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RESEARCH PAPER

Cellphone-based detection platform for rbST biomarker analysis in milk extracts using a microsphere fluorescence immunoassay

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Abstract Current contaminant and residue monitoring throughout the food chain is based on sampling, transport, administration, and analysis in specialized control laboratories. This is a highly inefficient and costly process since typically more than 99 % of the samples are found to be compliant. On-site simplified prescreening may provide a scenario in which only samples that are suspect are transported and further processed. Such a prescreening can be performed using a small attachment on a cellphone. To this end, a cellphone-based imaging platform for a microsphere fluorescence immunoassay that detects the presence of anti-recombinant bovine somatotropin (rbST) antibodies in milk extracts was developed. RbST administration to cows increases their milk production, but is illegal in the EU and a public health concern in the USA. The cellphone monitors the presence of anti-rbST antibodies (rbST biomarker), which are endogenously produced upon administration of rbST and excreted in milk. The rbST biomarker present in milk extracts was captured by rbST covalently coupled to paramagnetic microspheres and labeled by quantum dot (QD)-coupled detection antibodies. The emitted fluorescence light from these captured QDs was then imaged using the cellphone camera. Additionally, a dark-field image was taken in which all microspheres present were visible. The fluorescence and dark-field microimages were analyzed using a custom-developed Android application running on the same cellphone. With this setup, the microsphere fluorescence immunoassay and cellphone-based detection were successfully applied to milk sample extracts from rbST-treated and untreated cows. An 80 % true-positive rate and 95 % true-negative rate were achieved using this setup. Next, the cellphone-based detection platform was benchmarked against a newly developed planar imaging array alternative and found to be equally performing versus the much more sophisticated alternative. Using cellphone-based on-site analysis in future residue monitoring can limit the number of samples for laboratory analysis already at an early stage. Therewith, the entire monitoring process can become much more efficient and economical.

Keywords Cellphone · Microsphere immunoassay · Anti-rbST antibodies · Biomarker · Milk · Fluorescence imaging

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Introduction

Current contaminant and residue monitoring throughout the food chain comprises several steps that are standardized and require extensive administration. First, samples are taken

either at the farm, in the food industry, in retail, or even at the consumer's home. Samples are then transported to specialized control laboratories, where they are registered and stored. Thereafter, depending on the residue to be monitored, all samples are subject to an initial screening procedure, which identifies suspicious samples in a high-throughput manner. Any identified suspicious sample undergoes the subsequent confirmation procedure, in which the residue is unequivocally identified and, if necessary, quantified [1]. Throughout the entire residue monitoring process, the number of transported, analyzed, and administrated samples is enormous, since only after screening at a specialized laboratory they become narrowed down to the actual suspicious ones (Electronic Supplementary Material Fig. S1). This process is very inefficient and costly since typically more than 99 % of the samples are found to be compliant.

For future contaminant and residue monitoring, we propose a slightly different approach: the introduction of a simplified on-site prescreening step that limits the number of samples for the following steps already at a very early stage (ESM Fig. S1). Then, only the suspicious samples will be transported, administrated, and further analyzed in specialized control laboratories. Furthermore, the screening procedure in the specialized laboratory would only remain optional. This proposed approach is much more efficient in terms of transportation, administration, and use of equipment in highly specialized laboratories. For the proposed on-site prescreening procedure, a small attachment on a cellphone may be used and the administrative data together with the results can be transmitted wireless to a food quality and safety officer.

