Chapter 11

Microarray-Based Immunoassay for Parallel Quantification of Multiple Mycotoxins in Oat

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Abstract

Because multianalyte methods are highly desirable in order to keep analysis time and costs low, the biosensor development increasingly focuses on parallel analysis of several mycotoxins. Here, we describe an indirect competitive immunoassay on regenerable, reusable glass microchips for the parallel determination of aflatoxins, ochratoxin A, deoxynivalenol, and fumonisin B1 in oat extracts, using a fully automated flow-through device with chemiluminescence readout.

Key words Automated multianalyte immunoassay, Munich chip reader (MCR 3), Chemiluminescence microarray readout, Mycotoxins, Oat

1 Introduction

The mycotoxins are secondary metabolites from fungal species such as *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium* growing on agricultural commodities in the field or during storage [1]. They are known for their acute toxic, immunosuppressive, mutagenic, teratogenic, or even carcinogenic effects and therefore, maximum levels for several mycotoxins in food have been set by the European Community (EC) [2, 3]. For example, maximum levels of 4 μg/kg for the sum of aflatoxins B1, B2, G1, and G2 as well as 2 μg/kg for aflatoxin B1 and 5 μg/kg for ochratoxin A in cereals and all products derived from cereals are allowed by the Commission regulations (EC) No 1881/2006 and No 1126/2007. In contrast, higher limits for fumonisin B1 of 400–4000 μg/kg and for deoxynivalenol of 750–1750 μg/kg were set.

Mycotoxins constitute a very heterogeneous group of compounds that differ greatly in their chemico-physical properties and polarity, as well as in their distribution and concentration in food (Fig. 1). Because of this, particular extraction, cleanup, and detection

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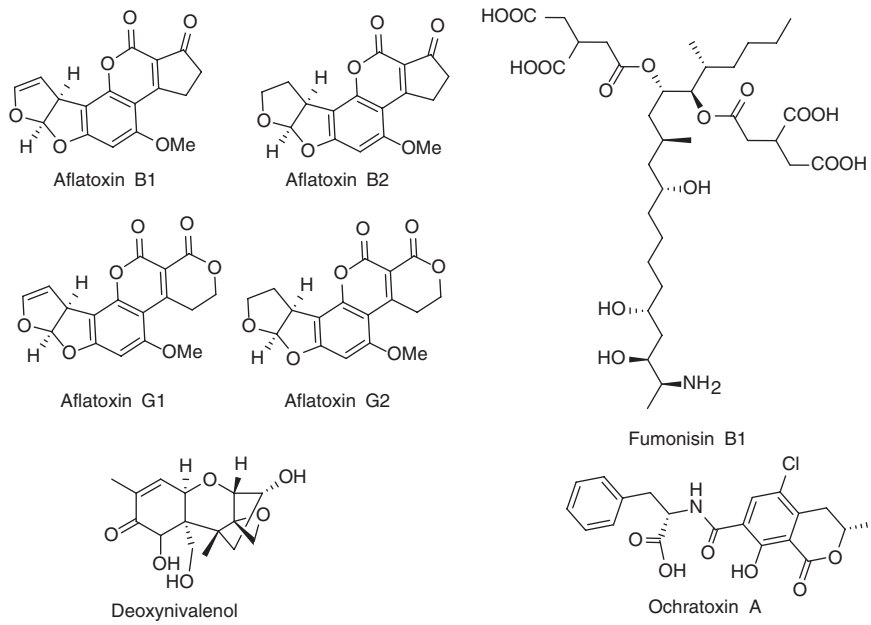

Fig. 1 The chemical structures of aflatoxins B1, B2, G1, G2, deoxynivalenol, fumonisin B1 and ochratoxin A

strategies have been developed. For the qualitative and quantitative analysis of mycotoxins in food traditionally high performance liquid chromatography (HPLC), gas chromatography (GC), and thin-layer chromatography (TLC) are used ([4–6]; and references therein). Over the last decade, HPLC hyphenated to tandem mass spectrometry (LC-MS/MS) have become the method of choice owing to the development of efficient electrospray (ESI) and atmospheric pressure chemical ionization (APCI) interfaces [7–10]. However, an entire sample preparation and especially extract cleanup is still required using, e.g., solid phase extraction (SPE) based on conventional reversed-phase materials, mixed-mode cartridges (MycoSep®), molecularly imprinted polymers (MIP), and immunoaffinity columns (IAC) [11, 12]. Even so, reliable quantification can often be achieved only by matrix-matched calibration or use of isotope-labeled internal standards [5, 11, 13].

