT, and amine groups are known to attract TNT (Gao et al. 2008), it is important

to verify that the specific binding between scFv and TNT, instead of nonspecific interactions between TNT and amine groups or biosilica, causes the PL response. The comparison between BSA-biosilica and scFv-biosilica serves this purpose. Even the interactions between TNT and bioslica or amine groups exist, it cannot survive the rinsing process to cause a significant PL response.

To test the selectivity of the scFv-biosilica biosensor, it was challenged by TNB, RDX, 2,6-DNT, as well as their mixture with TNT (Figure 6B). In addition, the PL responses after one rinse and three rinses were compared. The significance was tested by a two-way ANOVA test (Table S1). There is no significant difference of PL responses to all the TNT containing solutions. PL responses to TNB are the same with those to TNT. On the other hand, PL responses to RDX and 2,6-DNT are significantly lower than those to TNT. Only RDX shows a significant difference in PL response between one rinse and three rinses. There is no significant response to RDX after three rinses. These results demonstrate excellent selectivity towards both TNT and TNB, which is not surprising since the scFv was originally selected against TNB. No interference occurs when TNT was mixed with RDX and 2,6-DNT, which is of practical importance because these explosives are often blended in industry. RDX, although structurally similar to TNB (N is in 1,3,5 positions instead of C), only attaches to scFv through a weak binding which cannot survive 3 rinses. Previous study on the same scFv shows similar selectivity (Goldman et al., 2003 ).

**4. Conclusion**

This article describes a prove-of-principle study on a label-free biosensor based on TNT's PL quenching effect on biosilica. Quantitative and selective detection was achieved through specific

binding between TNT and scFv. Limitations like small signal intensity and background signal can be overcome by selection of biosilica and binding element, and application of genetical functionalization. In a broader sense, this research opens up numerous possibilities in selective detection of small moleculein water using diatom biosilica.

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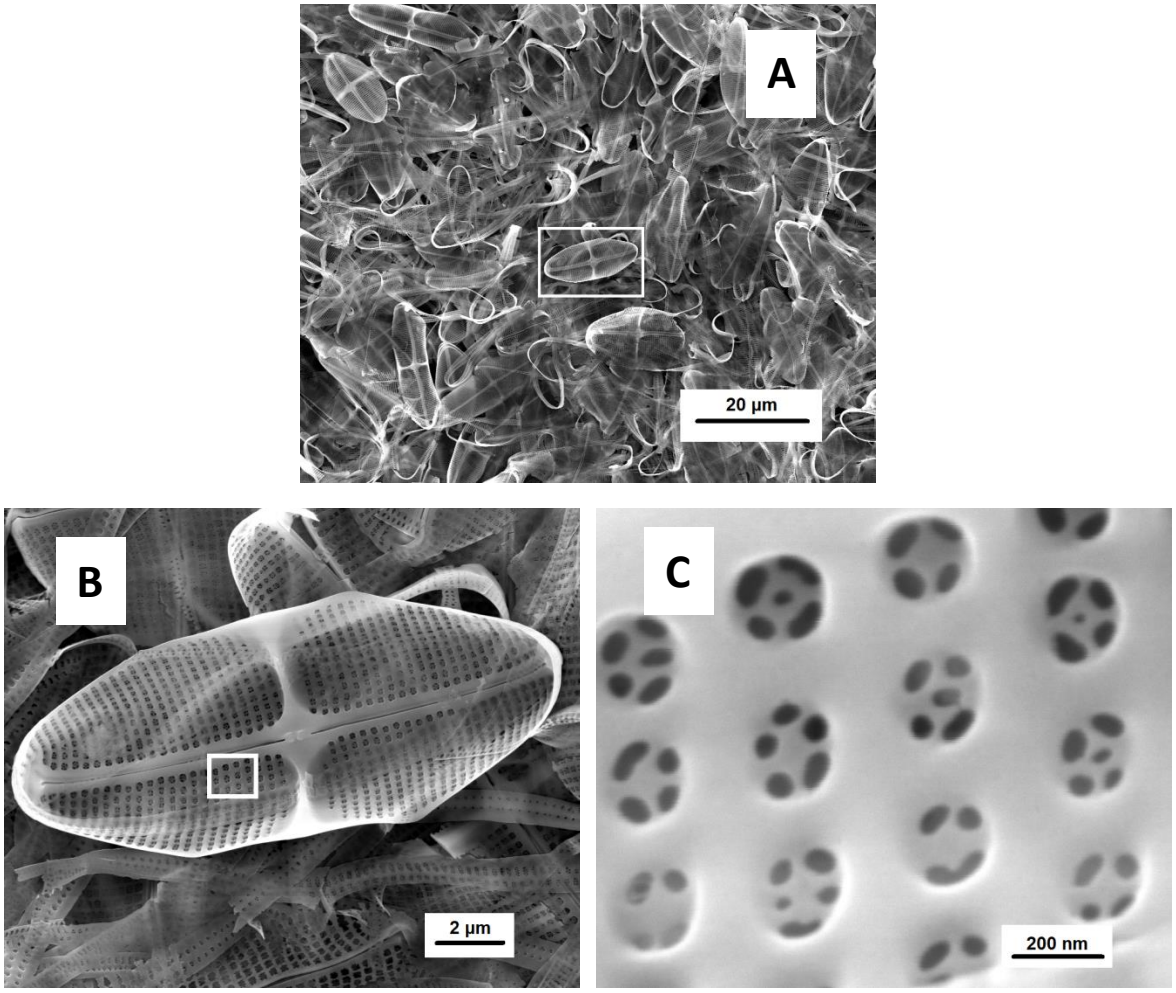


**Figure 1.** Scaning electron micrograph of biosilica film of Pinnularia sp. frustules. A) an overlook of the multilayer biosilica film; B) a single frustule valve and girdle bands (boxed region in A); C) a detail look at the pore structure on frustule (the boxed region in B).

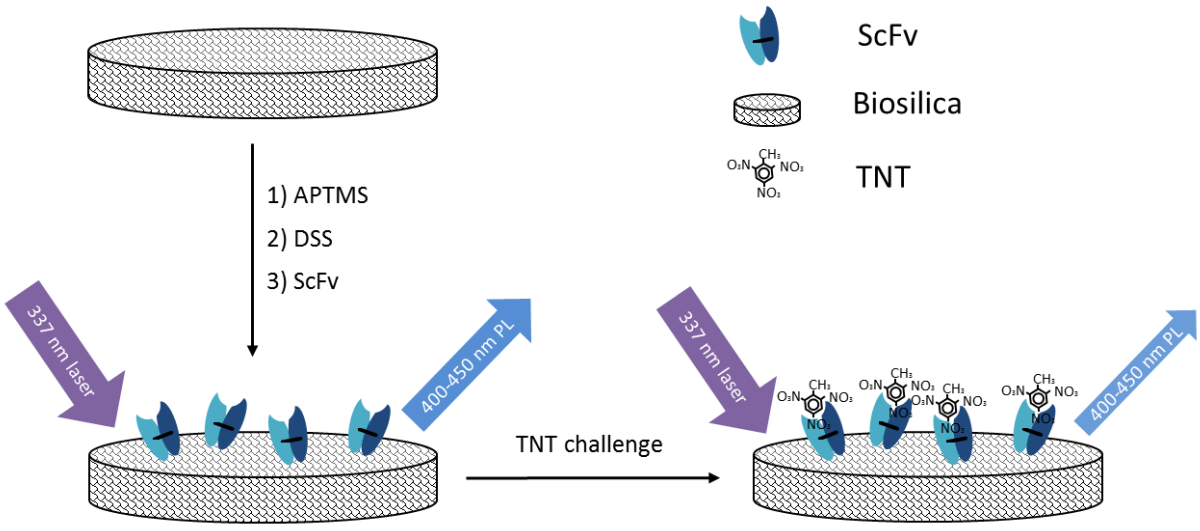


**Figure 2.** Schematic representation of the scFv-biosilica biosensor. The biosensor was made by immobolizing the scFv on biosilica following a three-step reaction: 1) anchoring primary amine groups on biosilica surface by reaction with APTMS; 2) binding crosslinker DSS to the amine groups on biosilica surface; 3) binding scFv covalenly to biosilica surface by the reaction between DSS and the primary amine groups on the surface of scFv. The biosensor emits blue photoluminescence (PL) that peaks between the wavelength of 400 to 450 nm when subjected 337 nm laser. This photoluminescence is quenched when scFv bind to TNT in a manner that's dependent on the TNT concentration. This phenomenon serves as the basis of TNT detection.