As a first step on the road toward future cellphone-based food analysis, we modified a cellphone attachment, originally designed for cell analysis [2–5]. For our attachment design, a dual imaging approach is followed. In this approach, two different light sources are used, viz. white light-emitting diodes (LEDs) for dark-field imaging and ultraviolet (UV) LEDs for fluorescence imaging. This newly developed cellphone attachment was applied as a detection platform for a microsphere fluorescence immunoassay using the analysis of recombinant bovine somatotropin (rbST) biomarker in milk extracts as a showcase. RbST is a proteohormone and increases milk production in dairy cows by 10–20 % [6]. While rbST use is approved by the Food and Drug Administration in the USA, it is banned in the European Union [7]. To implement European regulations and have accurate "rbST-free" labeling of milk in, for example, the USA, field monitoring of rbST use and abuse is necessary and would be greatly facilitated by the use of cellphone-assisted rapid screening assays even at farm settings. To screen for rbST, rbST-dependent protein biomarkers can be measured [8]. Protein biomarkers include antibodies, which are endogenously produced by the cow upon treatment with rbST. These anti-rbST antibodies are present not only in serum but also in milk [9]. Previously, a microsphere-based flow cytometric immunoassay (FCIA) method for the detection of anti-rbST antibody (rbST biomarker) in milk was developed [9]. In this previous method, rbST is covalently coupled to microspheres. After incubating the microspheres with a milk sample extract from an rbST-treated cow, the biomarker binds to the rbST on the surface of the microspheres. The presence of the rbST biomarker can then be detected by a fluorescently labeled anti-bovine-IgG detection antibody. Finally, the fluorescence on the microspheres is measured using a flow cytometer (used as a reference method in this paper) [9]. In the present work, we redesigned that rbST biomarker assay and combined it with a cellphone-based detection by imaging the total fluorescence on a number of microspheres. Cellphone-based devices for bioanalysis have been recently reviewed by Vashist et al. [10]. However, our approach combines for the first time a microsphere immunoassay for real-life samples with a cellphone-based readout platform. To this end, a low-cost opto-mechanical cellphone attachment was designed, which uses UV LEDs to excite fluorescent quantum dot (QD)-labeled anti-bovine-IgG detection antibodies and white LEDs for dark-field imaging of all microspheres present in the sample. An optical filter and an external lens in this attachment were used to image the emitted light onto the cellphone camera. A custom-developed Android application, which we term as "GotMilk," enabled image analysis to be performed on the same cellphone to obtain immediate results.

The developed cellphone detection platform was benchmarked against a newly developed transportable planar imaging array version of the original FCIA approach. The results of this comparison revealed that our cellphone-based approach could detect milk extracts from rbST-treated cows equally well. We believe that the cost-effective and field-portable design of our detection platform provides a good match for field testing of milk samples even in farm conditions and permits remote reporting and analysis of the acquired test results.

Materials and methods

Chemicals and instruments

Monsanto rbST standard was obtained from the National Hormone & Peptide Program of Dr. Parlow (Torrance, CA, USA). Posilac 500-mg single-dose syringes and syringes with only the slow-release formula were purchased from Monsanto Company (St Louis, MO, USA) and Ely Lilly and Company (Indianapolis, IN, USA). Sodium chloride (NaCl), monosodium phosphate monohydrate ($NaH_2PO_4xH_2O$), potassium dihydrogen phosphate (KH_2PO_4), Tween-20, sodium azide (NaN_3), and glass microscope cover slides (rectangular 24×32 mm, thickness 1; round Ø10 mm, thickness 1) were

obtained from VWR International (Amsterdam, The Netherlands) and off-the-shelf transparent nail polish was from Herome Cosmetics B.V. (Almere, The Netherlands). Sodium hydroxide (NaOH), disodium hydrogen phosphate dihydrate ($Na_2HPO_4x2\ H_2O$), and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was obtained from Serva (Heidelberg, Germany), and sulfo-*N*-Hydroxysuccinimide (Sulfo-NHS) from Fluka (Buchs, Switzerland). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 2-(*N*-morpholino)ethanesulfonic acid (MES hydrate), and bovine serum albumin (BSA) were purchased at Sigma-Aldrich (St. Louis, MO, USA). MagPlex™ microspheres (set 064) and the MagPix™ planar imaging array platform were from Luminex (Austin, TX, USA). Carboxylated paramagnetic polystyrene microspheres (diameter 8–9.9 μm) were obtained from Microspheres-Nanospheres (Cold Spring, NY, USA), and R-phycoerythrin (PE)-coupled goat anti-bovine immunoglobulins and biotinylated goat anti-bovine immunoglobulins were both from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein LoBind Tubes and 1.5-mL reaction tubes were from Eppendorf (Hamburg, Germany). Streptavidin-coupled quantum dots (QD; semiconductor CdSe crystal core coated with a semiconductor ZnS shell, a polymer coating, and streptavidin protein; total size 15–20 nm; emission at 625 nm) were from Life Technologies (Grand Island, NY, USA) and MultiScreen HTS filter plates were purchased from Millipore (Billerica, MA, USA). The 96-well plates were from Greiner Bio-One B.V. (Alphen aan de Rijn, The Netherlands), the magnetic separator was from Dexter Magnetic Technologies, Inc. (Elk Grove Village, IL, USA), and the orbital shaker was obtained from Salm en Kipp B.V. (Breukelen, The Netherlands). White light-emitting diodes (3 mm) were from Conrad Electronic Benelux BV (Oldenzaal, The Netherlands), and the Samsung Galaxy SII was obtained from Amazon.com, Inc. (Seattle, WA, USA). Ultrabright ultraviolet (380 nm) 5-mm LEDs were bought from Parts Express (Springboro, OH, USA), and 610-nm long pass filter (25 mm diameter) and aspherical lens (focal length 8 mm) were obtained from Thorlabs (Newton, NJ, USA). The battery compartment was obtained from DigiKey (Thief River Falls, MN, USA), and the 3D printer model Dimension Elite was from Stratasys (Eden Prairie, MN, USA).