To provide rapid and sensitive detection based on cost-effective and easy-to-use methods, which can be performed in a non-laboratory environment by non-specialists, a multitude of the so-called screening methods were developed. Generally, they do not require sample preparation other than extraction and dilution; however, they are less precise and sensitive than chromatographic methods. This field is dominated by immunochemical tests that use anti-mycotoxin antibodies in a variety of different assay formats like, e.g., enzyme-linked immunosorbent assay (ELISA), fluorescence polarisation immunoassay (FPIA), and kinetic FPIA, also

called stopped-flow FPIA (*aokinmycontrol*® kits, Aokin AG, Berlin, Germany) [4, 6, 14]. Notably, the need to provide even more simple, fast, robust, user-friendly, and cost-effective methods has led to a multitude of noninstrumental membrane-based lateral flow devices and gel-based flow-through tests with steadily improved detection capability and use of different labels ([15–23]; and references therein). Normally, these tests provide a "yes/no" result for a certain limit or range set previously; however, during the last years there is a tendency to develop modified devices enabling quantitative evaluation and multiplexing.

Because multi-mycotoxin methods are highly desirable in order to keep analysis time and costs low, the biosensor development increasingly focuses on parallel analysis of several mycotoxins, e.g., the MultiAnalyte Profiling (xMAP) technology from Luminex Corp. (Austin, TX) which combines a unique color-coded microsphere suspension array with a dedicated flow cytometer [24–27]. Here, we demonstrate an indirect competitive immunoassay on regenerable, reusable glass microchips for the parallel determination of four mycotoxins, i.e., aflatoxins, ochratoxin A, deoxynivalenol, and fumonisin B1 in cereal extracts on a fully automated flow-through device (MCR 3) with chemiluminescence readout (Fig. 1) [28].

2 Materials

1. Prepare all solutions using ultrapure water (prepared by reverse osmosis with UV treatment to attain a resistivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (RT) unless indicated otherwise. Diligently follow all waste disposal regulations when disposing waste materials. Mycotoxins are highly toxic. Especially, the aflatoxins and ochratoxin A should be handled with extreme care. Related contaminated material should be decontaminated with an aqueous solution of sodium hypochlorite (10 %).

2.1 Microarray Fabrication Components, Primary Anti-Mycotoxin Antibodies, and Mycotoxin Standard Solutions

1. Stand alone platform "Munich Chip Reader 3" (MCR 3) (GWK Präzisionstechnik GmbH, München, Germany).
2. Ochratoxin A (OTA) (Sigma-Aldrich, Steinheim, Germany). To prepare stock solution (100 µg/mL), add ~5 mL of acetonitrile to a 10-mL conical flask. Weigh 1.00 mg of OTA and transfer to the flask. Mix and make up to 10 mL with acetonitrile. Prepare 1:100 dilution (v/v) with methanol by the addition of 2 mL of methanol to a 5-mL conical flask followed by 50 µL of OTA stock solution. Mix, make up to 5 mL with methanol, and store at 4 °C.

