# Analytical Methods

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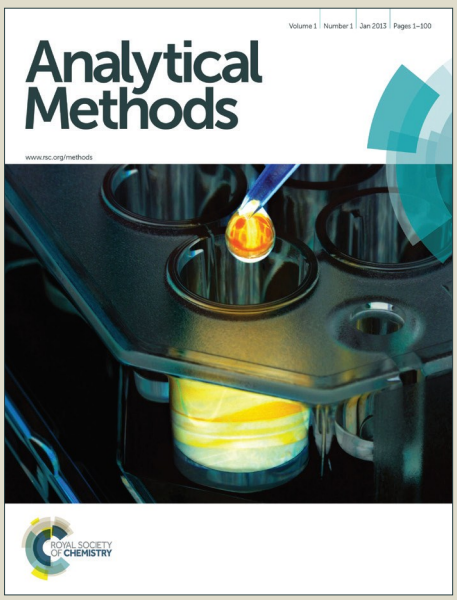


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Analytical Methods

ARTICLE

# Sensitive and homogenous immunoassay of fumonisin in foods using single molecule fluorescence correlation spectroscopy

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Fumonisin $B_1$ ($FB_1$) is considered to be the most prevalent mycotoxin in naturally contaminated cereals throughout the world and is potentially hazardous to humans and animals. Therefore, it is necessary to develop sensitive, fast and reliable detection methods of $FB_1$. In this paper, we reported a homogeneous immunoassay for sensitive detection of $FB_1$ in maize using single molecular fluorescence correlation spectroscopy (FCS). In this study, competitive immunoassay model was used, and $FB_1$ was labeled with fluorescent dye as a fluorescent tracer. The principle of competitive immunoassay is based on sensitively distinguishing the fluorescent tracer and tracer-antibody complex by FCS technique due to significant difference of the characteristic diffusion times between the tracer and tracer-antibody complex. We firstly synthesized the fluorescent $FB_1$ tracer using Alexa 488 as labeling probes, and then optimized the experimental conditions for competitive immunoassay. We observed good linear relations between the fraction of Alexa 488–labeled $FB_1$ immune complex in reaction solution and the $FB_1$ concentration in sample. Under optimal conditions, the linear range is from 1.0 μg $L^{-1}$ to 25.0 μg $L^{-1}$, and the detection limit is 1.0 μg $L^{-1}$ for $FB_1$. This method was successfully used for determination of $FB_1$ in spiked and natural samples. The results obtained by FCS are in good agreement with that by ELISA method. Our results demonstrate that the quantitative FCS method is rapid, simple and highly sensitive, and it can easily be extended to detect other chemical contaminants for food safety.

## 1. Introduction

Fumonisins are a class of mycotoxins primarily produced by Fusarium moniliforme. They are characterized by two tricarballylic acid chains esterified to a 20-carbon backbone having one or more hydroxyl groups and a single primary amine. The fumonisins were first isolated by Geiderblon et al. in 1988.[1] More than 11 structurally related fumonisins ($FB_1$, $FB_2$, $FB_3$, $FA_1$, $FA_2$, $FC_1$, $FC_2$, $FC_3$, $FC_4$, $FP_1$ and so on) have been found in several types of foods worldwide. Fumonisin $B_1$ ($FB_1$) is the most prevalent and also the most toxic of the Fumonisins family,[2] and $FB_1$ accounts for 70%-80% of the total amount of Fumonisins.[3] Fumonisins are mainly produced in maize and maize-based products.[4,5] Fumonisins are potentially hazardous to humans and animals, and can cause leukoencephalomalacia in horses, pulmonary edema in pigs,[6] nephrotoxicity, and liver cancer in rats.[7,8] Fumonisins in foods were related to the occurrence of esophageal cancer in the Transkei region of Africa,[9] Hebei, Henan and Hainan regions of China,[10,11] Iran,[12] Northern Argentima[13] and Brazil.[14] $FB_1$ is considered a possible carcinogen in humans, and is classified in group 2B by the International Agency for Research on Cancer.[15] In order to reduce the risk associated with the consumption of contaminated maize, many countries have regulated the levels of fumonisins in maize.[16] The [US] Food and Drug Administration has issued the maximum residue limits in maize and maize-based products (corn and corn products) in foods and animal feeds. The maximum residue limits are 2-4 ppm and 5-100 ppm total fumonisins ($FB_1$ + $FB_2$ + $FB_3$) in human foods and animal feeds, respectively.[17] The level of the single fumonisin should be even lower. Therefore, there is an increasing demand for a sensitive, reliable, simple, fast detection method of $FB_1$ in maize and maize-based products. So far, several analytical methods have been developed for detection of fumonisins, mainly including chromatography methods and immunoassays. Chromatography is a separation technique, and can be used to simultaneously detect different types of fumonisins. Currently, various chromatography methods are used to determination of fumonisins, which include thin layer chromatography (TLC),[18] liquid chromatography–mass spectrometry (LC–MS),[19,20] gas chromatography-mass spectrometry (GC–MS),[21] high-performance liquid chromatography (HPLC),[22] and capillary electrophoresis (CE).[23]