Sample material

Milk samples from two *Bos taurus* animal experiments were used, which had been analyzed previously with the FCIA method [9]. The following milk sampling time points were used in the present study: 1 week before the rbST or placebo treatment and 36 and 58 days after the start of the treatment. Twenty milk samples from untreated animals were randomly selected, and 20 milk samples from rbST-treated cows with known rbST antibody responses were tested.

Buffers and solutions

Buffers and solutions used were as follows: phosphate-buffered saline (PBS; 154 mM NaCl, 5.39 mM Na_2HPO_4, 1.29 mM KH_2PO_4, pH 7.4), PBST (PBS, 0.05 %*v*/*v* Tween-20), activation buffer (100 mM NaH_2PO_4, pH 6.2), MES buffer (50 mM, pH 5), blocking buffer (PBS, 0.1 %*w*/*v* BSA, 0.02 %*v*/*v* Tween-20, 0.05 %*w*/*v* NaN_3), and sample diluent (PBST, 0.1 %*w*/*v* BSA, 0.008 %*w*/*v* SDS).

Microsphere preparation

RbST was covalently coupled to carboxylated noncolored magnetic polystyrene microspheres for the cellphone platform using the two-step carbodiimide reaction as described previously [11]. All protocol steps were done in Protein LoBind Tubes to avoid protein loss. Briefly, 200 μL of microsphere suspension was used, and microspheres were washed in deionized water, activated with 10 μL of 50 mg mL^{-1} Sulfo-NHS in dH_2O, 10 μL of 50 mg mL^{-1} EDC in dH_2O, and 80 μL of activation buffer for 18 min, washed twice in 500 μL of MES buffer, covalently coupled with 0.1 mg mL^{-1} rbST in 500 μL of MES buffer for 2 h, blocked in 500 μL blocking buffer for 30 min, washed in 500 μL blocking buffer twice and stored in the dark in 500 μL blocking buffer until further use at 2–8 °C. The same protocol was followed for coupling rbST to color-encoded MagPlex microspheres (microsphere set number 064) for the planar imaging array platform.

Preparation of milk extracts

Before conducting the assay protocol, milk samples must be extracted to lower nonspecific binding to the microspheres. The extraction procedure is summarized in Fig. S2 of the Electronic Supplementary Material. Note that the development of a simplified on-site extraction method was not the objective of the present study yet. Therefore, filtration for removal of fat micelles was simply done by centrifugation at 3,000*g* for 5 min using 96-well filter bottom plates of 2-μm pore size.

Cellphone-based detection platform

Opto-mechanical attachment design

The cellphone attachment was designed specifically for an Android-based Samsung Galaxy SII cellphone and based on the model of the previously described fluorescence microscopy cellphone platform [2–5]. In the presented attachment here, however, two different light sources were incorporated for the

dual imaging approach, viz. dark-field and fluorescence imaging. Similar attachments can also be created for other smartphones. This cellphone attachment module (overall dimensions 88×73×31.25 mm) consisted of several parts (Fig. 1a–c):

- a cellphone holder to align all optical parts with the camera
- a sample tray to position the cover slides, having the microsphere suspension sandwiched in between
- twelve excitation light-emitting diodes (wavelength 380 nm) for exciting the QDs for fluorescence imaging. These LEDs were arranged on three of the four sides of the sample tray perpendicular to the glass cover slides, so that the glass slides could serve as planar waveguides for the excitation light
- two white light LEDs for dark-field imaging
- an optical filter (long pass 610 nm, 25 mm diameter) was placed in the sample tray for filtering the scattered excitation light
- an aspherical lens that provides ×2 demagnification of the microspheres and an increase of the imaging field of view
- a battery compartment
- a mechanical lid to protect fluorescence measurements from ambient light.