3. Aflatoxins B1 and B2 (AFB1 and AFB2) (Sigma-Aldrich) (*see* **Note 1**). Prepare stock solution in acetonitrile (100 µg/mL) by a validated method [28]. Add ~5 mL of acetonitrile to a 10-mL CERTAN® capillary bottle (Sigma-Aldrich) (*see* **Note 2**). Weigh about ~1.00 mg of aflatoxin and transfer to the bottle. Mix and make up to 10 mL with acetonitrile. Prepare 1:10 dilution with acetonitrile (v/v) using the same type of capillary bottle. To determine the concentration of aflatoxin, prepare further dilutions in the range of 1:5 to 1:15 (v/v) with acetonitrile and measure absorbance of aliquots (threefold determination) at the absorbance maximum between 355 and 360 nm in UV-transparent plastic cuvettes. The optimal absorbance should be 0.6 ± 0.2. Calculate the AFB1 and AFB2 concentrations using the validated molar extinction coefficients (ε) in acetonitrile of 20700 and 22500, respectively [29]. Store stock solution and dilutions at 4 °C.
4. Deoxynivalenol (DON) (Sigma-Aldrich). To prepare stock solution (1 mg/mL) add ~5 mL of acetonitrile to a 10-mL conical flask. Weigh 10.00 mg of DON and transfer to the flask. Mix and make up to 10 mL with acetonitrile. Prepare 1:10 dilution (v/v) with methanol by the addition of 2 mL of methanol to a 5-mL conical flask followed by 500 µL of DON stock solution. Mix, make up to 5 mL with methanol, and store at 4 °C.
5. HS-DON (3,15-*O*-dihemisuccinyl-DON) is prepared by the reaction of DON with succinic anhydride. 10 mg of DON is dissolved with 200 mg of succinic anhydride in 1 mL of pyridine and heated at 100 °C in a steam bath for 8 h. Pyridine is removed under N_2 pressure and the residue dissolved in 15 mL of methylene chloride. After extraction three times with 10 mL of 0.1 M HCl the organic phase is dried under vacuum in a rotary evaporator and the residue redissolved in 2 mL of methanol (*see* **Note 3**).
6. Fumonisin B1 (FB1) (Sigma-Aldrich). To prepare stock solution (150 µg/mL), add ~3 mL of methanol to a 10-mL conical flask. Weigh 1.50 mg of FB1 and transfer to the flask. Mix, make up to 10 mL with methanol, and store at 4 °C.
7. Mouse monoclonal anti-ochratoxin (OTA) antibody 5G9 (concentration of 1.0 mg/mL); (Soft Flow Biotechnology, Gödöllö, Hungary).
8. Mouse monoclonal anti-Aflatoxin antibody 1F2 (concentration 1.0 mg/mL).
9. Mouse monoclonal anti-Deoxynivalenol antibody 1H8 (concentration 0.72 mg/mL).
10. Mouse monoclonal anti-Fumonisin B1 antibody 1H4 (concentration 0.37 mg/mL).

11. Double-sided adhesive foil ARcare 90106 (Adhesive Research Ireland Ltd., Limerick, Ireland).
12. Carbonate buffer: Add about 80 mL water to a 100-mL conical flask. Weigh 0.159 g sodium carbonate and 0.29 g sodium bicarbonate and transfer to the flask. Mix and adjust pH to 8.5 with HCl. Make up to 100 mL with water. Store at 4 °C.
13. Spotting buffer: Transfer 300 µL dimethylsulfoxide (DMSO), 690 µL carbonate buffer, and 10 µL glycerol to a 1-mL conical flask. Mix and store at 4 °C.
14. BioOdyssey Calligrapher Miniarrayer (Bio-Rad Laboratories GmbH, München, Germany).
15. Solid printhead for microcontact spotting (Stealth printhead SNS12; Arrayit Corporation, Sunnyvale, USA).
16. T25 Ultra-Turrax® dispenser (IKA Labortechnik, Staufen, Germany).

2.2 Automated Chemiluminescence Immunoassay Components

1. Phosphate buffer saline (PBS, pH 7.6): Add about 100 mL water to a 1-L conical flask. Weigh 1.36 g potassium dihydrogen phosphate, 12.2 g dipotassium hydrogen phosphate, and 8.5 g sodium chloride and transfer to the flask. Make up to 1 L with water. Store at 4 °C.
2. Running buffer: PBS containing 0.5 % (w/v) casein. Add about 90 mL PBS to a 100-mL conical flask. Weigh 0.5 mg casein and transfer to the flask. Make up to 100 mL with water (*see* **Note 4**). Store at 4 °C.
3. Regeneration buffer: Add about 1.5 L water to a 2-L conical flask. Weigh 15.01 g glycine, 11.7 g sodium chloride, and 2 g sodium dodecyl sulphate (SDS) and transfer to the flask. Mix and adjust pH to 3.0 with HCl. Make up to 2 L with water. Store at 4 °C.
4. Wash buffer: Add 20 mL methanol to a 100-mL conical flask and make up to 100 mL with PBS. Prepare fresh and use on the same day.
5. Primary antibodies mixture (Ab-mix): Prepare mixture in running buffer. Add aliquots of primary antibody stock solutions to 30 mL of running buffer to obtain dilutions of 1:15,000 of anti-aflatoxin antibody, 1:5000 of anti-OTA antibody, 1:4000 of anti-FB1 antibody, and 1:3000 of anti-DON antibody (*see* **Note 5**).
6. Peroxidase-labeled horse anti-mouse IgG: Prepare ~1:1000 dilution in running buffer of by the addition of 50 µL of antibody stock solution to 50 mL of buffer.
7. SuperSignal ELISA Femto Substrate (Thermo Fisher Scientific GmbH, Dreieich, Germany). The kit constitutes of reagent *A* (luminol solution) and reagent *B* (H_2O_2 solution). The reagents are used without further treatment.