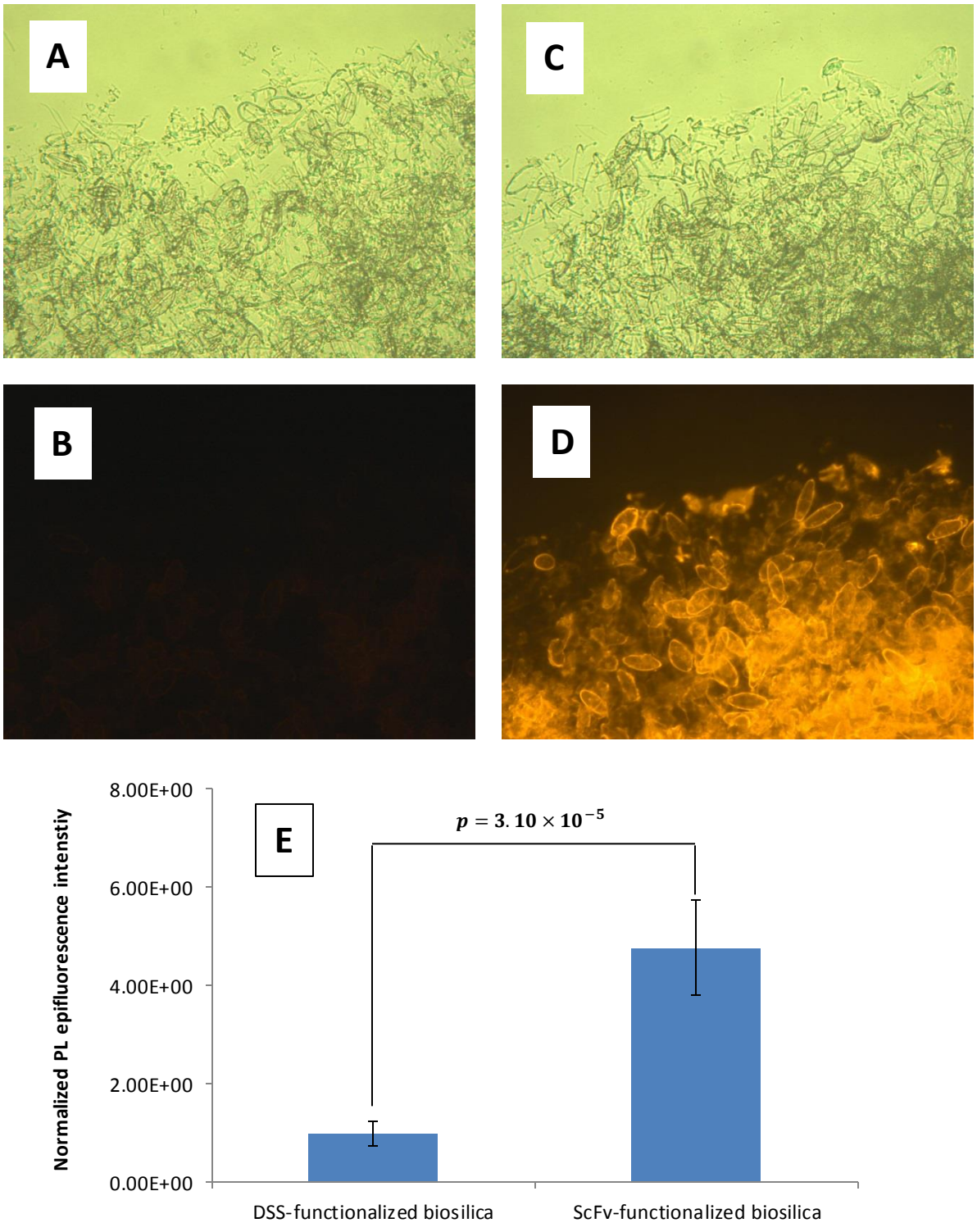


**Figure 3.** Comparison of epifluorescence of AF-TNB challenged DSS-biosilica and scFv-biosilica. DSS-biosilica with A) bright field imaging, B) epifluorescence imaging; scFv-biosilica with C) bright field imaging, D) epifluorescence imaging; and E) average normalized epifluorescence intensities, errors are reported in ±1 standard deviation, number of images n=5, p value obtained from Student's t test.

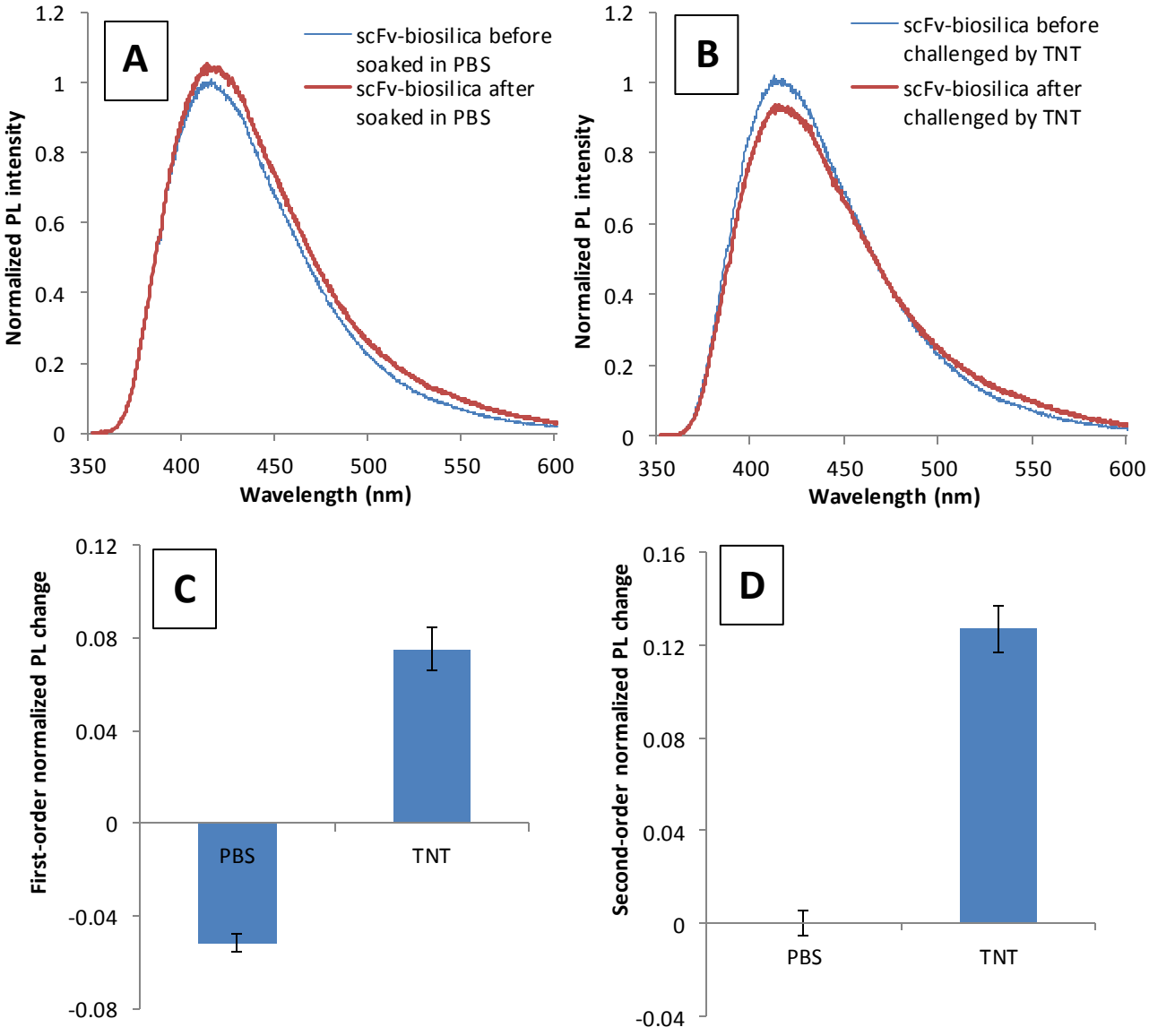


**Figure 4.** Quantitative representation of PL response to TNT challenge. A) Representative PL spectra of one scFv-biosilica film before and after soaked in PBS (normalized by the peak intensity before soaked); B) representative PL spectra of one scFv-biosilica film before and after challenged by TNT (normalized by the peak intensity before challenge); C) comparison of first-order normalized PL changes for PBS soaking and TNT challenge; D) comparison of second-order normalized PL changes for PBS soaking and TNT challenge. Sample size n=3, errors are reported in ±1 standard deviation, all samples made at the same time and under the same conditions.