The mechanical components of this attachment unit were made from thermoplastic using a 3D printer. The entire attachment was aligned with the cellphone in such a way that the center of the cellphone camera lens was in line with the center of the attachment's external lens. The costs of such an attachment are typically a few dollars only [3, 5].

Cellphone-based assay procedures

The cellphone-based assay procedures utilize the specific binding of the cows' endogenous rbST biomarker to rbST-coupled magnetic microspheres. After a washing step, a QD-labeled anti-bovine antibody is used to detect the presence of the rbST biomarker (Fig. 2a).

Following milk extraction, the immunoassay procedure is performed in transparent 96-well plates (ESM Fig. S2). For all dilutions and washing steps, PBST was used, and the procedure was performed at room temperature. While the FCIA reference method used R-PE as a fluorescence label, 625-nm emitting QDs were used for the cellphone-based detection because their excitation and emission spectra allow the use of a standard long pass filter (Fig. 1d) and QDs do not show photo-bleaching effects. The prepared sandwiched glass slides were slid into the cellphone attachment and a dark-field image was taken by using the white LEDs (Fig. 2b) and the internal

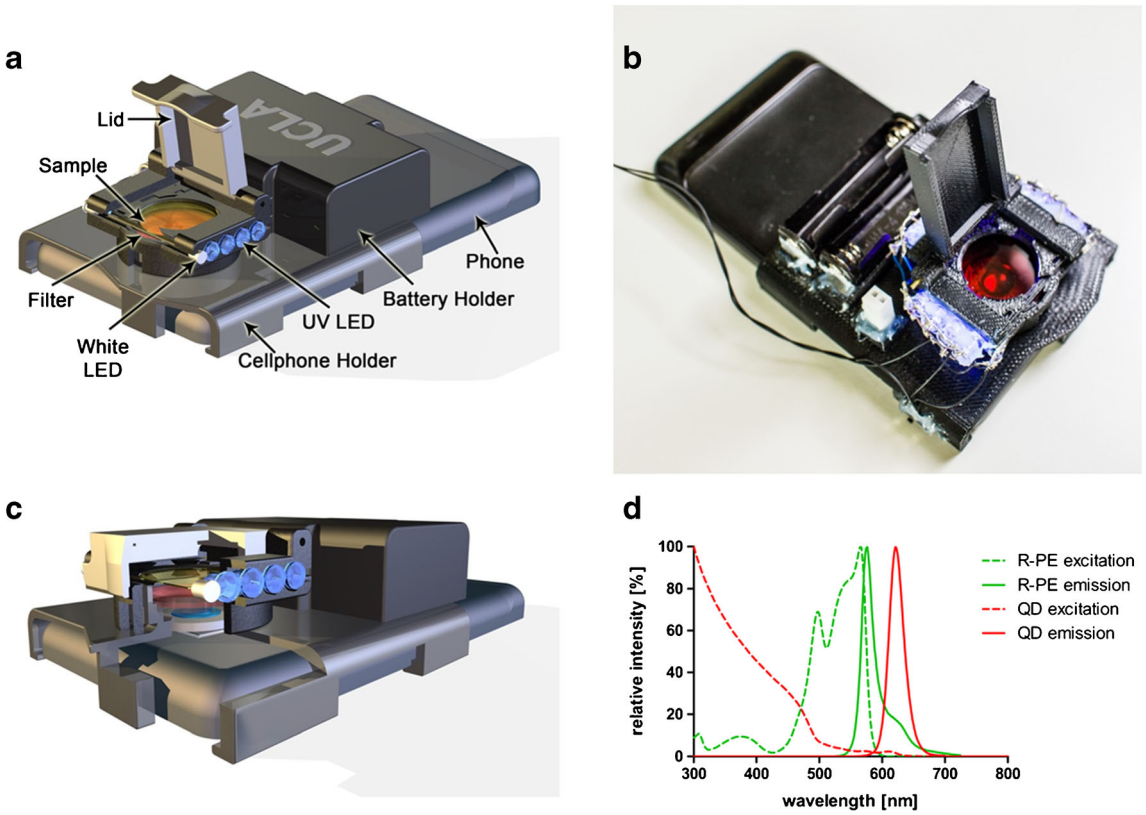

Fig. 1 Schematic overview (**a**, **c**) and a picture (**b**) of the cellphone attachment for the detection of rbST biomarker in milk extracts. Excitation (*dotted lines*) and emission (*solid lines*) spectra (**d**) of R-phycoerythrin (R-PE, *green*) and 625-nm emitting quantum dots (QD, *red*)