3 Methods

Carry out all procedures at RT unless otherwise specified. Before analysis, return refrigerated samples to RT before analysis.

3.1 Fabrication of Functionalized Glass Slides

1. For cleaning, put the glass slides (conventional microscopy glass slides (26 × 72 × 1 mm) into a slide chamber filled with 200 mL water and 4 mL of Hellmanex®III solution (Hellmanex GmbH & Co. KG, Müllheim, Germany) and incubate for 1 h in an ultrasonic bath, followed by slight orbital shaking overnight and repeated ultrasonication for 1 h. Wash the slides five times with 200 mL water and make final wash with methanol. Dry the slides carefully with a stream of nitrogen and perform visual surface inspection.
2. To activate the glass surface (to obtain many hydroxyl groups) incubate the slides with 200 mL methanol/HCl (1:1, v/v) for 1 h under shaking, followed by washing five times with 200 mL water and incubation for another 1 h with concentrated sulphuric acid. Wash sequentially with water and methanol and dry the slides under nitrogen flow.
3. The pretreated glasses are silanized by the addition of 600 µL of 3-glycidyloxypropyltrimethoxysilane (GOPTS) on the chip surface and covered with a second glass slide (sandwich technique). After incubation at RT for 3 h separate the slides from each other in pure ethanol and clean by sonication subsequently with ethanol, methanol, and ethanol each for 15 min.
4. After drying under nitrogen flow and heating at 98–100 °C in a drying oven for 5 min, cover the silanized glass slide with 1 mL molten diamino-poly(ethylene glycol) (DAPEG) (*see* **Note 6**) and incubate at 98–100 °C for 15 h by using the sandwich technique again. Finally, separate the slides and sonicate intensively with water for two times each 15 min and dry under nitrogen flow (*see* **Note 7**). Store the slides in a desiccator under vacuum, protected from light, until further use.

3.2 Microarray Construction

1. Apply the DAPEG-functionalized glass slides directly to the spotting process, using a Miniarrayer (Fig. 2) (*see* **Notes 8** and **9**). Dissolve 2.5 µL of an aqueous solution (10 mg/mL) of aflatoxin B2-carboxymethyl oxime (AFB2-CMO) in 45 µL of spotting buffer (*see* **Notes 10** and **11**). Dissolve 5 µL of OTA (5.5 mg/mL stock in acetonitrile) and 5 µL of FB1 (10 mg/mL stock in methanol), in 45 µL spotting buffer. Dissolve 20 µL of HS-DON (10 mg/mL stock in methanol) in 30 µL of spotting buffer. Use spotting buffer as a negative control.
2. To introduce an activated carboxyl group into AFB2-CMO, OTA, and HS-DON, 10 Eq of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 10 Eq

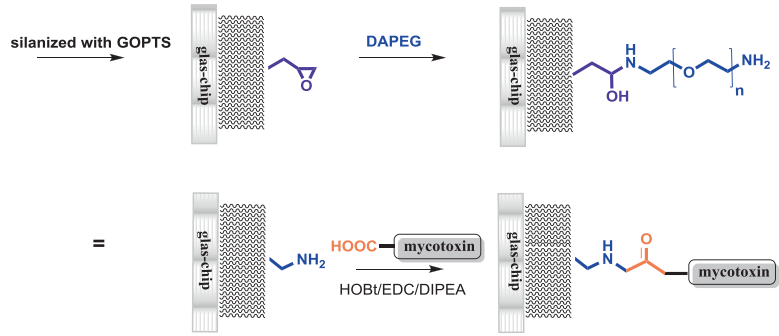

Fig. 2 Immobilization of mycotoxins to the modified glass-chip: After silanization with GOPTS, DAPEG is covalently bond to obtain free terminal amino groups. This is followed by coupling of the mycotoxins via available carboxyl functions