Immunoassay is considered to be a very important analytical method widely used in clinical diagnosis, food and environmental analyses and biomedical studies because of its extremely high selectivity and sensitivity. Up to now, certain immunoassays are developed for the determination of fumonisins, mainly including fluorescence polarization (FP),[24] immunosensors,[25] surface plasmon resonance (SPR)[26] and enzyme–linked immunosorbent assay (ELISA).[8,27] Due to its simplicity, selectivity and low cost, ELISA is

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† Footnotes relating to the title and/or authors should appear here.
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widely used for assay of fumonisins.[8,27] However, since it is heterogeneous assay mode, ELISA involves antibody immobilization, immune reaction and washing cycles, and thus this assay is labor-intensive and time-consuming. Compared to heterogeneous mode, homogeneous immunoassay is an attractive format due to its automation, less risk of contamination and short analysis time. In homogeneous immunoassay, the key strategy is how to quantitatively and sensitively distinguish antibodies (or antigen) and antigen-antibody complexes in the immunoreaction solution. So far, certain detection methods have been used in homogeneous immunoassays, mainly including fluorescence polarization,[28] fluorescence and chemiluminescence resonance energy transfer,[29] and time-resolved fluorescence.[30] However, these detection methods still show unsatisfactory sensitivity. Therefore, to develop sensitive and direct detection techniques, and design universal and homogenous strategies are very important topics in current immunoassays.

Fluorescence correlation spectroscopy (FCS) is a single molecule method, and its principle is based on measuring the fluorescence fluctuations in a highly-focused laser beam due to the Brownian motion of single fluorescent molecules. FCS has become a sensitive and efficient tool for studying molecular diffusion,[31] chemical kinetics,[32] and molecular interaction in vitro[33] and in vivo.[34] So far, FCS has been used to immunoassays,[35] drug screening,[36] and detection of drug-induced cell apoptosis.[37]

In this paper, we reported a homogeneous immunoassay for the sensitive detection of $FB_1$ in maize. In this study, competitive immunoassay model was used, and fluorescent labeled $FB_1$ was used as a tracer. The principle of competitive immunoassay is based on quantitatively and sensitively distinguishing the fluorescent tracer and tracer-antibody complex by FCS technique due to significant difference of the characteristic diffusion times between the tracer and tracer-antibody complex. The fraction of tracer-antibody complex (Y) in the mixed solution of the tracer and tracer-antibody complex can be obtained by non-linearly fitting of correlation curves. We expect that there is a quantitative relationship between Y and concentration of $FB_1$. We firstly synthesized the fluorescent $FB_1$ tracer, and then optimized the experimental conditions for competitive immunoassay. We observed the decrease of the Y value with an increase in concentration of $FB_1$, and obtained a good linear relation between Y and concentration of $FB_1$. Under optimal conditions, the linear range is from 1.0 μg $L^{-1}$ to 25.0 μg $L^{-1}$, and the detection limit is 1.0 μg $L^{-1}$ for $FB_1$.

Finally, we applied this method to determine the level of $FB_1$ in maize samples. Our results obtained by FCS are in good agreement with that by ELISA method.

## 2. Materials and methods

### 2.1 Chemicals and materials.

Fumonisins $B_1$ was purchased from J&K scientific Ltd (Shanghai, China). Rhodamine Green and Alexa Flour® 488 Succinimidyl Ester (Alexa 488) were purchased from Life Technologies Inc (Carlsbad, CA, USA). Monoclonal Mouse Anti-Fumonisin $B_1$ was obtained from Yiji Industries Co., Ltd (Shanghai, China). ELISA kits for Fumonisin $B_1$ were the product of QiYi Biological Technology Co., Ltd (Shanghai, China). Bovine serum albumin (BSA) was acquired from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical grade and used without further purification. All aqueous solutions were prepared with ultrapure water (18.2 MΩ) from a Millipore Simplicity System (Bedford, MA, USA).

