Microfluorimeter with disposable polymer chip for detection of coeliac disease toxic gliadin†

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Coeliac disease is an inflammatory disease of the upper small intestine and results from gluten ingestion in genetically susceptible individuals, and is the only life-long nutrient-induced enteropathy. The only treatment is a strict gluten-free diet and the longer the individual fails to adhere to this diet, the greater the chance of developing malnutrition and other complications. The existence of reliable gluten free food is crucial to the well-being of the population. Here we report on a microfluorimeter device for the *in situ* detection of gliadin in foodstuffs, which could be used for a rapid control of raw materials in food processing, as well as for process control of gliadin contamination. The microfluorimeter is based on a reflector that is used inside a microfluidic chip, exploiting various strategically placed reflective or totally metallised mirrors for efficient collection of the fluorescent light emitted in a large solid angle. The chip is capable of executing five assays in parallel and has been demonstrated to possess detection sensitivity applicable to fluoroimmunoassays. Various immunoassay formats exploiting fluorescence detection, using enzyme/fluorophore labels were developed and compared in terms of sensitivity, ease of assay, assay time and compatibility with buffer used to extract gliadin from raw and cooked foodstuffs, with the best performance observed with an indirect competition assay using a fluorophore-labelled anti-mouse antibody. This assay was exploited within the microfluorimeter device, and a very low detection limit of 4.1 ng/mL was obtained. The system was observed to be highly reproducible, with an RSD of 5.9%, for a concentration of 50 ng/mL of gliadin applied to each of the five channels of the microfluorimeter. Biofunctionalised disposable strips incorporated into the microfluorimeter were subjected to accelerated Arrhenius thermal stability studies and it was demonstrated that strips pre-coated with gliadin could be stored for $\approx$ 2 years at 4 °C, with no discernable loss in sensitivity or detectability of the assay. Finally, the microfluorimeter was applied to the analysis of commercial gluten-free food samples, and an excellent correlation with routine ELISA measurements was obtained. The developed microfluorimeter should find widespread application for on-site execution of fluoroimmunoassays.

Introduction

Coeliac disease is an inflammatory disease of the upper small intestine and results from gluten ingestion in genetically susceptible individuals, and is the only known life-long nutrient-induced enteropathy. The magnitude of the prevalence of coeliac disease has only recently been recognised, and a widespread screening study has demonstrated the prevalence to be as high as 1%.[1] Coeliac disease is not an allergy; it is an auto-immune disease, which means that the body produces antibodies that attack its own tissues. For people with coeliac disease this attack is triggered by gluten, a protein found in wheat, rye and barley.[2] Gluten consists of gliadins (the alcohol-soluble fraction) and glutenins (solubilised in the presence of reducing agents).

The only treatment for coeliac disease is a strict gluten-free diet, and the longer the individual fails to adhere to this diet, the greater the chance of developing malnutrition and other complications. Thus, the existence of reliable gluten-free food is crucial to the well-being of the population. The official limits to consider foods as "gluten-free" are described in the Codex Alimentarius[3] and new standards for gluten-free foods have recently been approved (July 2008).[3] The new benchmark states that foods labelled "gluten-free" may not contain wheat, rye, oats or barley and the gluten level may not exceed 20 ppm (parts per million). Foods that have been processed to reduce gluten content to a level between 20–100 ppm cannot be labelled gluten-free but may be called "low gluten" or "reduced gluten".

The most common technique for measuring gluten in foodstuffs is an enzyme-linked immunosorbent assay (ELISA) and commercial kits available are generally based on sandwich assays.[4–9] There have also been reports of alternative techniques for the detection of gluten using methods such as real time PCR,[10–13] immuno-PCR,[14,15] fluorescence[16,17] or mass

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spectroscopy.[18,19] However, all the techniques developed to date are necessarily laboratory based, and apart from qualitative immunochromatographic strips, there is no possibility to measure the gliadin content of foodstuffs on-site, and this is of tremendous importance to food industries who suffer from unnecessary lag times when testing raw materials, or checking for contamination of food products during the production process.