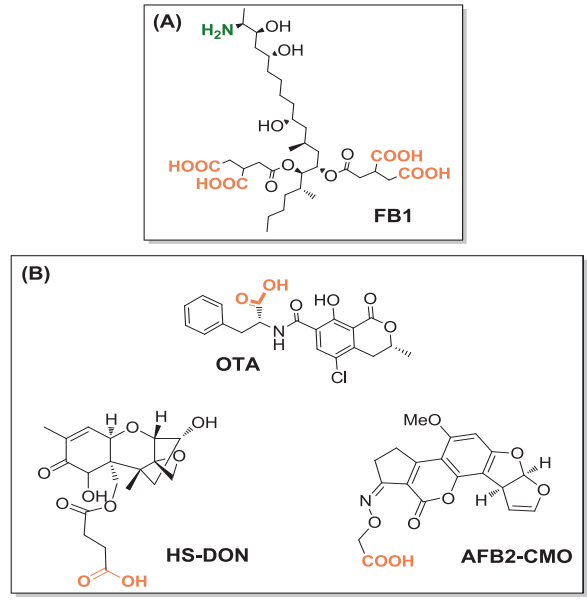

Fig. 3 Structures of immobilized mycotoxins: (**a**) FB1—direct binding via carboxyl groups; (**b**) OTA and derivatized toxins DON and AFB2—binding via activated carboxyl functions

1-hydroxybenzotriazole hydrate (HOBt), and 50 µL *N*,*N*-diisopropylethylamine (DIPEA) are added to the spotting buffer containing the individual mycotoxin derivative and incubated for 3 h under shaking at RT (Fig. 3) (*see* **Note 12**).

3. Transfer 25 µL of each of the spotting solutions (use plain spotting buffer as the negative control) to different cavities of a 384-well low binding microtiter plate and spot aliquots on the DAPEG glass slide in six replicates. During spotting temper the glass slide at 20 °C and set spotting chamber humidity

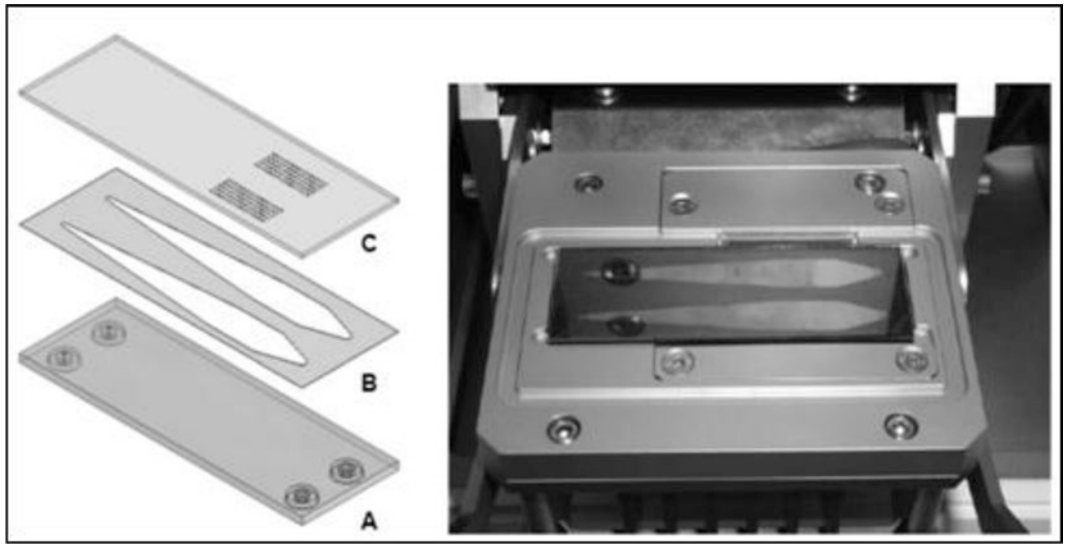

Fig. 4 *Left*: The flow cell construct forming two diamond-shaped chambers, consisting of a black poly(methyl methacrylate) (PMMA) opaque slide that contains two openings at opposite ends for the inlet and the outlet of the flow cell (*A*), double-sided adhesive sheet (*B*), and spotted glass slide (*C*). *Right*: MCR 3 flow cell holder

to 50%. Incubate the slides for 15 h at RT, then wash with PBS, sonicate with water for 15 min, and dry under nitrogen flow (*see* **Note 13**).

4. Glue the slides to a double-sided adhesive sheet where the shape of the flow cell had been previously carved out by laser-cutting. Finish the assembled chip and flow cell by placing a double-sided adhesive sheet in between the spotted glass chip (on top) and a black poly(methyl methacrylate) (PMMA) opaque slide (at the bottom) which contains two openings at opposite ends for the inlet and the outlet of the flow cell (Fig. 4).