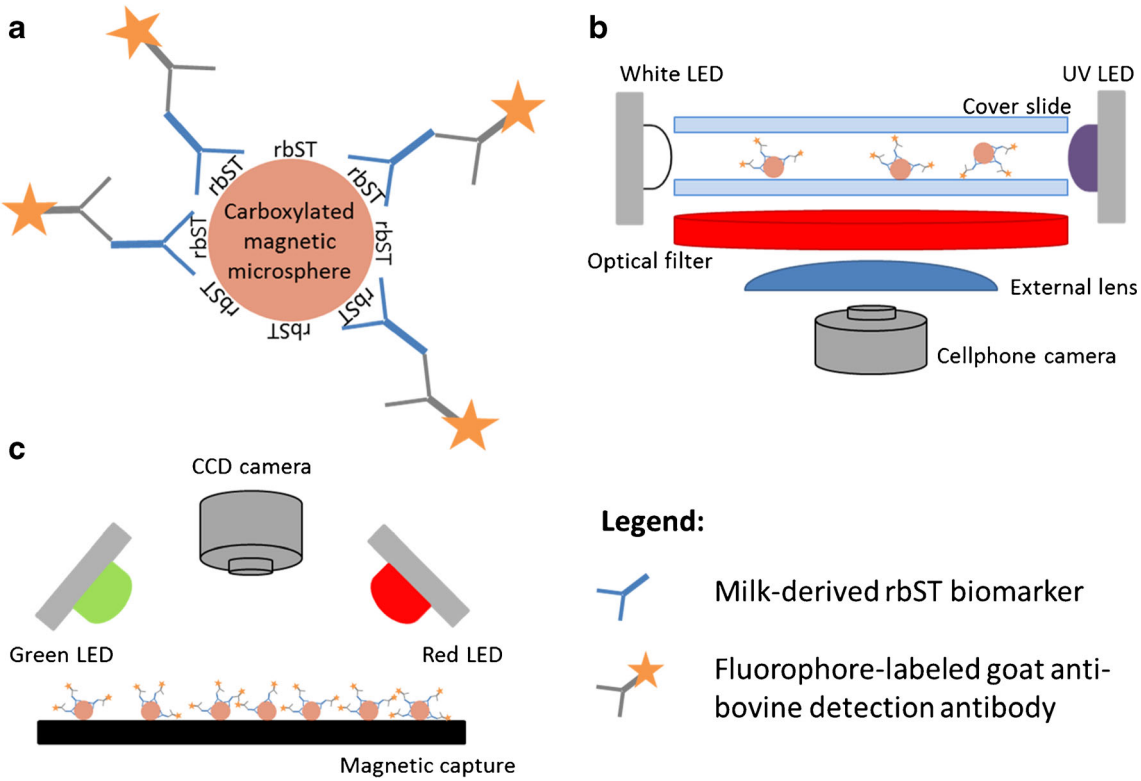

Fig. 2 **a** Assay principle for rbST biomarker detection, **b** detection setup for the cellphone-based platform, and **c** detection setup of the planar imaging array platform

camera of the cellphone operated in the "night mode" for increased sensitivity. Thereafter, the white LEDs were switched off and the UV LEDs were switched on (Fig. 2b) for taking a fluorescence image with the same settings on the cellphone. The acquired fluorescence images were analyzed by using a custom-designed Android application, termed GotMilk. Using this smart application, it is possible to analyze images located at the internal memory of the cellphone or to capture images with the camera and analyze them immediately. After a region of interest was selected (unless otherwise specified, by default the center region of the image was analyzed), total microsphere count, average fluorescence intensity, and its standard deviation were given as the result of the measurement (ESM Fig. S3). This analysis procedure was possible for both fluorescence images and dark-field images; in the latter, the number of total microspheres was counted, which was used for normalization in the fluorescence image analysis.