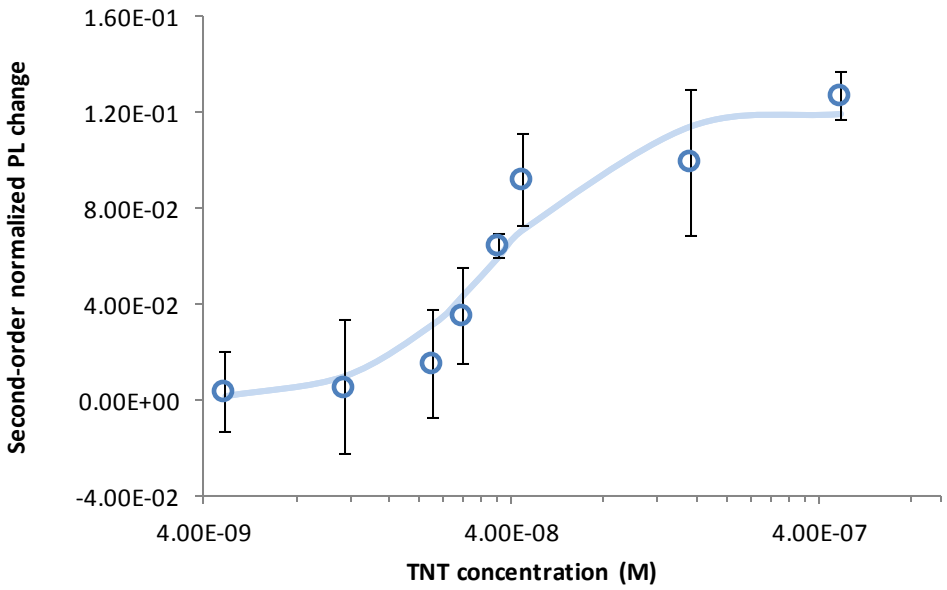


**Figure 5.** Dose response of scFv-biosilica films to TNT. Errors are presented in ±1 standard deviation. Trend line represents the best fit to equation (3), with estimated parameters $Q_{max}$=0.12, Kd=$4.04\times10^{-16}$ $M^{2.07}$, n=2.07. The half saturated concentration for dose response curve is $3.66\times10^{-8}$ M. Errors are reported in ±1 standard deviation, 3 samples for each point.

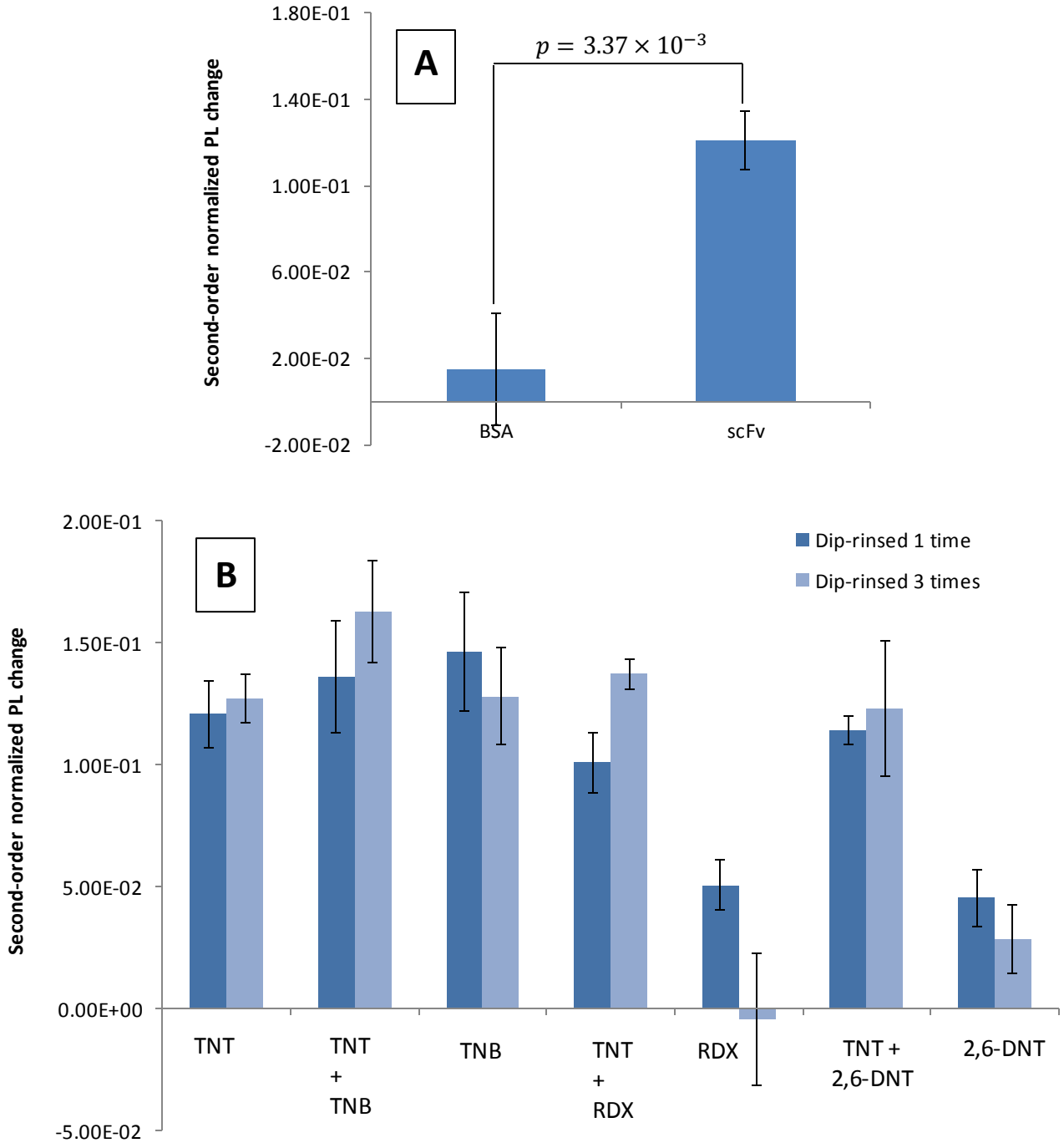


**Figure 6.** Specificity scFv-biosilica PL response. A) PL responses of BSA-bioslica and scFv-bioslica to TNT challenge. B) PL responses of scFv-biosilica to competitive antigen. Errors are reported in ±1 standard deviation, 3 or 4 samples for each point.