3.3 Sample Preparation

1. For extraction, in a beaker mix 25 g of oat sample (*see* **Note 14**) with 5 g of sodium chloride and 100 mL of methanol/water (80:20, v/v) with a dispenser for 5 min while cooling on ice (*see* **Note 15**).
2. Filter the extract through a folded filter (210 mm), and dilute fourfold with PBS (add 20 mL of extract to 60 mL PBS in a 100-mL beaker) to give a final methanol content of 20% (*see* **Note 16**). The diluted samples should be analyzed within 24 h (*see* **Note 17**).

3.4 Performance of the Automated Chemiluminescence Immunoassay (CLISA)

1. Before starting the measurements, pump 3×1 mL running buffer with 200 µL/s through the flow cell (Fig. 5) (*see* **Note 18**).
2. The sequence of the performed immunoassay is programmed as follows:

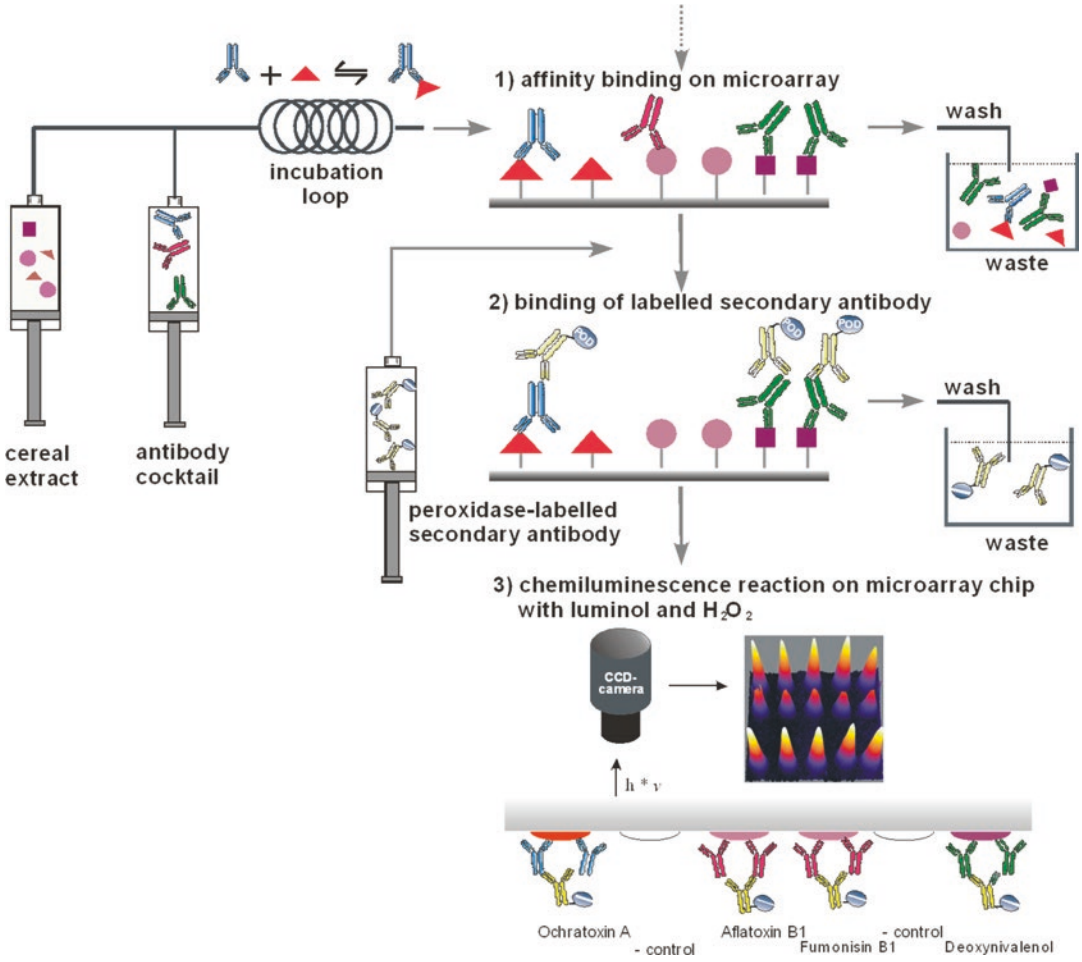

Fig. 5 Principle of the indirect competitive chemiluminescence ELISA using the microarray chip. The mycotoxins are immobilized in a defined grid pattern. After primary and secondary antibodies bound to the chip surface, chemiluminescence reagents (luminol/H_2O_2) are added and the light signal is detected with a CCD camera. The light intensity is inversely proportional to the concentration of mycotoxins in the sample

(a) Submit 1 mL of running buffer to the chip with a flow speed of 500 µL/s.