Planar imaging array detection platform

The FCIA reference method described by Ludwig et al. [9] was slightly modified in a way that for all tested platforms in this work, the same sample extraction procedure could be applied (ESM Fig. S2). Next, the assay principle of the FCIA reference method was transferred to a newly developed transportable planar imaging array. For this, color-encoded MagPlex microspheres (microsphere set number 064) were coupled with rbST as described in the "Microsphere preparation" section, milk samples were extracted as detailed in the "Preparation of milk extracts" section, and the samples were prepared following the procedures shown in ESM Fig. S2. Since color-encoded microspheres and the photolabile fluorophore R-PE were used for this platform, all the assay procedures were performed in the dark. The readily prepared microspheres in the 96-well plate were put into the planar imaging array instrument for detection (Fig. 2c). The color code of the microspheres was identified after excitation with a red LED (621 nm), and the signal was detected with a CCD camera and two optical filters. The amount of fluorescence (i.e., the signal) for each microsphere was quantified after excitation with a green LED (511 nm) and detected with the CCD camera and an optical filter (590 nm). The quantified fluorescence signal was reported as the median fluorescence intensity (MFI) of the particles.

Validation

For the detection of the biomarker anti-rbST antibodies, no conventional in-house validation is possible. Anti-rbST antibodies are endogenously produced by the cow upon rbST treatment. No protein standard is available for this biomarker, and therefore, no calibration curve, limit of detection, and limit of quantification can be determined. Alternatively, the different detection methods are validated by analyzing 20 milk

sample extracts from rbST-treated and 20 milk sample extracts from untreated cows.

To determine the sensitivity (true-positive rate) and the specificity (true-negative rate) for all platforms, receiver operator characteristic (ROC, ESM Table S1) curves [12] were plotted based on the results obtained from the tested milk sample extracts (ESM Fig. S4). The cutoff value that achieved the highest accuracy for each platform was selected as the decision limit (ESM Table S2). A sample was considered as being suspicious for rbST treatment when its test result was higher than the selected decision limit.

Results

Cellphone-based detection

In this work, a cellphone-based detection method for the detection of anti-rbST antibodies (rbST biomarker) was developed, following the capture of rbST biomarker by rbST-coupled magnetic microspheres. The binding event was detected using a QD-labeled detection antibody together with the specific dual imaging design of the cellphone attachment.

Cellphone-based fluorescence and dark-field imaging

The developed cellphone attachment for visualizing the presence of rbST biomarker is a lightweight and low-cost device, which can easily be attached to and detached from the cellphone. Its compactness, light weight, and low-power consumption make it a versatile tool, suitable for laboratory and field use. It can be adapted to any available cellphone that has a camera module by simply modifying the dimensions of the cellphone holder and 3D-printing another one accordingly. Our specific attachment was designed for the excitation and emission light spectrum of the 625-nm emitting QDs (Fig. 1d), which were used as a label in the rbST biomarker assay. Therefore, UV LEDs (at 380 nm) were used for the excitation of these QDs, and a 610-nm long pass filter was used for filtering the emission light. Additionally, white LEDs were used for dark-field imaging of all microspheres present in the sample irrespective of their fluorescence characteristics. Since the long pass filter was not removed for dark-field imaging, the microspheres in dark-field images appeared also in red color. The two different light sources incorporated in the same attachment allowed dual imaging without moving the sample. Therewith, it was ensured that in both light modes, the same field of view was analyzed. Furthermore, an external aspherical lens was used for demagnification of the microspheres such that a large field of view of 80 mm^2 was imaged by the cellphone camera. The cellphone holder and the sample tray positioned all the optical parts correctly and aligned the external and camera lenses for imaging. Using this cellphone attachment setup, dark-field (Fig. 3(A.1 and B.1)) and fluorescence (Fig. 3(A.2 and B.2)) images were captured, where a custom-designed application (GotMilk) was used to process these images to count the number of microspheres and determine their mean fluorescence intensity.