**Supplementary Information**

```
%DoseModelIter2

%Initial TNT concentration [M]:
Ci=[4.8e-9; 1.2e-8;2.4e-8;3e-8;4e-8;4.8e-8;1.6e-7;4.8e-7];
%Second-order normalized proportion of quenching [dimensionless]:
Q=[3.41e-3;5.42e-3;1.51e-2;3.55e-2;6.44e-2;9.16e-2;9.92e-2;1.27e-1];
%Initial guest of Qmax and Kd:
beta0=[0.127 36e-16 2];
%Amount of scFv on biosilica film=1.61e-6 g=5.37e-11 M
q_max=5.37e-11;
%Volume of the TNT solutions= 0.015L
V=0.015;
%Count data points
n=numel(Ci);
%Model function (Langmuir Isotherm):
modelfunc=@(b,x)b(1)*x.^b(3)./(b(2)+x.^b(3));
%Estimate model parameters taking its affect on Cf into consideration.
iter=1; es=10; ea=100; C(:,iter)=Ci; beta(iter,:)=beta0;
while(1)

%Initial estimation of Qmax , Kd and N_antigen:
    beta(iter+1,:)=nlinfit(C(:,iter),Q,modelfunc,beta(iter,:));

%If concentration change less than 5% due to antibody-antigen binding,
%       end the loops.
    if max(ea)<=es||iter>=5,break,end

%Concentration adjustment:
   for i=1:n
      func=@(x)q_max*beta(iter+1,3)/V*x^beta(iter+1,3)/(beta(iter+1,2)+...
          x^beta(iter+1,3))+x-C(i,iter);
      C(i,iter+1)=fzero(func,C(i,iter));
      ea(i)=abs((C(i,iter+1)-C(i,iter))/C(i,iter+1))*100;
   end
      iter=iter+1;

end
[alpha,R,J,Cov,MSE] = nlinfit(C(:,iter),...
    Q,modelfunc,beta(iter+1,:));
disp('The estimation of parameters');
fprintf('%8.2e\n',alpha);
disp('95% confidence intergrals:');
ci = nlparci(alpha,R,'covar',Cov);
fprintf('%8.2e\n',ci);
disp('Adjusted TNT concentrations:')
Cf = C(:,iter)';
fprintf('%8.2e\n',Cf);
```

**Figure S1.** Matlab code for the fitting of dose response curve.

```
% m file to coverge PL signal to TNT concentration for the
% estimation of method detection limit.

%Initial spike concentration:
Ci=3.5e-8;
%Second-order normalized proportion of quenching [dimensionless]:
Q=[7.15e-02;3.47e-02;7.02e-02;7.81e-02;7.43e-02;8.36e-02;3.41e-02];
%Count data points
n=numel(Q);
%Parameters for the model fuction;
b=[1.20e-01 4.04e-16 2.07e+00];

for i=1:n
%Model function (Langmuir Isotherm):
  func=@(x)b(1)*x.^b(3)./(b(2)+x.^b(3))-Q(i);
%Estimate TNT concentration:
  C(i)=fzero(func,Ci);
end

disp('The estimation of TNT concentrations:');
fprintf('%8.2e\n',C);
```

**Figure S2.** Matlab code for converting PL response to TNT concentration.

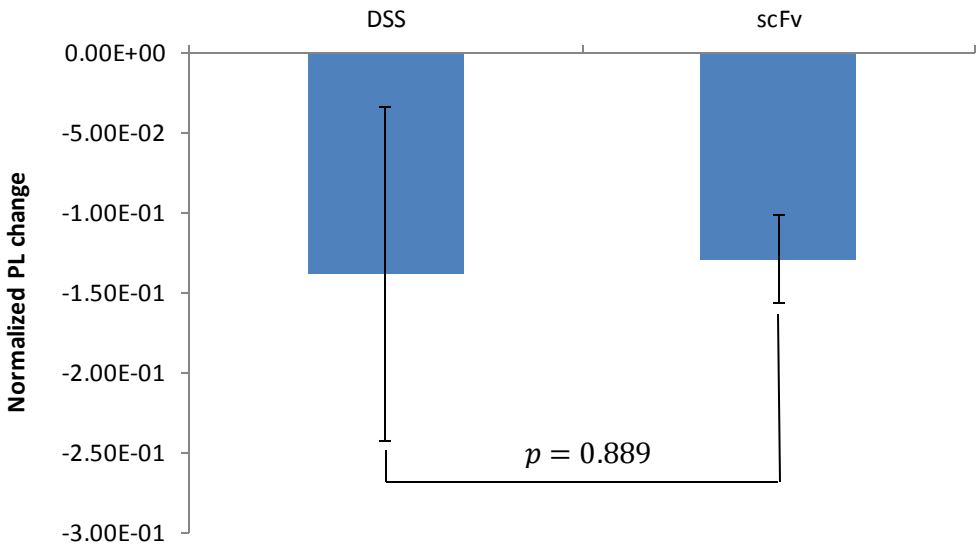


**Figure S3.** PL changes DSS-biosilica and scFv-biosilica after soaked in PBS. Errors are reported in ±1 standard deviation, number of samples n=3, p value obtained from Student's t test. (Note: DSS-biosilica was partially washed off after soaking in PBS. In order to exclude the effect of wash-off on the PL change, APTMS-bioslica was soaked in PBS, whose PL change was subtracted from that of the DSS-biosilica. While scFv-biosilica withstood the soaking perfectly (probably because of extra strength provided by the binding of adjacent frustules to the same scFv molecule), no background was subtracted. Wash-off and error propagation can explain the relatively large standard deviation of DSS-bioslica compared to that of the scFv-biosilica.)

**Table S1.** T values of multiple comparisons from ANOVA test for competitive antigen data.

| Washes & Comparate | TNT | TNT+TNB | TNB | TNT+RDX | RDX | TNT+DNT | DNT |
|---|---|---|---|---|---|---|---|
| 1 wash, control | (control) | -1.06 | -1.75 | 1.45 | 4.81* | 0.444 | 5.53* |
| 3 washes, control | (control) | -2.44 | -0.0775 | -0.706 | 9.63* | 0.277 | 6.76* |
| 1 wash, 3 washes | -0.429 | -1.81 | 1.33 | -2.67 | 4.03* | -0.595 | 1.23 |

* Significant at 95% confidence level (critical t value determined by Bonferroni method : 3.26)

# APPENDIX

## Appendix 1: Procedures

### A1.1 Autoclave method for Artificial Seawater Medium (ASM) Preparation

**Le Zhen (Revised on September 07, 2013)**

*Specialized Equipment*

- Autoclave (Gleeson Hall basement)
- Laminar flow hood
- 50 ml plastic graduated cylinder (1)
- 100 ml glass graduated cylinder with aluminum foil (1)
- 250 ml plastic graduated cylinder (1)
- 1 L Kimax bottles (5)
- 2 L Kimax bottle (1)
- 500 ml flasks with foam stoppers (according to need)

*Reagents*

- ASM Stock Solutions (I, II, III, V)

*Procedure*

1. Label all the bottles according to contents
2. Add 280 mL of Stock I into a 1 L Kimax bottle, and dilute to 500 mL with distilled water
3. Add 100 mL of Stock II into a 1 L Kimax bottle and dilute to 500 mL with distilled water
4. Add 50 mL of Stock V into a 1 L Kimax bottle and dilute to 500 mL with distilled water
5. Add 50 mL of Stock V and 20 mL of Stock III to a 1 L Kimax bottle and dilute to 500 mL with distilled water

6. Add 300 mL of distilled water to a 1 L Kimax bottle
7. Autoclave all the glassware listed in "Equipment" for 45 minutes using the fluid cycle
8. Remove all the glassware from the autoclave
9. Place all bottles into the laminar flow hood using aseptic technique
10. Loosen the caps and allow the bottles to cold down for 1~2 hours
11. Combine the diluted stock solutions in the sterile 2 L Kimax bottle
12. Fill the 2 L Kimax bottle to the 2 L mark using the sterile distilled water
13. Mix the ASM rigorously
14. Remove all the 1 L Kimax bottles from the laminar flow hood
15. Place the autoclaved aluminum foil capped graduated cylinder and four of the autoclaved 500 ml flasks into the laminar flow hood using aseptic technique
16. Add 90 ml ASM into each of the 500 ml flasks with aseptic technique
17. Repeat step 15 and 16 until all the flasks are filled
18. Incubate the ASM in room temperature for 1~2 days before use for subculturing
19. Prepared media should be used within 4 weeks of preparation

## A1.2 *Pinnularia Sp. Culture Maintainance for Biosilica Harvest*

**Le Zhen (revised September 7, 2013)**

*Specialized Equipment*

- 500 mL flasks with foam stoppers (10)
- Incubator with 22 ºC temperature control, 150 µE light on a 14/10, light/dark cycle
- Autoclave

*Reagents*

- 1 L Harrison's Artificial Seawater Medium (ASM), refer to procedure *Autoclave method for Artificial Seawater Medium*.
- 200 mM Si Stock Solution (VI)

*Procedure*

1. Complete the following steps in the laminar flow hood using sterile technique.
2. Combine four flasks from previous subculture. This culture is 30 days old.
3. Transfer four autoclaved flasks each containing 90 ml Harrison's ASM into the lamina flow hood.
4. Add 250 µL of 200 mM Si stock solution to each of the flasks, shake the flasks to insure well-mixed.
5. Transfer 10 mL of combined old culture into each of the flasks containing ASM.
6. Label each flask with the following: PI2 - Initials - Subculture Number – Date of subculture-flask number.
7. Store these flasks in the incubator at 22ºC, on a 14/10 light/dark cycle, under 150 µE of light from 9W fluorescent lights.