Here we report on a microfluorimeter, capable of simultaneous multiple sample analysis, which we apply to the highly sensitive, cost-effective and rapid detection of gliadin using an indirect one-step competition fluoroimmunoassay. The measurement principle of the device relies on detecting the emission of a fluorophore-labelled antibody upon being excited with light. The emitted fluorescence photons are distributed statistically and the main technical hurdle for the detection of a weak fluorescence signal, *e.g.* due to low analyte concentration, is the efficient collection of the emitted photons by the detector, which effectively defines the detection limit of the system. Previous reports have detailed the use of different optical elements such as lenses, gratings or prisms to increase the efficiency of collecting the emitted photons.[20–23] Additionally, there are some reports of microsystems or microchips that exploit fluorescence detection. In one configuration, highly sensitive detection of laser induced fluorescence (LIF) for chip-capillary electrophoresis was achieved by detecting fluorescence emitted in a microchannel through the sidewall of the chip, thus reducing the effect of scattered light interference from the laser source.[24] Using a collection angle of 45°, a detection limit of 1.1 pM of fluorescein was achieved. Detection limits of 1.7 pM rhodamine 6G and 8.5 pM rhodamine B have been reported for chip-based capillary electrophoresis with a confocal LIF detector,[25] and confocal epifluorescence microscope systems for microchip LIF detection have achieved limits of detection of just 300 fM and 9 pM for fluorescein[26] and Cy5,[27] respectively. Whitesides' group reported the use of a microavalanche photodiode detector integrated into a microfluidic device, orthogonally positioned relative to an excitation fibre optic, which enabled fluorescence detection due to the close proximity between the detector and the sample.[28] Krogmeier *et al.* claim the first report of an integrated optical microfluidic device that does not depend on an independent objective lens;[29] the fluorescence based device uses a small illuminator lens to deliver excitation light and a collector, which reflects the fluorescence over a large collection angle (representing 71% of a hemisphere) toward a single photon counting module for the single molecule detection of λ-phage DNA molecules. In a further report, Kaigala *et al.*, reported on an inexpensive, portable microchip, which integrates RT-PCR and capillary electrophoresis, using a CCD camera positioned above the microchip for the detection of DNA fragments.[30]

Within the microfluorimeter we report here, a reflector is used in a microfluidic chip, exploiting various strategically placed mirrors for efficient collection of the fluorescent light emitted in a 360° solid angle.[31] The concept of the chip-based multiparameter analysis system includes a novel integrated reflector arrangement for the detection of fluorescence (Fig. 1a) using 45°-tilted mirrors.[32] The mirrors can be realised as total reflection or metallised optical surfaces, which allow for collection of up to 85% of the emitted photons to a solid angle of about 60°, this higher yield of emitted light reaching the detector facilitates lower detection limits. For the fluorescence excitation, the beam is guided through the detection cell directly to the detector using 45°-tilted mirrors located in the direction of the beam (Fig. 1b), which serve as reflectors in combination with a polymer covered foil. Fluorescence detection is achieved by a mirror arrangement oriented in parallel to the detection cell which collects the emitted light and concentrates it onto the detector (Fig. 1c). The microfluidic analytical chip integrated with the fluorescence detection unit has five separate channels for fluorescence measurement (Fig. 2a and b), into which streptavidin functionalised polymer strips were inserted and used as a support for immobilisation of biotinylated biocomponents. To demonstrate the functionality of the microfluorimeter and its applicability to *in situ* analysis, the

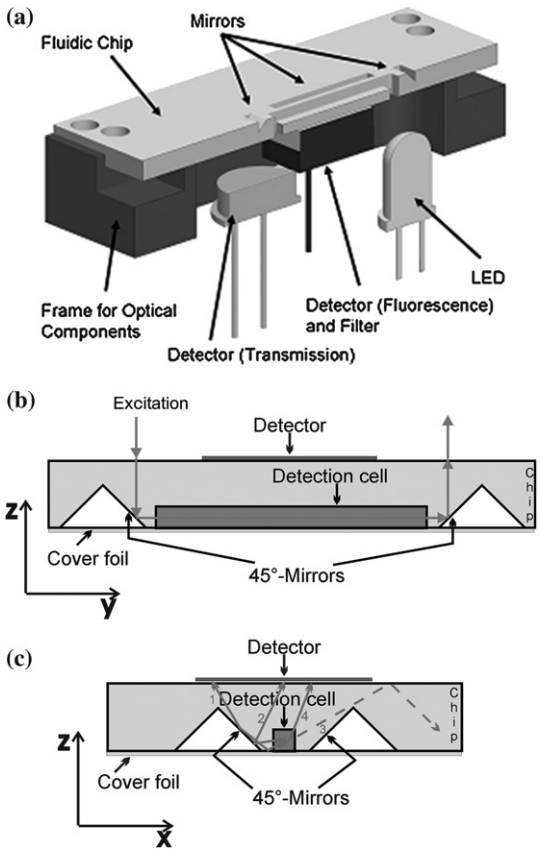

Fig. 1 (a) Schematic of a novel integrated reflector system based on 45°-tilted mirrors used for optical detection based on fluorescence. (b) The grey arrows indicate the optical path for excitation. Excitation light coming from a LED enters the chip from the bottom side, passes through the detection cell parallel to the flow direction and exits from the chip after reflection on the 45° mirror. Polymer strips with immobilised antibodies are inserted at the bottom of the detection channel. (c) Design of the fluorescence read-out from the chip. The grey arrows 1–4 indicate the optical path for the measurement of fluorescence, and a large Si detector for the read-out of the florescence is placed on the bottom side of the chip. Fluorescence photons transmitted in the direction of the detector arrive directly (beam 4). The majority of the rest of the photons arrive indirectly by reflection at the 45° mirrors on the sidewalls of the channel and at the cover foil (beams 1, 2). Some of the fluorescence photons are handicapped by the total reflection and cannot leave the plastic chip due to the variation of refraction indices of both media (beam 3). The flow direction of the liquid is perpendicular to the drawing plane.