(b) Pump 500 µL of oat sample and same volume of Ab-mix simultaneously into an incubation loop at a flow rate of 60 µL/s.

(c) Push the sample/antibodies mixture over the chip with 1 mL of running buffer at a flow rate of 100 µL/s.

(d) Wash with 2 mL of running buffer at a flow rate of 500 µL/s.

(e) Submit 1 mL of diluted peroxidase-labeled horse anti-mouse IgG (secondary antibody) to the chip in two steps: 200 µL at a flow rate of 100 µL/s and followed by 800 µL at a flow rate of 10 µL/s (*see* **Note 19**).

(f) Wash with 2 mL of running buffer at a flow rate of 500 µL/s.

(g) Submit 200 µL of chemiluminescence substrate solution at a flow rate of 150 µL/s to the chip (see Note 20).

(h) Stop flow and take image for 60 s by the installed CCD camera.

(i) Regenerate the chip exhaustively using a total volume of 22.5 mL of regeneration buffer at a flow rate of 100 µL/s (see Note 21).

3.5 Data Evaluation

1. The text files generated by the CCD camera are evaluated with the Spot Image Processor (*see* **Note 22**). Electronic artifacts of the CCD camera are automatically eliminated and all spots are identified regarding signal intensity and consistency with the spotted grid. Those spots that do not fit one of these criteria are identified as outliers, and the integrated area is moved to the identified grid. The software integrates the pixel intensities within a square of a given side length around each spot center. A 9×9 pixel square around each spot center, which is up to an area of 0.13 mm^2, is integrated, and the average value over all pixels in this area is the spot intensity.

4 Notes

1. The standard solutions for AFB1 and AFB2 are prepared same.
2. Use this or comparable type of bottle to avoid evaporation of solvent.
3. The HS-DON was prepared as described in the literature [30].
4. To improve solubility, heat the running buffer up to 80 °C, filtrate and cool down the filtered solution to RT.
5. In principle, anti-mycotoxin antibodies from other sources (suppliers) can be used providing that the specificity and required stability in appropriately diluted organic solvents acetonitrile and methanol is given. The individual dilutions in running buffer have to be identified.
6. Melt DAPEG in a drying oven at 98–100 °C.
7. The slides can be stored for further use under vacuum but should be used within 1 month.
8. In principal, any spotting machine equipped with heating and humidity adjustment is applicable.
9. The delivered volume is ~5 nL.
10. Use 0.5-mL polypropylene Eppendorf Safe-Lock Tubes, no. 0030121.023, Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany.

11. The synthesis of AFB2-carboxymethyl oxime (AFB2-CMO) should be performed as follows. Add 30 mg of *O*-(carboxymethyl)hydroxylamine hemihydrochloride (Sigma-Aldrich) and 10 mg of AFB2 dissolved in 12 mL of methanol/water/pyridine (4:1:1, v/v/v) to a 50-mL round flask and reflux for 3 h at 85 °C. During reaction, the colourless solution shows yellow fluorescence. After reaction, remove the solvent with a rotary evaporator and redissolve the residue with 5 mL of dichloromethane. Wash with 15 mL of 5% acetic acid that is accompanied by dark-green coloring of the organic phase. After separation of the two phases with a separatory funnel remove the organic solvent with rotary evaporation. Redissolve the black residue with 4 mL acetonitrile (0.1% trifluoroacetic acid)/water (0.1% trifluoroacetic acid) (2:1, v/v) and centrifuge for 5 min at 1789 rcf (Hettich Universal Centrifuge 30F, Andreas Hettich GmbH, Tuttlingen, Germany). Dispense the supernatant into two identical aliquots and purify with preparative HPLC using acetonitrile/water as mobile phase and C18 column (*00F-4435-N0* Gemini® 5 µm C18 110 Å, LC Column 150×10 mm, Phenomenex, Aschaffenburg, Germany). Start with 10% acetonitrile and increase to 90% within 10 min and keep for further 10 min. Use UV/vis detection at wavelength of 360 nm. Collect fraction between 10 and 14 min and remove solvent with rotary evaporator.
12. Add 7.00 mg HOBt, 8.00 mg EDC and 50 µL DIPEA to 1.00 mL of spotting buffer. After short sonication (15 s) add the specified volumes of mycotoxin solutions. The FB1 contains several carboxyl groups and primary amino group. To avoid intermolecular cross-linking of this mycotoxin, it was immobilized on the chip surface without activation.
13. The slides can be stored for further use under vacuum but should be used within 1 month.
14. Oat, flour, and oat flakes are applicable.
15. Other commercial dispersers are applicable; avoid solvent loss.
16. This corresponds to a 16-fold dilution of the mycotoxin concentration in the original sample.
17. Store samples at 4 °C if not analyzed immediately. Before analysis, return samples to RT.
18. This step is important to displace possibly existing air bubbles.
19. Horseradish peroxidase-labeled anti-mouse IgG antibodies from other suppliers and species (e.g., goat) can be used. The individual dilutions in running buffer have to be identified.
20. Alternately 50-µL aliquots of reagents *A* and *B* are pumped into coil and total volume of 400 µL is pushed over the chip.