8. Swirl cultures twice daily for 5 seconds.

9. Allow the diatoms to grow for 21 days before hydrogen peroxide treatment.

## A1.3 $H_2O_2$ Treatment of *Pinnularia sp.* for Functionalization Experiments

**Le Zhen (revised September 07, 2013)**

*Specialized Equipment*

- 250 mL flask with foil top
- Heated Shaker controlled at 80ºC
- IEC centrifuge
- 50 mL centrifuge tubes

*Reagents*

- Hydrogen peroxide, 30 %
- HCl, 37.5%
- 2 flasks of 100 mL, 21 day old culture.
- Deionized water

*Procedure*

1. Transfer contents of culture flasks into 50 mL centrifuge tubes.
2. Centrifuge at 3,000 rpm for 10 minutes.
3. Pour off supernatant.
4. Replace supernatant with fresh DI water.
5. Invert to mix fresh water and culture pellet.
6. Repeat steps 2-5, twice, ending with step 3.
7. Add 100 mL hydrogen peroxide into a 250 mL flask.
8. Add 200 µl of 37.5% HCl to the flask, mix the solution well.
9. Transfer 40 ml of the solution above from the flask to a 50 ml centrifuge tube.

10. Pour culture pellet made in step 1~6 from the centrifuge tube to the flask containing 60 ml $H_2O_2$.
11. Rinse the centrifuge tube with $H_2O_2$ from step 9, pour the solution in to the flask.
12. Swirl to mix.
13. Cover the top with foil.
14. Place in heated shaker and allow shaking for 24 hours.
15. Remove flask from shaker.
16. Pour contents into 50 mL centrifuge tubes.
17. Repeat steps 2-4, three times, ending with step 3.
18. Replace supernatant with anhydrous ethanol and store.

**A1.4 Amine Functionalization of $H_2O_2$ Treated Diatom Frustules with 3-aminopropyltrimethoxysilane (1000x dilution APS Functionalization)**

**Le Zhen (revised September 08, 2013)**

*Specialized Equipment*

- Reacti-therm
- Reactitherm 3.0 mL reactivial
- Reactivial conical stirbar to fit reactivial

*Specialized Equipment*

- 5 mg of diatom biosilica which has been treated with hydrogen peroxide according to procedure

*Reagents*

- 3-aminopropyl trimethoxysilane (APS) (Sigma Aldrich 09326-100ML), $H_2N(CH_2)_3Si(OCH_3)_3$, 179.29 g/mol, density 0.946 g/mL
- Anhydrous Reagent grade ethanol, $C_2H_2O$, 46.06 g/mol

*Procedure*

1. Complete all Procedure steps in a laminar flow hood wearing eyewear and gloves.
2. Suspend 5 mg of hydrogen peroxide treated diatom frustules in a 1.0 mL of AR-grade ethanol in a 3.0 mL reactivial with a reactivial stir bar.
3. Add 2.5 µl of APS in 1 ml anhydrous ethanol.
4. Add 1 mL of the APS ethanol solution to the reactivial containing 1 mL of ethanol and 5 mg of frustules.

5. Place reactivial in reactitherm in laminar flow hood and turn heat on "high" at setting 1.75 and turn on stirring mechanism. This setting is calibration to 80ºC.
6. Allow frustule and APS mixture to stir at 80ºC for 2 hours, periodically double checking the temperature.
7. After 2 hours, turn off the heating without removing the reactivial from the reactitherm.
8. Allow the frustule and APS mixture to stir for another 22 hours without any heating.
9. After the 24 hour reaction time, wash the biosilica with ethanol and by centrifugation and pipetting off the ethanol.
10. Store the aminated biosilica in anhydrous ethanol.

## A1.5 Diatom Frustule Film Preparation for Photoluminescence Measurement

**Le Zhen (revised September 8, 2013)**

*Specialized Equipment*

- Eppendorf 5414C microcentrifuge
- VWR* Micro Cover Glasses, Round, No. 1, 18 mm diameter, ($^{23}/_{32}$) inch diameter (#48380-046)
- VWR* Micro Cover Glasses, Square, No. 2, 22×22 mm, (#)
- 10 μL pipette (Fihserbrand) and tips
- One piece of laminated green engineering paper
- One plastic clipboard, one wooden clipboard.
- 37×42 cm Kimwipes (KIMTECK, #34256)
- Laminar Flow Hood
- Lab Oven (90 $^{o}$C)

*Reagents*

- 1 mg/mL $H_2O_2$ treated diatom frustules suspension or APTMS functionalized diatom frustules in deionized water in a 1.5 mL microcentrifuge tube.
- 70% ethanol

*Procedure*

1. Complete the following process in laminar flow hood, wearing lab coat and gargle.
2. Spray the top and bottom side of the plastic clipboard with 70% ethanol.

3. Clip the laminated engineering paper on the clipboard with grid side up, the ethanol on the surface of the clip board will stick the engineering paper and the clipboard together.
4. Arrange one round cover glass (or square cover glass) on the grid side of engineering paper with one grid in the center of the coverslip.
5. Spray the cover glass with 70% ethanol, some of the ethanol will fill the gap between cover glass and the laminated engineering paper and prevent the cover glass from moving.
6. Wipe the surface of the cover glass to remove ethanol and dust, allow the surface to dry, adjust the position of cover glass to insure one grid in the center of the glass.
7. Invert frustule suspension in the microcentrifuge to ensure a homogenous suspension.
8. Pipette 10 μL of the frustule suspension onto the center of the glass coverslip, the circle formed by the drop of suspension should inscribe the grid in the center.
9. Transfer the cover glass on a folded 37×42 cm Kimwipes clipped on the wooden clipboard.
10. Transfer the wooden clipboard into 90 $^{o}$C lab oven, the frustule suspension will dry and become a white film in 3 minutes.
11. Transfer the wooden clipboard back into the laminar flow hood, repeat step 7 through 10 three times. At the last time leave the film in the oven for 1 hour.
12. Remove the cover glass from the oven, store it in a mailer in room temperature for 2 days before measuring photoluminescence.
13. Before measuring photoluminescence label each samples with a sample name with the format "Diatom intial-student initial-generation-sample number-last step of functionalization" on the upper part of the cover glass, and the date of measurement on the lower part of the sample.

14. Draw a straight line intersect with parameter of the cover glass between the sample name and date if round cover glass is used. This line will be used to lock the position of the cover glass in photoluminescence measurement.
15. Refer to Figure 1 below to see how to position cover glass on green engineering paper and how to label it.