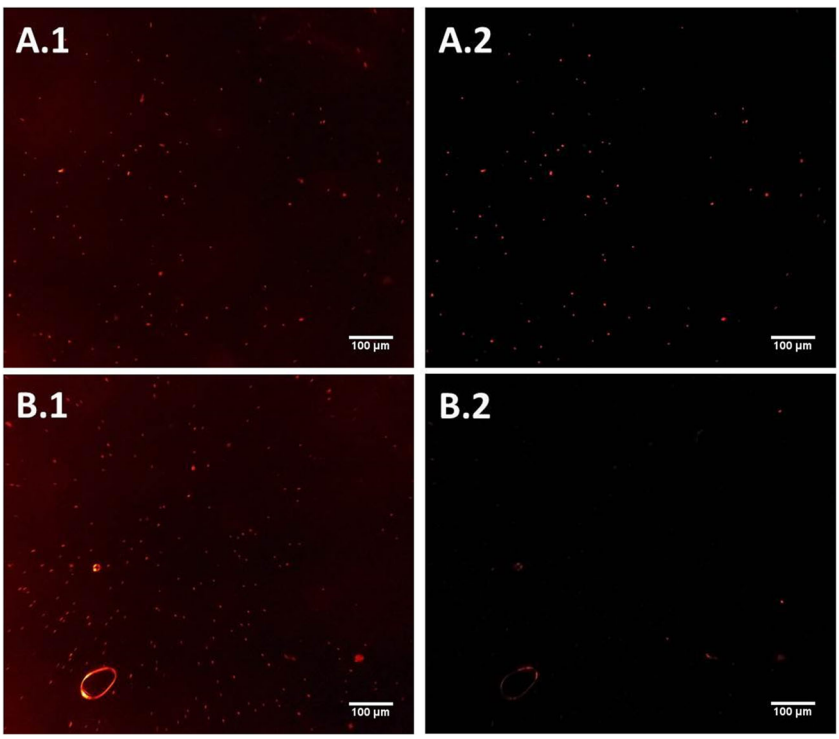

Fig. 3 Sample images obtained using the presented cellphone-based detection platform. Dark-field (*A.1, B.1*) and fluorescence images (*A.2*, *B.2*) are shown for a milk extract from an rbST-treated animal (*A.1*, *A.2*) and a milk extract from an untreated animal (*B.1*, *B.2*). Note that the dark-field images also appear in *red color* due to the long pass filter present in the optical path

Cellphone image data analysis

To be able to discriminate samples derived from rbST-treated and untreated animals, two different image analysis approaches, namely the intensity analysis approach and the microsphere count-based approach, were tested for reproducibility and linearity. For reproducibility, the same milk extract was analyzed four times, and for linearity, samples with no, low, and high rbST biomarker levels were correlated to the results of the FCIA reference method. Note that it is not possible to measure absolute rbST biomarker concentrations due to a lack of a suitable standard protein. In the intensity analysis approach, the average intensity values of the detected microspheres and the standard deviations calculated by the GotMilk application were used. This approach was expected to deliver similar results as the planar imaging array platform ("Planar imaging array detection platform" and "Planar imaging array assay" sections), in which also MFIs are obtained. However, this approach was found less useful in our cellphone platform: first, the dynamic range of the obtained intensities varied between 0.09 and 0.22 on a scale from 0 to 1.00 (Fig. 4(A.1)). Second, the standard deviation of the microspheres from the same sample was approximately 25 % of the total intensity, which is high and makes the discrimination between milk samples from rbST-treated and untreated animals difficult. Third, there was no obvious biomarker-dependent increase in signal observed (see, for example, Fig. 4(A.1 and A.2)).