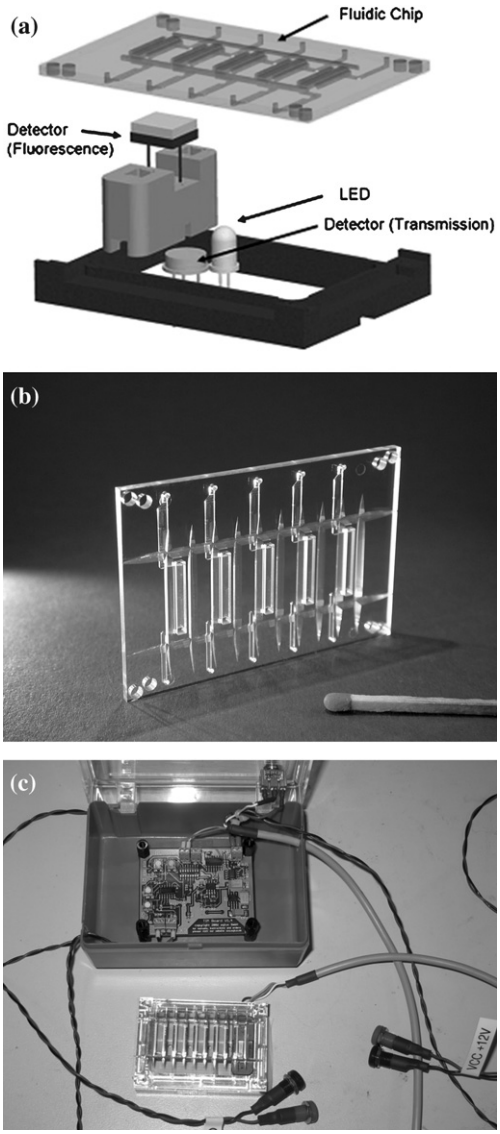

Fig. 2 (a) Schematic of the microfluorimeter. The detection chip is mounted between two metal frames. The chip is adjusted by positioning pins. The optical components (LED, filter, fluorescence and transmission detector) are mounted in a holder. (b) Picture of the optical detection chip. 5 vertical fluidic channels allow 5 different measurements within 1 chip. The 2 horizontal channels are the mirrors for transmission measurements and for the in-coupling of light for fluorescence excitation. Vertical channels between the fluidic channels show the mirrors that reflect emitted fluorescence photons towards the detector. (c) Picture of actual experimental set-up used for measurement of gliadin in foodstuffs.

device was used for the detection of coeliac disease toxic gliadin in foodstuffs, which could eventually be used for a rapid screening of raw materials and for process control.

Experimental

Materials & methods

Reagents. Monoclonal anti-gliadin antibodies were kindly supplied by The Rayne Institute at St. Thomas' Hospital (King's College London). Anti-rabbit IgG peroxidase conjugate (anti-Rb IgG-HRP), anti-mouse IgG alkaline phosphatase conjugate (anti-IgG-ALP), anti-mouse IgG fluorescein conjugate (anti-IgG-FITC), streptavidin, tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 4-methylumbelliferyl phosphate (MUP), phosphate buffered saline with Tween 20, 3-(4-hydroxyphenyl)-propionic acid (HPPA) and 3,3′,5,5′-tetramethyl-benzidine (TMB) liquid substrate system for colour development (containing TMB and H_2O_2 in an slightly acidic buffer) were obtained from Sigma (Barcelona, Spain). Biotin hydrazide was supplied from Pierce, and the biotinylated-target conjugate were desalted using G-25 columns (Amersham-Pharmacia, Barcelona, Spain) and concentrated using Microcons (Millipore, Madrid, Spain). N-Hydroxysuccinimide (NHS), N-morpholinoethane sulfonic acid (MES) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) were purchased from Acros Organics (Geel, Belgium). Sodium di-hydrogen phosphate (NaH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) were purchased from Panreac Química (Barcelona, Spain). Sodium chloride (NaCl) was obtained from Scharlau (Barcelona, Spain). Deionised water was produced with a Milli-Q RG system (Millipore Ibérica, Madrid, Spain). 4-Methylumbelliferone was from Sigma. 5,6-Carboxy-fluorescein (5,6-FAM) and 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS) were obtained from Fluka. Magnesium dichloride ($MgCl_2$) was purchased from Roth.