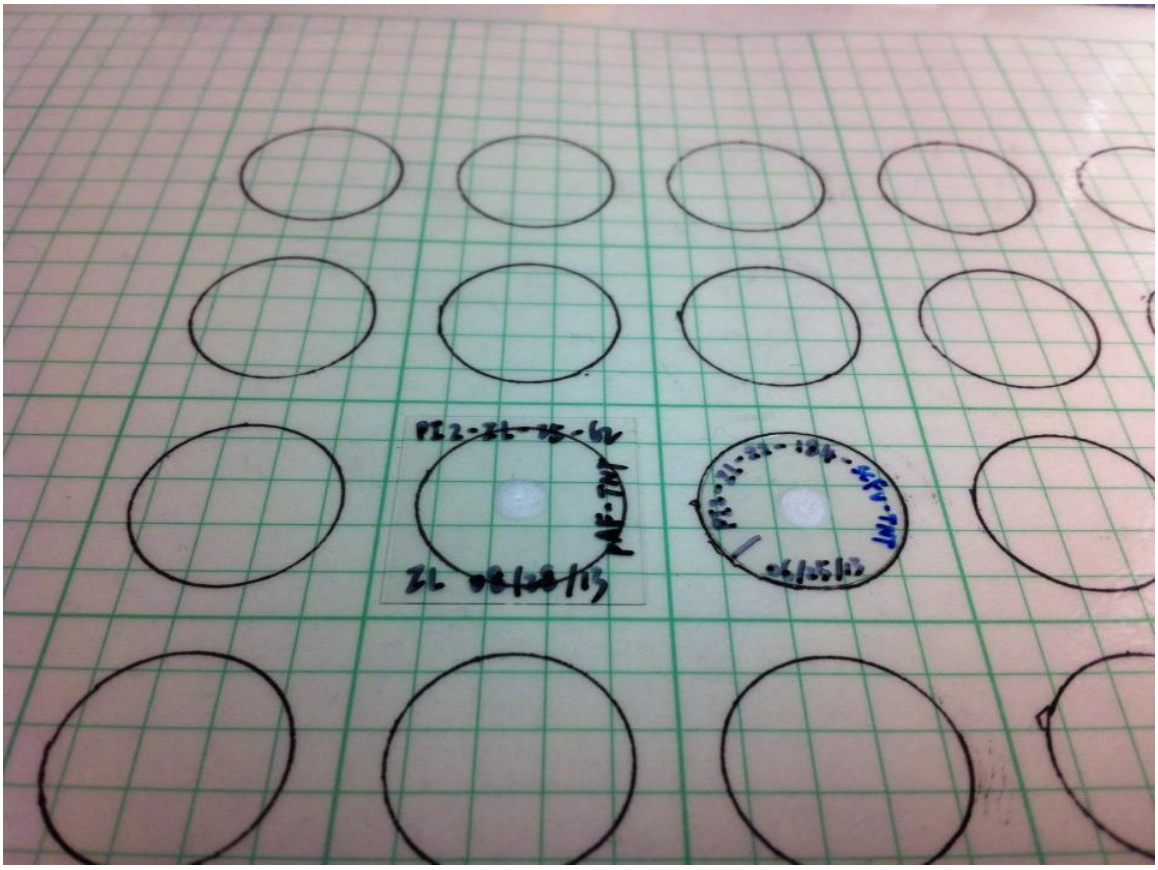

**Figure A1.** The position and label of two kinds of cover glasses on and biosilica films on engineering paper for guided deposit of frustule suspension.

**A1.6 Anti-TNT Single Chain Fragment Variable (scFv) Functionalization on 3-aminopropyltrimethoxysilane (APTMS) Functionalized Diatom Frustule Films on Round Coverslips**

**Le Zhen (September 03, 2013)**

*Specialized Equipment*

- Petri Dish (60×15 mm)
- Kimwipes
- VWR shaker
- 1.5 mL microcentrifuge tube
- 10 μL pipette (Fihserbrand)

*Reagents*

- Phosphate buffered saline (PBS) made according to the procedure *Phosphate Buffered Saline Preparation, pH 7.4, 0.01M Phosphate, 0.15 M NaCl 08-25-09*
- Acetonitrile (EMD$^{TM}$, HPLC grade, Lot#47295, dried with 0.4 nm molecular sieves for 2 days)
- Anti-TNT andtibody, single chain fragment variable (scFv derived from 2G5B5) , MW = 30,000 g/mol, 1.66 mg/ml.
- Disuccinimydyl suberate (DSS), MW=368.35, (Pierce Biotechnology, Lot#NJ176028, Prod#21655), or bis[sulfosuccinimidyl] suberate (BS3), MW=572.43, (Pierce Biotechnology, Lot#NC169133, Prod#21580)

*Sample*

- 3-aminopropyltrimethoxysilane (APTMS) Functionalized Diatom Frustule film on a round coverglass slip, 0.04 mg $H_2O_2$ treated frustules in a 5 mm diameter film, see Procedure *Diatom Frustule Film Preparation*

*Procedure*

1. Dissolve 1 mg DSS in 1 ml anhydrous acetonitrile. (If BS3 is used, replace solvent with PBS.)
2. Deposit 10 µl of DSS solution on each frustule film. (For BS3 solution, this step needs to be done within 1 minute after dissolved.)
3. Cover the films with a cap for pipette tip box, place the films on 100 rpm shaker and allow reaction for 10 minutes. (For DSS the solvent could dry out in 6 minutes, add 10 µl solvent on the films at the $5^{th}$ minute to keep the reaction going, remove the cap at the $8^{th}$ minute, and allow the solvent to dry out at the $10^{th}$ minute.)
4. Mix 20 µl 1.66mg/ml scFv solution with 30 µl PBS in a 1.5 ml centrifuge tube. (Ajust the amount of scFv and PBS according to the concentration of the initial scFv solution and the amount of final solution needed, make sure the final concentration is higher than 0.2 mg/ml.)
5. Diprinse the frustule films in PBS at the end of the 10-minute crosslinker functionalization, remove excess PBS on the films with kimwipe, and immediately deposit 10 µl scFv solution made in step 4 on the frustule films.
6. Fold a kimwipe and fit it into a 60×15 mm petri dish, wet the kimwipe with deionized water.
7. Put the frustule films in the petri dish, cap the dish, put the dish on 100 rpm shaker, and allow react for 2 hours.

8. Diprinse the frustule films in PBS solution 3 times.
9. Allow the frustule films to dry in stagnant air and room temperature for 1 hour.
10. Measure the photoluminescence spectra of the scFv functionalized frustule films at the end of 1-hour drying.

**A1.7 Immunocomplex formation on Anti-TNT scFv functionalized biosilica films on round cover slips**

**Le Zhen (September 5, 2013)**

*Specialized Equipment*

- 1.5 mL microcentrifuge tube
- 50 mL centrifuge tube
- Leica DMIL microscope
- CY3 filter for Leica DMIL microscope (excitation: 530/50 nm, emission: 610/75 nm)
- Petri Dish (60×15 mm)
- VWR shaker, 100 rpm
- six-well plate

*Reagents*

- Phosphate buffered saline made the procedure *Phosphate Buffered Saline Preparation, pH 7.6, 0.01M Phosphate, 0.15 M NaCl 08-25-09*
- 0.0785g/L TNT solution in deionized water
- 226 µg/L AF-TNB solution in deionized water
- 1,3,5-trinitrobenzene (1000 µg/ml in acetonitrile, SUPLECO, Lot #LB91318)
- 2-Amino-4,6-dinitrotoluene (1000 µg/ml in acetonitrile, SUPLECO, Lot #LB85132)
- 4-Amino-2,6-dinitrotoluene (1000 µg/ml in acetonitrile, RESTEK, Lot #A095420)

*Sample*

- 3-aminopropyltrimethoxysilane (APS), Bis[sulfosuccinimidyl]suberate (BS3) , scFv Functionalized Diatom Frustule film on a round coverglass slip, 0.040 mg $H_2O_2$ treated frustules as a 5 mm diameter film

**Antigen:** 0.0785g/L TNT solution in deionized water

**The following procedure is for a TNT molar concentration of $4.83\times10^{-7}$ M, however, all of the following TNT molar concentrations were used: $4.84\times10^{-6}$ M, $4.84\times10^{-8}$ M, $1.70\times10^{-9}$ M, $9.68\times10^{-10}$ M, $4.83\times10^{-10}$ M, $6.01\times10^{-11}$ M, $6.01\times10^{-12}$ M.**

*Procedure*

1. Add 15 mL of PBS to one Petri dish.
2. Add 21 µl 0.0785g/L TNT solution into the same Petri dish.
3. Place the scFv functionalized diatom frustules film covered coverslip film side down on the TNT solution. The surface tension of the solution will prevent the coverslip from sinking.
4. Place the petri dish on the shaker at 100 rpm for 2 hours.
5. Using tweezers carefully remove the coverslip.
6. Dip the coverslip in PBS to remove excess TNT.
7. Remove excess PBS on frustule film with kimwipe.
8. Place the frustule film in stagnant air under room temperature, allow to dry for 1 hour.
9. Measure photoluminescence signal at the end of 1-hour drying.