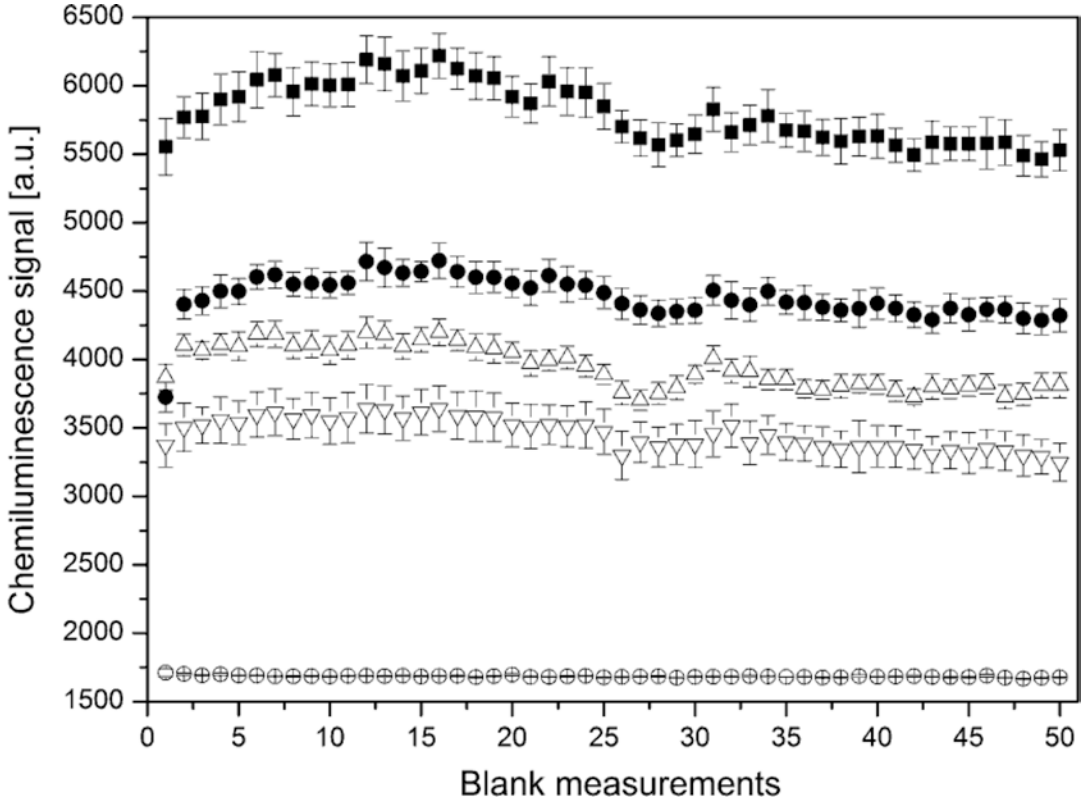

Fig. 6 The performance of 50 regeneration cycles using oat extract as a sample (blank): (*filled square*) aflatoxin B2, (*filled circle*) ochratoxin A, (*triangle*) DON-HS, (*inverted triangle*) fumonisin B1, (*open circle*) negative control. The between-spot standard deviations (1s; $n=6$) are indicated as error bars

21. On the same day, the chip can be used for at least 50 cycles of sample loading and regeneration (Fig. 6).
22. The text files generated by the CCD camera are evaluated with the Spot Image Processor SIP 0.4 (Karsunke Softwarebüro, Wolnzach, Germany). Appropriate microarray image analysis software from other sources could be applicable.

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