### 2.2 Apparatus.

FCS measurements were carried on a home-built FCS system,[38] and its setup used is shown in Fig. S1. Briefly, the setup of FCS was based on an inverted Olympus IX 71 microscope (Japan). The laser was provided by a 488 nm laser beam from an argon ion laser (Ion Laser Technology, Shanghai, China). After passing through a series of optical elements, the laser was focused on the sample solution (about 40 μL) by a water immersion objective (UplanApo, 60× NA 1.2, Olympus). The resulting excitation volume was 0.44 fL (shown in Supporting Information). The fluorescence signal was collected by the objective after passing though the dichroic mirror (505DRLP, Omega Optical, USA) and then was filtered by a band-pass filter 530DF30 (Omega Optical, USA). Finally, the fluorescence signal was collected after passing the pinhole by a real time digital correlator (Flex02–12D/C, Correlator. Com, USA).

### 2.3 Preparation of alexa 488-labeled $FB_1$

The reaction of the primary amine of $FB_1$ with Alexa 488 succinimidyl ester produces a fluorescent tracer and the procedure is shown in Fig. 1. Briefly, $FB_1$ (10.0 μg in 1 μL of DMF) was mixed with 2 μL of 1 M sodium carbonate buffer, and Alexa 488 (200.0 μg in 20 μL of DMF) was added. The reaction mixture was shaken and incubated overnight at ambient temperature and diluted to 200 μL with 1 M sodium carbonate buffer. The crude product was purified and characterized by HPLC–MS (Fig. S2) and the purified product was stored at –20 °C for further use.

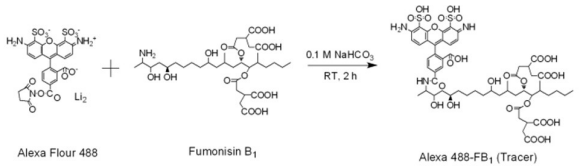


**Fig. 1** The procedure for the preparation of Alexa 488-labeled $FB_1$.

### 2.4 $FB_1$ extraction of samples

Samples of whole kernel corn were ground using a shatter machine. A portion was then extracted with water and the methanol/water (7/3, v/v) for FCS, respectively. The extracts were used in ELISA assay according to the operational manuals of ELISA kits. 0.2 g of ground maize was mixed with 1 mL of the methanol/water or water and then was sufficiently shaken for 3 min. The miscible liquid was then centrifuged for 5 min. The mixture was then filtered through a 0.2 μm filter membrane and stored frozen for further use.

### 2.5 FCS assay

$FB_1$ standard solutions were prepared by diluting the $FB_1$ stock solution (100.0 μg $L^{-1}$ in 10 μL of DMF) with PBS. Eighteen μL of



 

antibody solution (diluted with PBS) was placed into a 200 µL centrifuge tube. Five µL of BSA was added to suppress the nonspecific adsorption and improve the reproducibility of the measurements. Nine µL of fumonisin $B_1$ standard solution, or sample extract, was added and then incubated at 37 °C for 5 min. Next, 18 µL of Alexa 488–labeled $FB_1$ stock solution (diluted with PBS) was added and mixed, then incubated at 37 °C for 5 min again. Finally, the samples were measured by FCS. FCS measurements were carried out over a period of 60 s at room temperature, and were repeated 3 times.

### 2.6 ELISA assay

To investigate the reliability of this method, samples were analyzed by conventional ELISA method according to the manufacturer's manual of ELISA kits. We measured the absorbance of 450 nm for $FB_1$ using a UV/Vis-3501 spectrophotometer.