The chip material, cyclic olefin copolymer (COC) was purchased from Topas Advanced Polymers, Höchst Germany and unstructured, blank chips were fabricated by injection moulding from Roth, Langen Germany.

Buffer preparation. Phosphate buffered saline (10 mM PBS, pH 7.4) was prepared from the phosphate salts, NaH_2PO_4 and Na_2HPO_4, to a concentration of 10 mM and then NaCl was dissolved up to a concentration of 50 mM. PBS-Tween (0.01 M, pH 7.4) was prepared by dissolving the contents of one phosphate buffered saline with a Tween 20 pouch in 1 L of deionised water, and measuring the pH. An ammonia buffer solution (pH 11) was prepared by mixing 0.6 g NH_4Cl and 41.4 ml 15 mol/L ammonia and diluting it to 100 mL. All reagents used were of analytical reagent grade.

Equipment. All the fluorescent assays were performed with a fluorescence spectrophotometer, Cary Eclipse from Varian with Cary temperature controller.

Design and fabrication of the optical detection chip. The microfluidic analytical chip, which includes 5 separate channels for fluorescence measurement, is shown in Fig. 2b. The fluid channels have a cross section of 1×1 mm^2 and 1×2 mm^2 in the detection zone. The whole detection cell has a length of 16 mm. Chips were made of COC and open channels were sealed temporarily using adhesive tape. Rapid prototyped chips were used, manufactured by milling (Charlyrobot, Charly2U). Ultra-precise milling (Precitech Precision Nanoform 350), was also used for the fabrication of chip-integrated mirrors. The miniaturised read-out device used for fluorescence measurements consisted of an optical detection unit and an electronic controlling board implemented in a plastic box for the protection of the board (Fig. 2a and 2c).

For surface modification and introduction of functional groups for chemical crosslinking to streptavidin, polystyrene foil was attached to adhesive tape and cut into strips (2×15 mm^2) using a dicing saw (Disco DAD 2H6/T). Carboxylation of the strip surface was achieved using an O_2-plasma in a Tegal Plasmaline 515 oven for 10 min at 300 W with an O_2 flux of 2.6853 cubic meter/min.

Fabrication of the microchip device. The detection unit (Fig. 3) consists of a LED ($\lambda = 477$ nm) and amplified detector with a large active area (5.5×6.1 mm^2) PR33-2CER (Silicon sensor GmbH) placed in a milled plastic frame, that has the option for passive positioning of optoelectronic components to the multifunctional chip, with a positioning accuracy better than 50 μm. This allows a high positioning tolerance in accordance with a small beam diameter of about 0.5 mm and a 1.5×1.5 mm^2 effective area of the mirror. The variable amplification resulted from a Multifunctional 2 - Channel Amplifier Board (Sylux GmbH) with the adjustable gain in the range 10^5–10^7 V/A with a fine-tuning option. The BrightLine interference filter set form FF01-482/35-25 (edge wavelength = 482 nm) for LED and FF536-40-25 (edge wavelength = 536 nm) for detector, was used for the separation of excitation and emission photons. The typical spectral response was 0.3 A/W at 500 nm. The transimpedance amplifier board (Digiboard from Sylux) supplied an amplification of 10^6 V/A. The signals were read by a multimeter with a range between 1 and 4000 mV, and thus, the minimum spectral response of the complete system is 0.3×10^6 V/W. 10^{10} photons were counted, with the integration time of the measurement being 1 s. For guidance of the light no extra beam forming optical components such as lenses were used, and only planar surfaces acting as mirrors were integrated into the fluidic chip.

A housing for the set-up was fabricated in house. Care was taken to ensure that the optical beam for excitation hits the detection chip in a perpendicular fashion to minimise loss of intensity by reflection at the phase boundary between air and chip. An internal adjustment of the mirrors within the chip is not required since all components are fixed after their fabrication either by milling or ultraprecise milling. The chip and detection unit were mounted in a metal frame that was fabricated in house, and positioning pins allowed easy adjustment of the chip to the frame and the detection unit. Test measurements were performed in a dark environment, but a double-insulated dark box could be used for *in situ* analysis.