**Antigen:** Alex Fluor 555 Conjugated 1,3,5-trinitrobenzen, 226 µg/L, excitation: 555, emission: 565.

**Samples:** scFv funtionalized diatom frustule film, BS3 functionalized diatom frustule film soaked in PBS for 2 hours.

**The following procedure is for an Alex Fluor 555 [1] Conjugated 1,3,5-trinitrobenzen (AF-TNB) mass concentration of 5.56 µg/L.**

1. Add 1.95 mL of PBS to two wells of a six-well plate.
2. Add 50 µL of 226 µg/L AF-TNB in each of the wells.
3. Place the scFv functionalized and BS3 functionalized diatom frustule films coverslip film side down on the PBS, FITC-anti-Rabbit IgG solution. The surface tension of the PBS, anti-IgG mixture will prevent the coverslip from sinking.
4. Cover the 6 well plate in foil to protect the AF-TNB from photobleaching.
5. Place the 6 well plate on the shaker at 50 rpm for 15 minutes.
6. Using tweezers carefully remove the coverslip.
7. Dip the coverslip in PBS to remove excess antigen
8. Place the coverslip film side up on a piece of paper for drying in a dark location for 2 hours.
9. Using the DMIL microscope equipped with a CY3 filter view the film using 10x or 40 x. The 100x objective will not focus on the film due to the requirement of oil immersion.
10. Record fluorescent image using a 10 second exposure time.

**Antigen:** 2,4,6-trinitrotoluene (TNT)**,** 1,3,5-trinitrobenzene (TNB), 2-Amino-4,6-dinitrotoluene (2ADNT), 4-Amino-2,6-dinitrotoluene (4ADNT).

**The following procedure is for a competitive immunocomplex formation in $4.83\times10^{-7}$ M TNT and $4.84\times10^{-7}$ M 2ADNT mixture. However, immunocomplex formations are also performed in $4.79\times10^{-7}$ M TNB solultion, $4.84\times10^{-7}$ M 4ADNT solution, $4.84\times10^{-7}$ M 2ADNT solution, and the mixture of the solution above and $4.83\times10^{-7}$ M TNT solution.**

1. Add 4.3 µL of 1000 µg/ml 2ADNT acetonitrile solution into 50 ml centrifuge tube.
2. Allow solvent to dry out.
3. Add 45 ml PBS in the centrifuge tube and allow the 2ADNT to dissolve. (may take several days).
4. Add 63 µL of 0.0785g/L TNT solution in to the centrifuge tube.
5. Inverse the tube for 30 times.
6. Pipette 15 ml of the 2ADNT and TNT mixture solution in to Petri dish.
7. Place the scFv functionalized diatom frustules film covered coverslip film side down on the TNT solution. The surface tension of the solution will prevent the coverslip from sinking.
8. Place the petri dish on the shaker at 100 rpm for 2 hours.

9. Using tweezers carefully remove the coverslip.
10. Dip the coverslip in PBS to remove excess TNT.
11. Remove excess PBS on frustule film with kimwipe.
12. Place the frustule film in stagnant air under room temperature, allow to dry for 1 hour.
13. Measure photoluminescence signal at the end of 1-hour drying.

**Reference:**

**(1) Berlier, J. E.; Rothe, A.; Buller, G.; Bradford, J.; Gray, D. R.; Filanoski, B. J.; Telford, W. G.; Yue, S.; Liu, J.; Cheung, C.-Y.; Chang, W.; Hirsch, J. D.; Haugland, J. M. B. R. P.; Haugland, R. P. *J. Histochem. Cytochem.* 2003, *51*, 1699–1712.**

**A1.8 Measurement of Protein Concentration Using Bradford Test**

**Le Zhen (September 03, 2013)**

*Specialized Equipment*

- Spectrophotometer (SHIMAZU, MultiSpec-1501)
- 1.5 mL disposable cuvettes
- Parafilm (BEMIS)
- Finnpipette Digital 40-200 µl (A08458)
- 15 ml centrifuge tube
- 1.5 ml centrifuge tubes

*Reagents*

- Phosphate buffered saline (PBS) is made following the procedure *Phosphate Buffered Saline Preparation, pH 7.4, 0.01M Phosphate, 0.15 M NaCl 08-25-09*
- Quick Start™ Bradford 1× dye reagent (BIO-RAD, Cat#500-0205)
- Quick Start™ bovine serum albumin (BSA) standard (2mg/ml, Cat#500-0206)

*Sample*

- scFv samples in PBS solution before and after immobilization on biosilica, refer to procedure *Measurement of the Amount of Anti-TNT ScFv immobilized on biosilica Using Bradford Test_2013-09-04*

*Procedure*

1. Turn on the spectrophotometer 1 hour before the measurement for the machine to warm up, make sure the following parameters are properly set:

   D2-blue (which means the light source D2 is off);

   WI-green (on);

   EX-gray (off);

   Measure Mode: Abs (which means absorbance will be measured);

   Lamp Mode: D2+WI;

   View Intensity Position: High 2.0, Low 0.0;

   Wavelength Range: 450-800 nm;

   Sampling Interval: 1.0

   Trigger Mode: Start Button.

2. Pour out 10 ml of Bradford reagent into 15 ml centrifuge tube, allow to warm up to room temperature.
3. Pipette 40 µl of PBS solution into each of the five 1.5 ml micro-centrifuge tubes.
4. Pipette 40 µl of 2 mg/ml standard BSA into one of the micro-centrifuge, make sure it mix well with the PBS solution, this is the 1mg/ml standard solution.
5. Pipette 40 µl of 1mg/ml standard solution made in step 3 into the second micro-centrifuge tube to make 0.5 mg/ml standard solution.
6. Repeat step 5 to make 0.25, 0.125 and 0.0626 mg/ml serial standard solutions.
7. Pipette 1ml of Bradford reagent in to each of the six 1.5 ml cuvettes.

8. Pipette 20 µl of BSA standards made in step 4 to 6 into five different the cuvettes, seal the cuvettes with parafilms and inverse it 10 times to insure well mixed, incubate the solutions in room temperature for 5 minutes.
9. Pipette 20 µl of PBS into the sixth cuvette, mix it and measure with spectrophotometer as background.
10. Measure the five BSA standard solutions challenged Bradford solutions with spectrophotometer.
11. Plot the concentrations of BSA standard solutions against the absorbance at 595 nm to make the standard curve.
12. Measure samples with similar method as those for measuring standards, make sure the absorbance fall within linear region of the standard curve.
13. Extract the sample concentrations from standard curve.

For more information on the Bradford test, please refer to *Quick Start™ Bradford Protein Assay Instruction Manual (http://kirschner.med.harvard.edu/files/protocols/BioRad_proteinassay.pdf )*.

## A1.9 Photoluminescence Spectroscopy Measurement and Data Handling

**Le Zhen (Revised on September 10, 2013)**

*Specialized Equipment*

- VSL-337 Nitrogen Laser (Spectra-Physics, maximum power output: 2.0mW, peak output power: 40 kW, pulse length: 3 ns, pulse energy: 120μJ, wavelength: 337 nm, repetition rate: 10 Hz, beam dimension: 3mm×8mm.)
- 337 nm band pass filter
- Slit (1mm×1mm)
- Sample holder
- Focusing lens
- Acton SP2150 Spectra Detector (Priceton Instrument) with filter (position 1, allow wavelength longer then 360 nm) and slit (1mm)
- PIXIS 100 CCD Detector
- 26 cm×23 cm Optical Box
- 15 cm×15 cm Optical Plate

*Software:*

- WinSpec/32

*Reagents*

- $H_2O_2$ treated biosilica films, APS functionalized biosilica films, BS3 or DSS funtionalized films, scFv functionalized biosilica films, TNT challenged scFv functionalized biosilica films.