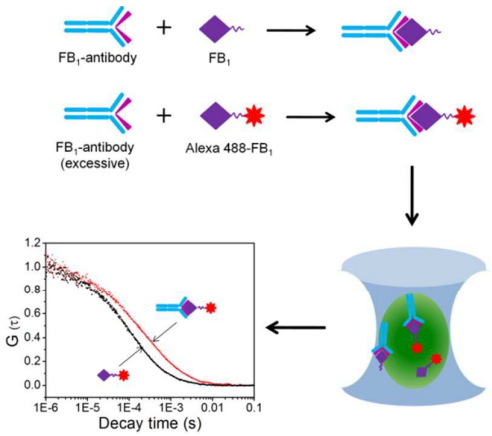


**Fig. 2** The principle of homogeneous competitive immunoassay by FCS.

## 3. Results and discussion

### 3.1 Principle of homogeneous competitive immunoassay

The principle of homogeneous immunoassay by FCS is illustrated in Fig. 2. In the procedure of immunoassay, competitive mode was used and FCS was used to distinguish the free fluorescent tracer and tracer-antibody complex. The immunoreaction (1) and competitive immunoreaction (2) are described by the following equations.

$$A + B = AB \quad (1)$$

$$A^* + B = A^*B \quad (2)$$

Where $A$ is $FB_1$, $A^*$ is fluorescent labeled $FB_1$ (the tracer), $B$ is antibody, and $A^*B$ is tracer-antibody complex. In this system, there are two fluorescent components $A^*$ and $A^*B$. The fraction (Y) of $A^*B$ in two fluorescent components ($A^*$ and $A^*B$) is expressed:

$$Y = \frac{[A^*B]}{[A^*] + [A^*B]} \quad (3)$$

From equations (1-2), when the amounts of $A^*$ and $B$ are kept constant, Y value will decrease with an increase of $A$ component ($FB_1$). In this method, FCS can be applied to distinguish free $A^*$ (Alexa 488–labeled $FB_1$) and $A^*B$ complex (Alexa 488–labeled $FB_1$–antibody complex) in solution due to the significant difference in their characteristic diffusion times. The fraction Y can be measured by two-component fitting procedure. The equation of two-component model:

$$G(\tau) = \frac{1}{N}\left[1 - T + T\exp\left(\frac{-\tau}{\tau_T}\right)\right]\left[\frac{1-Y}{\left(1+\frac{\tau}{\tau_{free}}\right)\sqrt{1+\frac{\omega_o^2}{z_o^2}\frac{\tau}{\tau_{free}}}} + \frac{Y}{\left(1+\frac{\tau}{\tau_{bound}}\right)\sqrt{1+\frac{\omega_o^2}{z_o^2}\frac{\tau}{\tau_{bound}}}}\right] \quad (4)$$

Where $N$ is the total average number of fluorescent molecules ($A^*$ and $A^*B$) in the detection volume, $\omega_0$ and $z_0$ are the distances from the center of the laser beam focus in the radial and axial directions, respectively. $T$ is the average fraction of fluorescent molecules in the triplet state with relaxation time $\tau_T$. $\tau_{free}$ and $\tau_{bound}$ are characteristic diffusion times of free $A^*$ and binding complex $A^*B$. The $\tau_{free}$ and the $\tau_{bound}$ as 91.3 and 216.0 µs, respectively. Y can be obtained by non-linearly fitting of FCS curves. FCS data were analyzed with the standard equation for two-component model (Eq. 4) and non-linearly fitted with the Microcal Origin 8.0 software package based on the Levenberg-Marquardt algorithm.

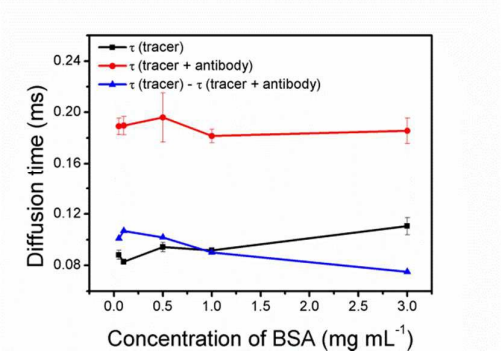


**Fig. 3** Effects of the BSA concentration added. Five different BSA concentrations (0.05, 0.1, 0.5, 1.0, and 3.0 mg $mL^{-1}$) were used in this study. The reaction and detection buffer were 10 mM PBS buffer (pH 7.3). The concentration of Alexa 488–labeled $FB_1$ was 11.0 nM and the concentration of $FB_1$–antibody was 55.0 nM.