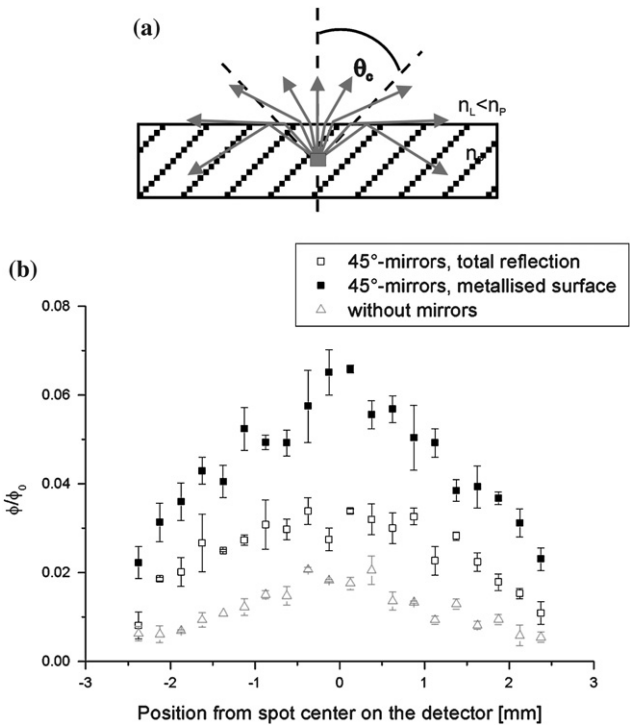

Fig. 3 (a) Schematic of the out-coupling of fluorescence emission from a polymer chip without additional techniques to compensate for the different refraction indices at the boundary of the chip. The green box (in the centre) represents the fluorescent detection cell and red arrows show possible optical paths of fluorescent light in the direction of the detector. (b) Simulated distributions of the optical power arriving at the detector read-out from the chip without mirrors compared to the design with total-reflective or metallised mirrors.

Ray tracing simulation. The read-out of the fluorescence signal from a plastic chip is usually handicapped by total reflection at the plastic–air boundary caused by differences in the refraction indices of the two media (Fig. 3a). A ray tracing simulation was carried out to model the optimum position of the tilted mirrors for maximal efficiency in collection of emitted photons, as well as to compare a non-reflective cover foil, a reflective cover foil, and a total reflecting mirror placed on top of the chip. OptiCAD software (OPTOcon GmbH) was used for the ray tracing simulation of the reflection and refraction of the excitation beam and the emitted light (Fig. 3a). OptiCAD is a non-sequential, geometrical ray tracing program. Only the fluorescence detection was simulated. The studied emitting zone on the chip was $1 \times 1 \times 3$ mm^3 and was presented as a cube emitting *ca.* 100 rays per surface statistically over the whole solid angle. The parameters employed for simulation are: refractive index of chip material (PMMA): 1.4838; medium filled in the micro channel: water ($n = 1.35$); emission beam assumed to be a bundle of geometrical rays with a wavelength of 520 nm. A detector area of 36 mm^2 was used at a distance of 0.5 mm underneath the chip. The simulation was performed comparing a non-reflective and a reflective cover foil or with a total reflecting mirror placed on top of the chip. As can be seen in Fig. 3b, due to the redirection of emitted rays using total reflecting or metallised mirrors, the angular distribution of the emitted rays can be changed and these rays concentrated within a smaller solid angle, and due to this, about 85% of emitted rays could be detected with the reflective cover foil and metallised mirrors and up to 60% with the non-reflective surfaces using total reflection.

Experimental protocols

Biotinylated-gliadin conjugate synthesis. 1 mg of gliadin was dissolved in 1 mL MES buffer (0.1 M, pH 5.0). 25 μL of 1.25 mM biotin hydrazide in DMSO and 12.5 μL of 5 mM ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) were added to the protein solution and mixed. The reaction was incubated overnight at room temperature under vigorous shaking conditions. Finally, precipitated protein, and non-reacted biotin hydrazide

and EDC by-products were removed by passing the mixture through a Sephadex G-25 desalting column. The biotinylated gliadin was then concentrated using a 30 kDa MWCO Microcon to a final concentration of 0.2 mg/mL.

Competition ELISA: colorimetric detection. The prepared biotinylated gliadin (500 ng/mL, 10 nM) was immobilised by incubation of 50 μL in each well of a streptavidin-coated microtitre plate for 1 h at 37 °C in PBS-Tween (0.01 M, pH 7.4). At the same time, 60 μL of a range of gliadin concentrations (from 18000 to 0 ng/mL), 60 μL of monoclonal anti-gliadin antibody diluted (1 : 3000) and 60 μL horse radish peroxidase-labelled rabbit anti-mouse IgG, (HRP-anti-IgG, 1 : 3000) were added to an Eppendorf tube and allowed to pre-incubate for the same hour at 37 °C. Following this first incubation, the plate was thoroughly washed with PBS-Tween buffer, and 50 μL of the pre-incubated solution was introduced into each well. After 10 min incubation at 25 °C, the plate was washed 3 times with PBS-Tween and 50 μL of tetramethylbenzidine (TMB) substrate was added to each well. Color development was monitored at 650 nm, stopped after 20 min using 0.1 M H_2SO_4, and finally measured at 450nm.