*Procedure*

*Data Collection*

1. Turn the key on the rear panel of the VSL 337 laser to the ON position.
2. Open the beam shuttle in front of the laser.
3. Keep the optical box closed and allow the laser to warm up for 10 minutes.
4. Turn on WinSpec/32, check the following parameters:

   [spectrograph]->[move]: grating 600BLZ=300, move to:480, speed: 100nm/min

   [Aqusiton]->[experiment set up]->[Main]: exposure time:5 seconds

5. Go to [Aqusiton]->[experiment set up]->[data correction], uncheck [background].
6. Go to [Aqusiton]->[experiment set up]->[data file], choose a output file, and name the first read BACK.
7. Acquire a spectrum without sample, that will be the background for this batch of measurments.
8. Go to [Aqusiton]->[experiment set up]->[data correction], check [background] and select the *BACK* file collected in step 7 as background.
9. Open the optical box, mount the standard sample onto sample holder, make sure the mark on the sample overlap the mark on the sample holder, close the optical box.
10. Go to [Aqusiton]->[experiment set up]->[data file], name the second read *Standard*.
11. Measure the spectrum of the standard, record the peak intensity, if two batches of measurements are needed to be comparable, the standard peak intensity of the two batches need to be less than 100 counts away from each other.

12. Go to [Aqusiton]->[experiment set up]->[data file], name the follow reads with sample names.
13. Measure spectra of samples.
14. Close and save all the spectra when measurements are finished.
15. Go to [tools]->[convert to ASCII], click [choose file] and selected all the spectra just acquired, click [choose output directory] and choose a file to shore the converted data, click [convert to ASCII], all the data will be converted to txt files.

*Data Handling*

**When only peak intensity data are needed, open the spectra file in WinSpec, click on the peak position, read and record the peak intensity directly. However, when comparing two spectra with only small difference, such as comparing the spectra of scFv functionalized biosilica film before and after challenged with TNT, the following data handling procedure need to be applied.**

1. Copy the data from txt files into one Excel file.
2. Label the wavelength region from 400 nm to 450 nm, this is the peaking region of the spectra.
3. Integrate the intensity of the peaking region using ellipsoidal rule, for each sample before and after TNT challenge, we obtain a peaking area $A_{before}$ and $A_{after}$ to represent the signal.
4. Normalized each sample's change in signal with that sample's singal before challenge following formular:

Normalized signal change= $(A_{before} - A_{after}) / A_{before.}$

## A1.10 Photoluminescence Spectroscopy Measurement Using Raman Microscope

**Le Zhen (Revised on September 10, 2013)**

*Specialized Equipment*

- LabRAM HR Raman Microscope (HORIBA JOBINYVON)
- Synapse CCD (HORIBA JOBINYVON)
- 325 nm He-Cd Laser

*Software:*

- LabSpec 5

*Reagents*

- $H_2O_2$ treated biosilica films, APS functionalized biosilica films, BS3 or DSS funtionalized films, scFv functionalized biosilica films, TNT challenged scFv functionalized biosilica films prepared following procedures except that **only 10 µl of 1mg/ml frustule are deposit to make the films and use silicon as substrate.**

*Procedure*

1. Turn the key on the rear panel of the He-Cd laser to the ON position, allow to warm up for 1 hour before taking measurement.
2. Slid open the panel on the top of the LabRAM HR, lift out the black cover.
3. Turn the rod attached to the mirror in front of the laser entrance counter-clockwise and slide the mirror down, which allows the 325 laser to enter.
4. Install 325nm interference filter.
5. Switch notch pin to size 11.

6. Put the black cover back and slide close the panel.
7. Push the rod on the right-hand side of the sample plate in to switch to UV mode.
8. Change the objective to 40X UV objective.
9. Turn of LabSpec5.
10. Go to [options]->[unit], select “nm”.
11. Check to make sure the following parameters are correct:

    Wavelength range:350-800 nm

    Laser: 350 nm

    Filter:--

    Hole: 200 μm

    Grading: 300

    Objective: 40x

    Integration time: 10sec

    Accumulation: 3 times

12. Turn on white light source and camera, focus the laser on the frustules of interest.
13. Turn off whith light source, camera, and take the measurement.

## Appendix 2: Tabular Data

**Table A1.** Epifluorescence Intensities of AF-TNB Challenged DSS-biosilica and ScFv-biosilica

| Image Names | DSS-7 | DSS-6 | DSS-5 | DSS-4 | DSS-1 | scFv-7 | scFv-6 | scFv-5 | scFv-4 | scFv-1 |
|---|---|---|---|---|---|---|---|---|---|---|
| Sum of Sampled Intensity ($\times 10^{-5}$) | 2.47 | 2.47 | 2.55 | 2.62 | 1.62 | 13.0 | 10.6 | 10.2 | 11.7 | 10.3 |
| Average Sum of Intensity ($\times 10^{-5}$) | | | 2.35 | | | | | 11.2 | | |
| Standard Deviation ($\times 10^{-5}$) | | | 0.41 | | | | | 1.18 | | |

**Table A1.** (Continued)

| Group Name | DSS-functionalized biosilica | ScFv-functionalized biosilica |
|---|---|---|
| Normalized Sum of Intensity | 1.00 | 4.77 |
| Standard Deviation | 0.247 | 0.974 |
| Number of samples | 5 | 5 |

**Table A2.** Saturated PL Response of PBS-soaked and TNT-challenged ScFv-biosilica

| Challenge | PBS | TNT |
|---|---|---|
| First Order Normalization ($\times 10^2$) | -5.19 | 7.51 |
| Standard Deviation ($\times 10^2$) | 0.392 | 0.936 |
| Second Order Normalization ($\times 10^2$) | 0.00 | 12.7 |
| Standard Deviation ($\times 10^2$) | 0.554 | 1.01 |
| Number of Samples | 3 | 3 |

**Table A3.** Dose Response of ScFv-biosilica to TNT Challenge

| $C_{TNT,i}$(M) | 4.80E-09 | 1.20E-08 | 2.40E-08 | 3.00E-08 | 4.00E-08 | 4.80E-08 | 1.60E-07 | 4.80E-07 |
|---|---|---|---|---|---|---|---|---|
| $Q^0$ | 3.41E-03 | 5.42E-03 | 1.51E-02 | 3.55E-02 | 6.44E-02 | 9.16E-02 | 9.92E-02 | 1.27E-01 |
| stdev. | 1.70E-02 | 2.78E-02 | 2.22E-02 | 2.00E-02 | 5.13E-03 | 1.92E-02 | 3.04E-02 | 1.01E-02 |
| $C_{TNT,f}$(M)[a] | 4.71E-09 | 1.15E-08 | 2.23E-08 | 2.77E-08 | 3.67E-08 | 4.40E-08 | 1.53E-07 | 4.73E-07 |
| $Q^0_{predicted}$[b] | 1.70E-03 | 1.00E-02 | 3.17E-02 | 4.32E-02 | 6.02E-02 | 7.13E-02 | 1.14E-01 | 1.19E-01 |

[a] Final concentration considering the change from immunocomplex formation, estimated with matlab code shown in Fig. S1, $C_f$ are x values in Fig. 5;

[b] Predicted PL responses using the model equation (3), with parameters $Q_{max}$=0.12, $K_d$=4.04×$10^{-16}$ $M^{(n)}$, n=2.07(estimated with matlab code shown in Fig. S1), corresponded to the trend line shown in Fig. 5.

**Table A4.** PL Responses of BSA-biosilica and ScFv-biosilica to TNT Challenge.

| | BSA | scFv |
|---|---|---|
| mean | 1.52E-02 | 1.21E-01 |
| stdev. | 2.60E-02 | 1.36E-02 |
| n | 3 | 3 |

**Table A5.** PL Responses of ScFv-biosilica to Competitive Antigens.

| Antigen | TNT | | TNT+TNB | | TNB | | TNT+RDX | | RDX | |
|---|---|---|---|---|---|---|---|---|---|---|
| Times of Rinse | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 3 |
| Normal. PL Response (×10) | 1.21 | 1.27 | 1.36 | 1.63 | 1.46 | 1.28 | 1.01 | 1.37 | 0.506 | -0.0432 |
| Stdev.(×$10^2$) | 1.36 | 1.01 | 2.31 | 2.10 | 2.45 | 1.97 | 1.22 | 0.612 | 1.02 | 2.72 |

**Table A5.** (Continued)

| Antigen | TNT+2,6-DNT | | 2,6-DNT | |
|---|---|---|---|---|
| Times of Rinse | 1 | 3 | 1 | 3 |
| Normal. PL Response (×10) | 1.14 | 1.23 | 0.453 | 0.284 |
| Stdev.(×$10^2$) | 0.577 | 2.76 | 1.15 | 1.39 |