### 3.2 Optimization of immunoassay conditions

We firstly investigated the effects of the reaction time between Alexa 488–labeled $FB_1$ and $FB_1$–antibody, and the results are shown in Fig. S2. The results illustrated that the binding of Alexa 488–labeled $FB_1$ to $FB_1$-antibody was a fast process, and within 1 min the binding reaction reached the equilibrium in this case. Therefore, we

chose the reaction time as 5 min at 37 °C in order to fully achieve equilibrium of the immune reaction.

In immunoassay, BSA is usually used to suppress the nonspecific adsorption and improve the reproducibility of the measurements. In this study, we investigated effects of the BSA concentration in the reaction solution. Fig. 3 shows the characteristic diffusion times of free Alexa 488–labeled $FB_1$ and its immune complex under different BSA concentration. The characteristic diffusion times of its immune complexes were basically kept constant in the range of BSA concentration studied. However, the characteristic diffusion time of the free Alexa 488–labeled $FB_1$ slightly increased with an addition of BSA concentration. We observed that the difference in the characteristic diffusion time between the immune complex and the free Alexa 488–labeled $FB_1$ was the maximum in the presence of 0.1 mg $mL^{-1}$ BSA. Therefore, we chose 0.1 mg $mL^{-1}$ BSA in further experiments.

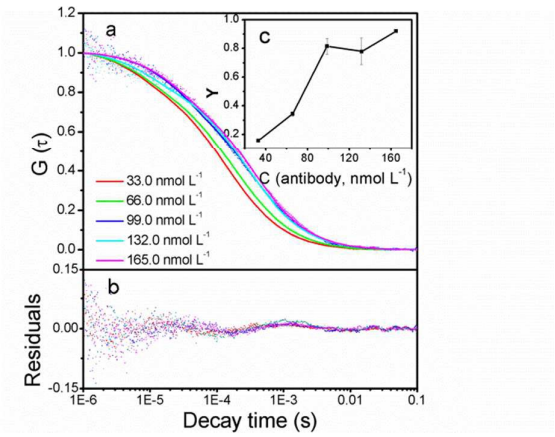


**Fig. 4** (a) Normalized correlation curves and their fitting curves of Alexa 488–labeled $FB_1$ and its binding complex at different concentration of $FB_1$–antibody. (b) The fitting residuals. (c) The relations between the bound ratio of Alexa 488–labeled $FB_1$ to $FB_1$–antibody (Y) and $FB_1$–antibody concentration (C). The reaction and detection buffer were 10 mM PBS buffer (containing 0.1 mg $mL^{-1}$ BSA, pH 7.3). The concentration of Alexa 488–labeled $FB_1$ was 70.0 nM.

In competitive immunoassay, the molar ratio of antibody to fluorescent tracer significantly affects the formation of the immunocomplex and sensitivity of immunoassay.[39] We also investigated the effects of the ratio of antibody to Alexa 488–labeled $FB_1$. In the measurements, various concentrations (from 33.0 nM to 165.0 nM) of antibody were added to a fixed concentration (70.0 nM) Alexa 488–labeled $FB_1$ solution. Fig. 4a shows typical normalized autocorrelation curves. The results illustrated that the autocorrelation curves were well fitted by Eq. 4. Fig. 4b showes that their fitting residuals are less than 0.15. As shown in Fig. 4a, we observed that correlation curves shifted to the right with the increase of antibody concentration, indicating the increase in the fraction of the immune complex. When the concentration of the antibody reached 100.0 nM, the correlation curves almost did not move to right, indicating that the fraction of the immune complex did not increase because almost all Alexa 488–labeled $FB_1$ bound to antibodies. Fig. 4c reflected the relationship between the fraction of the immune complex and the concentration of the antibody, and further demonstrated that when the concentration of the antibody reached 100.0 nM the fraction of the immune complex almost did not change. Therefore, the optimal ratio of antibody to Alexa 488–labeled $FB_1$ (the optimal concentration of the antibody was 100.0 nM) was 1.4 when the concentration of the Alexa 488–labeled $FB_1$ was 70.0 nM. Then, we investigated the optimal ratio of $FB_1$–antibody to Alexa 488–labeled $FB_1$ with different concentrations of Alexa 488–labeled $FB_1$ solution. The optimal ratio of $FB_1$-antibody to Alexa 488–labeled $FB_1$ was 10.0, 5.4, 3.8, 2.5 and 1.4 for 3.2, 6.1, 13.0, 33.0 and 70.0 nM of Alexa 488–labeled $FB_1$ solution, respectively (shown in Fig. S4).