A similar protocol was followed for the FITC, HRP and ALP-labelled antibodies for fluorescent detection, except for the final step, where in the case of FITC ($\lambda_{ex} = 490$ nm; $\lambda_{em} = 520$ nm), no substrate addition is required; for the HRP, HPPA substrate was added, and for the ALP, MUP substrate was added, and the fluorescent products measured ($\lambda_{ex} = 324$ nm; $\lambda_{em} = 409$ nm and $\lambda_{ex} = 360$ nm; $\lambda_{em} = 450$ nm, respectively.

Gliadin detection using the microfluorimeter. Carboxyl derivatised polymer strips were located in each channel of the detection chip. The strips were washed in an ultrasonic bath containing phosphate buffered saline (PBS) 0.1 M at pH 7.4. Subsequently, the carboxylic groups were activated by addition of 50 μL EDC/NHS (100 mM/50 mM), and left to react for 30 min at room temperature followed by addition of 50 μL of 4.5 μM streptavidin solution in acetate buffer (0.1 M, pH 5.0) and the protein left to interact for 1 h. After thorough washing with PBS-Tween (0.01 M, pH 7.4), the gliadin was immobilised on the strips by adding 50 μL of 10 μg/mL of the prepared biotinylated gliadin into each channel and incubating for 1 h at 37 °C. At the same time, 60 μL of a range of concentrations of the gliadin from 0 to 18000 ng/mL were pre-incubated with 60 μL of 1 μM monoclonal anti-gliadin antibody and 60 μL 1 μM of FITC-labelled anti-IgG antibody, for 1 h at RT. 50 μL of the pre-incubated solutions were introduced to each channel, and incubated for 10 min at RT, after washing with PBS-Tween. 50 μL of HEPES buffer 10 mM (pH 8.4) was added to the channels. Finally, a monochromatic light was applied to the fluorescent solution using an external power source and the potential of each solution was measured with a micro-potentiostat. The entire process takes place at room temperature and in a dark room. The concentration of reagents used was optimised using checkerboard assays, both for the macroscale assay using microtitre plates, and for the microscale assay using the streptavidin functionalised strips.

Stability studies. A batch of 10 carboxyl derivatised polymer strips were biofunctionalised with streptavidin and biotinylated gliadin immobilised on the surface. Five of the biofunctionalised strips were then tested in a fluoroimmunoassay set-up, as previously described, whilst the other 5 were stored in a sealed bag, in the presence of desiccant, at 37 °C for the period of one month (30 days). After this period, the stored biofunctionalised polymer strips were then used for analysis using the described fluoroimmunoassay set-up, and the sensitivity and limit of detection were compared to the values obtained at day 0. Using these accelerated Arrhenius thermal stability studies, storage at 37 °C for 30 days is approximately equivalent to a period of 2 years of real-time storage at 4 °C, which is the typical storage time required for bioanalytical reagents used in ELISA, *e.g.* capture antibody/antigen pre-coated onto microtitre plates.

Gliadin detection from real samples using the microfluorimeter. Gluten from a commercial gluten-free sample, Harisín dietetic pasta (from SANAVI, S.A., Spain), as well as from a gluten-containing sample, "Tostagrill" toasted bread (from Diatosta España, S.L.), was extracted with an extraction buffer consisting of 60% v/v ethanol in the presence of TCEP in Tris-HCl (50 mM, pH 7.4). The sample was blended to homogeneity and 75 mg of this mixture added to 750 μL of the extraction buffer. The mixture was then vortexed before heating under stirring conditions at 60 °C for 10 min and finally centrifuging for 5 min at RT. The supernatant was recovered and diluted to fall within the dynamic linear range of the ELISA/FLISA.

Results and discussion

Design and fabrication of microfluorimeter

The vast majority of the applications targeted by microsystems necessitate the chips to be disposable, inherently requiring the use of inexpensive materials. Additionally, no extra components should be integrated and a complicated fabrication process should be avoided. The optical set-up reported here addresses these needs, without loss of sensitivity. Optical components such as the light source or the detector are not disposable, and the mirrors that guide the light within the chip system can be fabricated in the same injection moulding step as the chip production, requiring no additional effort, this reducing fabrication costs and complexity. Adaptation of the microchip to other applications is easily achievable as the length of the detection cell is variable. This offers the opportunity for the measurement of higher concentrations with a reduced path length or lower concentration using a longer detection channel. Additionally, an exchange of light sources and filter components is easily performed, allowing a high variability of the applicable wavelength.