### 3.2 Optimization of working curves

Under optimal conditions, various concentrations (from 1.0 μg $L^{-1}$ to 25.0 μg $L^{-1}$) of $FB_1$ were added to a fixed concentration (33.0 nM) of $FB_1$–antibody solution and then added a fixed concentration (3.2 nM) of Alexa 488–labeled $FB_1$. Fig. 5a shows an example of the normalized correlation curves. The results illustrated that the autocorrelation curves were well fitted by Eq. 4. Fig. 5b shows their fitting residuals were less than 0.15. With an increase of standard $FB_1$, the autocorrelation curves resulted in the shift to the left due to the increase of free Alexa 488–labeled $FB_1$, indicating a decrease in the fraction of Alexa 488-$FB_1$ immune complex (Y). Fig. 5c reflectes a good liner relations between the fraction of Alexa 488–labeled $FB_1$ immune complex (Y) and the concentration of standard $FB_1$ ($R^2$ = 0.986). The calibration curve of $FB_1$ has a linear range from 1.0 μg $L^{-1}$ to 25.0 μg $L^{-1}$, which covers the FDA approved range of

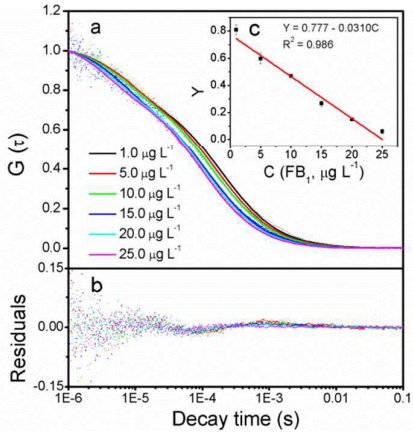


**Fig. 5** (a) Normalized correlation curves and their fitting curves of Alexa 488–labeled $FB_1$ and its binding complex at different concentration of $FB_1$. (b) The fitting residuals. (c) The relationship between the ratio of Alexa 488–labeled $FB_1$ to $FB_1$–antibody (Y) and the $FB_1$ concentration. The concentration of Alexa 488–labeled $FB_1$ was 3.2 nM and the concentration of $FB_1$–antibody was 33.0 nM.

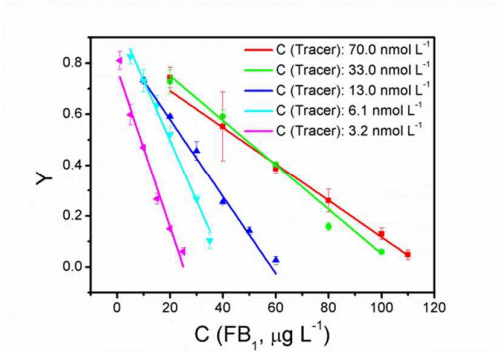


**Fig. 6** The working curves at different concentration of Alexa 488–labeled $FB_1$. The concentrations of $FB_1$-antibody were 33.0, 33.0, 66.0, 82.5 and 100.0 nM when the concentrations of Alexa 488–labeled $FB_1$ (the tracer) were 3.2, 6.1, 13.0, 33.0 and 70.0 nM, respectively.

$FB_1$. The detection limit is 1.0 μg $L^{-1}$ for $FB_1$. We investigated the working curve with different concentration of Alexa 488–labeled $FB_1$ solution (from 3.2 nM to 70.0 nM) respectively (shown in Fig. S5). We summed all the working curves to a graph, shown in Fig. 6. From the Fig. 6, we found that the slope of the working curve increased with the decrease in concentrations of the $FB_1$-antibody and Alexa 488–labeled $FB_1$, that is, high sensitivity can be obtained in the low concentration of the $FB_1$-antibody and Alexa 488–labeled $FB_1$. Therefore, we used the working curve in the presence of 33.0 nM $FB_1$-antibody and 3.2 nM Alexa 488–labeled $FB_1$ in the following research.