The focus of the microfluorimeter presented herein was to integrate cost effective components that provide a sensitivity suitable for detection of gluten in foodstuffs. An approximation of costs for the optical components (including light source, detector, filter and electronics) is less than 350 €. If required, a higher sensitivity could be achievable by forming the beam using additional lenses. Costs for the microfluidic chip with integrated mirror surfaces and inserted strips with immobilised antibodies for the ELISA reaction are approximately 1 €/chip.

Fluorescence measurements

The optical path for fluorescence measurements requires one small mirror for excitation and a sensor with a very large surface area was chosen and placed directly underneath the fluidic chip to collect photons that are directly emitted to the bottom of the chip. Two mirrors parallel to the fluidic channel reflect light that is emitted to the side and guide it to the detector. Using this arrangement of mirrors most of the emitted light can be detected. In future modifications of the microfluorimeter, the yield could be even further improved by using a planar mirror on top of the chip. Ray tracing simulations obtained a detection of up to 85% of the photons including a reflecting cover or of up to 60% with a non-reflecting cover. A study of the optimal mirror geometries was not carried out, as using the refractive indices of air and poly(methylmethacrylate), (PMMA), a mirror angle of 45° is revealed to be the easiest geometry for fabrication, whilst being suitable for the total reflection into the detection chamber or from there to the detector. To avoid loss of photons by reflection at the boundary surface between the chip and air the exciting light enters the chip perpendicularly.

To test the sensitivity of the fluorescence set-up 5,6-carboxy-fluorescein (5,6-FAM) was used for test measurements of a dilution series between 0.011 μM and 26.6 μM. With a background signal of 106.76 ± 0.26 mV, the theoretical limit of detection of the microfluorimeter set-up was calculated as 0.78 mV. The measurements were highly reproducible, with an RSD% = 0.25 (n = 5) and a detection limit of 5.6 nM, demonstrating that the sensitivity of the microfluorimeter is suitable for the concentration levels anticipated in immunoassay measurements.

ELISA detection of gliadin

The one-step indirect competition system used as a model assay to test the microfluorimeter was previously developed and optimised at our laboratory,[33] and has been applied to the analysis of a large number of real samples, with a detection limit of 6.4 ng/mL gliadin, and is highly reproducible, with an RSD% of 4.2 (n = 3), for a concentration of 50 ng/mL gliadin.

In order to optimise conditions of the fluorescent immunoassay to be carried within the channels of the microfluorimeter, the assay was first carried out using a routine laboratory spectrofluorimeter. Alkaline phosphatase (ALP), horse radish peroxidase (HRP) and fluorescein isothiocyanate (FITC) antibody labels of the secondary anti-mouse IgG antibody were compared in terms of sensitivity and detection limit. Table 1 summarises the limit of detection (LOD), IC_{50} and hillslope obtained and as can be seen, the LOD obtained are 4.8, 0.7 and 0.1 ng/mL gliadin for the HRP, ALP and FITC labels, respectively. The reproducibility of each system was also evaluated with RSD% values of 4.3, 4.9 and 8.3 for the slope of the linear range, n = 3, being obtained for the HRP, ALP and FITC labels, respectively. The compatibility of each of the assays with extraction buffers typically used for extraction of gliadin from foodstuffs was gauged: the FITC label was compatible using a 1 in 3 dilution of the extract, whereas both of the enzyme labels required a 1 in 10 dilution factor to avoid denaturation by the components of the extraction buffer. Based on these results, and taking into consideration that no substrate is required for the FITC label, the optimum assay set-up for fluorescence detection was deemed to be that using the FITC-labelled anti-mouse IgG as the secondary labelled antibody, and was thus the assay evaluated using the microfluorimeter.

Microfluorimeter based fluoroimmunoassay

The indirect competition assay was carried out within the channels of the microfluorimeter, using streptavidin functionalised polymer strips that were easily inserted into each of the channels, as shown in Fig. 2. As described in the experimental section, biotinylated gliadin was immobilised on the inserted streptavidin polymer strips. Gliadin, anti-gliadin monoclonal antibody and the labelled anti-mouse IgG antibody were pre-incubated outside the microfluorimeter for 60 min, and the mixture then added to each channel for 10 min, prior to a rapid wash and detection, allowing the entire assay to be carried out in 75 min.

As can be seen in Fig. 4, the detection limit obtained was similar to that obtained using the standard laboratory spectrofluorimeter, with an LOD of 4.1 ng/mL being obtained. The reproducibility of the system was evaluated using 50 ng/mL of gliadin by carrying out the fluoroimmunoassay in each of the 5 channels of the microfluorimeter. An RSD of 5.9% was obtained, demonstrating the reproducibility of the system.