Table. 1 Recovery results of samples measured by FCS and ELISA kits

| | FCS ($CH_3OH/H_2O$ (7/3, v/v)) | | | FCS ($H_2O$) | | | ELISA ($CH_3OH/H_2O$ (7/3, v/v)) | | |
|---|---|---|---|---|---|---|---|---|---|
| | S1[a] | S2[a] | S3[a] | S1[a] | S2[a] | S3[a] | S1[a] | S2[a] | S3[a] |
| Original amount[b] | 1.8 | 2.8 | 1.8 | 2.2 | 3.1 | 2.3 | 2.4 | 3.1 | 2.6 |
| RSD (%)[c] | 10.0 | 13.6 | 13.7 | 7.1 | 10.2 | 4.1 | -[d] | -[d] | -[d] |
| Added amount[b] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Founded amount[b] | 7.1 | 7.4 | 6.7 | 7.3 | 8.1 | 7.6 | 7.2 | 7.5 | 7.3 |
| RSD (%)[c] | 6.4 | 2.1 | 7.0 | 2.7 | 5.8 | 3.1 | -[d] | -[d] | -[d] |
| Recovery (%) | 105.0 | 93.2 | 97.6 | 102.0 | 101.0 | 106.0 | 96.2 | 89.6 | 95.2 |

[a]Sample. [b]The unit is μg $g^{-1}$. [c]The measurements were repeated 3 times. [d]Not measured.

### 3.2 Analysis of $FB_1$ in maize samples

Acetonitrile/water and methanol/water are commonly used extraction solvents for assays of $FB_1$ in foods.[40] Under optimal conditions, we used methanol/water (7/3, v/v) and water as extraction solvents for FCS assays. The results (shown in Table. 1) illustrated that the detection value of methanol/water (7/3, v/v) as the extraction solvents was less than that of water as the extraction solvents. The contrast experiments were preformed with ELISA kits, and the results obtained with the FCS using water as extraction solvents showed better agreement with ELISA assays. Therefore, we selected water as extraction solvent in the following research. As shown in Table. 1, the RSDs of FCS assays using water as extraction solvents were less than 11.0 %, and the recoveries of this method were from 101.0%–106.0%, which further illustrated that our method was reliable.

Under optimal conditions, six varieties of maize samples from Heibei (China) were tested and the results were shown in Fig. 7. The $FB_1$ contents were from 1.8 μg $g^{-1}$ to 3.6 μg $g^{-1}$, and the RSDs of the measurement were from 8.3% to 16.5% (n = 3). The results obtained by FCS method were in good agreement with that of by ELISA assays.

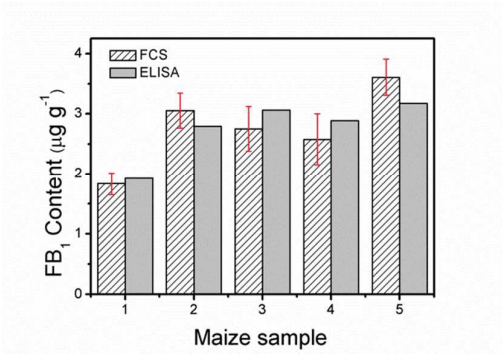


**Fig. 7** $FB_1$ contents in different varieties of maize samples from Heibei. The concentration of Alexa 488–labeled $FB_1$ was 3.2 nM and the concentration of $FB_1$–antibody was 33.0 nM.

## Conclusions

In conclusion, we reported a homogeneous immunoassay for sensitive detection of $FB_1$ in maize using single molecular fluorescence correlation spectroscopy. In comparison to conventional ELISA, our method shows high sensitivity, simplicity, a short analysis time, and low reagent and sample requirement. By replacing the target of interest, the FCS method can easily be extended to detect other chemical contaminants and thus represents a versatile strategy for food safety analysis.

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# Sensitive and homogenous immunoassay of fumonisin in foods using single molecule fluorescence correlation spectroscopy

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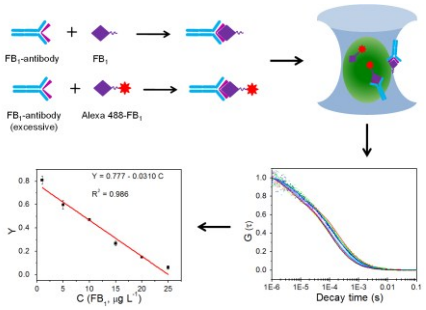


In this paper, we reported a new homogeneous immunoassay for sensitive detection of $FB_1$ in maize using single molecular fluorescence correlation spectroscopy (FCS). Our results demonstrate that the quantitative FCS method is rapid, simple and highly sensitive, and it can easily be extended to detect other chemical contaminants for food safety.