In a brief stability study, polymer strips with immobilised biotinylated gliadin were stored at 37 °C for one month and shown to retain 100% of biological activity, which using thermal accelerated Arrhenius stability studies extrapolates to greater than 2 years stability when stored at 4 °C, highlighting the possibility to use pre-coated polymer strips that could be inserted into the channels of the microfluorimeter at the time of use (Fig. 4 Inset).

Finally, the system was tested using real samples, which represented gluten-free raw materials, processed foods, and

Table 1 Comparison of assay performance for routine colorimetric immunoassay, macro-fluorescence immunoassay and microfluorimeter immunoassay

		Statistical parameters							
Methodology	Label	EC50 (ng/ml)	Hillslope	LOD (ng/ml)	Top	Bottom	Linear range (ng/ml)	RSD%	r^2
colorimetric	HRP	76.32 ± 1.1	−1.29 ± 0.10	6.48	2002	0.1832	8.6–2757.6	4.2	0.9959
spectrofluorimeter	HRP	52.65 ± 1.1	−1.39 ± 0.11	4.82	28.05	2.739	4.8–448.46	4.3	0.9912
	ALP	12.30 ± 1.1	−0.88 ± 0.09	0.67	1100	48.97	0.67–233.55	4.9	0.9806
	FITC	1.48 ± 1.1	−0.95 ± 0.08	0.12	20.44	2.363	0.12–39.75	8.3	0.985
optical device	FITC	46.6 ± 1.1	−1.24 ± 0.13	4.17	835.7	180.5	4.1–393.04	5.9	0.9843

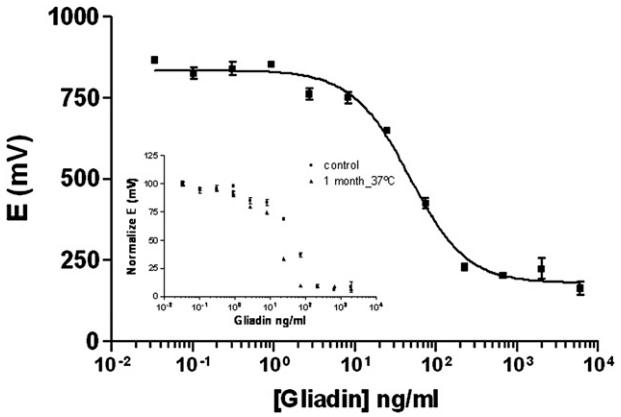

Fig. 4 Competition assay between attached and free gliadin, using a-Ab-FITC label on the optical micro-device. Error bars represent reproducibility obtained with three assays. Inset: evaluation of stability of disposable strips with pre-coated biotinylated gliadin using accelerated Arrhenius thermal stability studies storing the strips for one month at 37 °C, approximately equivalent to real time storage of 2 years at 4 °C.

Table 2 Validation of fluoroimmunoassay carried out in microfluorimeter with routine colorimetric ELISA for detection of gliadin in commercial gluten-free raw and processed foodstuffs

Sample	Microfluorimeter (ppm)	ELISA (ppm)
"Harisín" dietetic pasta (from SANAVI, S.A., Spain)	13.4 ± 0.9	11.2 ± 1.3
"Mix B" bread mix (from Dr Schär Srl, Italy)	2.3 ± 0.1	1.6 ± 0.4
"Damhert Nutrition" cake mix (from Damhert NV, Belgium	1.7 ± 0.2	1.65 ± 0.04

a gluten-containing food. As can be seen in Table 2, an excellent correlation with the colorimetric ELISA is obtained, demonstrating the usefulness of the developed microfluorimeter for *in situ* analysis of gliadin in raw materials and as a control of contamination along the food production line, for use by food industries.

Conclusion

A microfluorimeter device has been developed that can be integrated into a lab-on-a-chip system. The application of the detection of coeliac disease toxic gluten in foodstuff in laboratories of food producers requires a low budget device to ensure a wide acceptance. Further requirements are disposable components including all optical parts that come into contact with the sample as well as an easy optical adjustment of the detection chip, which were achieved by the integration of mirrors that work on the basis of total reflection, into the detection chip.

The device has been applied to the detection of gliadin in foodstuffs for use *in situ* by food industries and an excellent performance of the system was demonstrated. Different assay formats were tested, and the optimum performance in terms of assay time, detection limit and compatibility with extraction buffer obtained with a system using a FITC-labelled secondary antibody. Using the microfluorimeter, each of the channels were fitted with gliadin coated polymer strips and 5 simultaneous assays could be realised, with the only required end user intervention being mixing of the immunoreagents, addition to microfluorimeter and a rapid wash prior to detection. The microfluorimeter was applied to the analysis of commercial gluten-free food samples, and an excellent correlation with routine ELISA was obtained. The developed microfluorimeter should find widespread application for on site execution of fluoroimmunoassays.

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