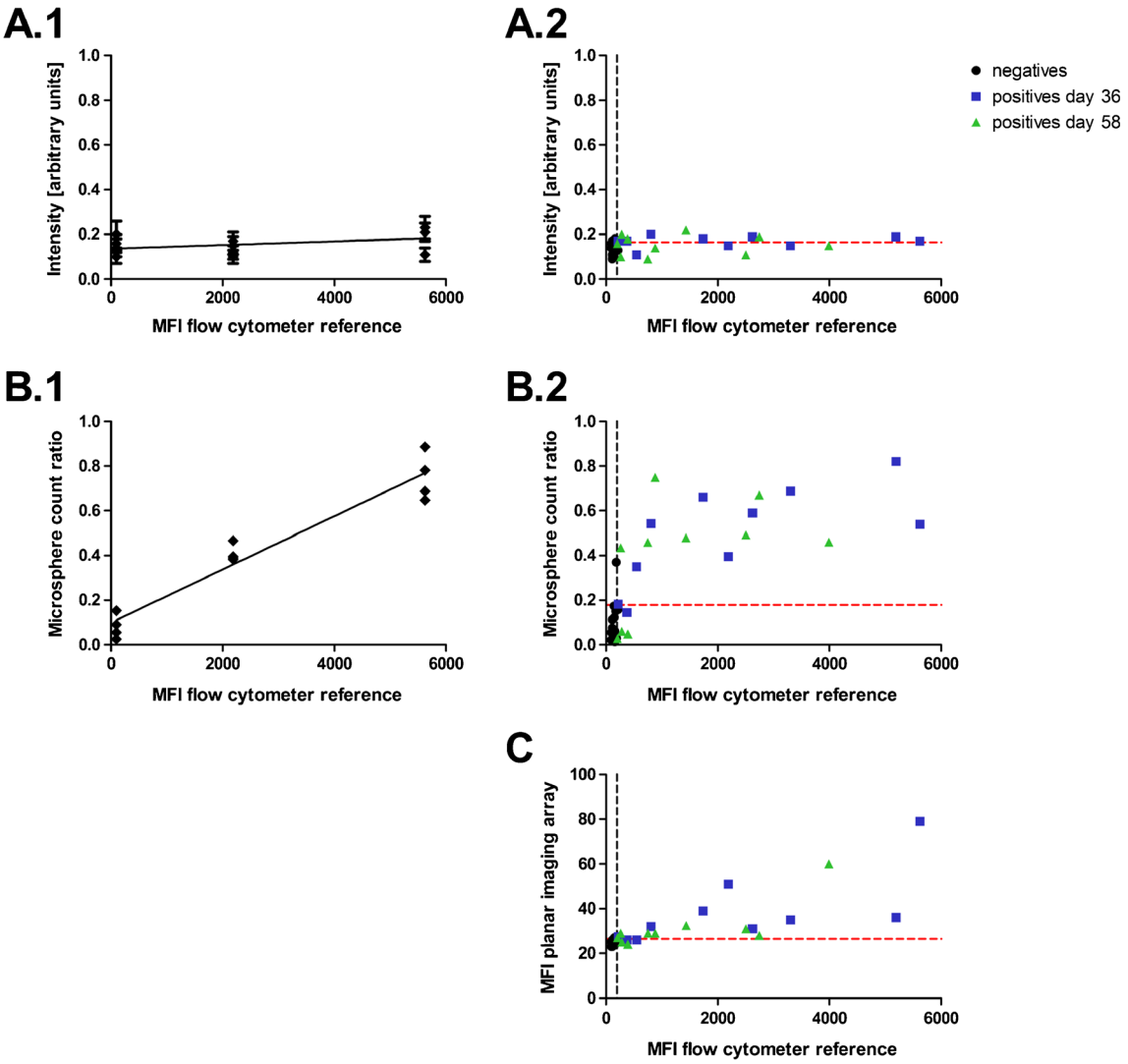

Fig. 4 The signal intensity obtained using the cellphone platform with (*A.1*, *A.2*) the intensity analysis approach, and (*B.1*, *B.2*) the microsphere count ratio obtained with the microsphere count-based approach. Reproducibility and linearity (*A.1* and *B.1*) and individual results for milk sample extracts from rbST-treated (*squares* and *triangles*) and untreated (*circles*) animals (*A.2* and *B.2*). (*C*) Correlation of MFI results obtained using the planar imaging array detection approach compared to the reference FCIA method. The decision limit, above which a sample was considered as "suspicious for rbST treatment" is marked as *red dotted line*. The decision limit of the flow cytometer reference method is marked with a *black dotted line*. *MFI*, median fluorescence intensity

As an alternative method, a microsphere count-based approach was tested, in which the total microsphere count of the fluorescence image was normalized against the total microsphere count of the dark-field image, and this ratio was used for analysis. Using this approach, an increased dynamic range (0.01 to 0.88 on a scale from 0 to 1.00) and an improved reproducibility could be obtained as illustrated in Fig. 4(B.1). Furthermore, this microsphere count ratio correlated quite well with the biomarker presence in the milk extracts. Therefore, this approach was selected to analyze 20 milk sample extracts derived from rbST-treated cows and 20 milk sample extracts from untreated cows.

Results of milk sample extracts analyzed with the cellphone-based detection platform

For evaluation of the developed cellphone-based rbST biomarker detection platform, 20 milk sample extracts from untreated cows and 20 further milk extracts from rbST-treated cows, having anti-rbST antibody response according to FCIA, were tested. In both cases, the microsphere count-based approach as detailed in the previous subsection was applied. Examples of dark-field and fluorescence images of milk samples taken from rbST-treated and untreated cows can be seen in Fig. 3. In the dark-field images (Fig. 3(A.1 and B.1)), microspheres are well visible as red dots. In the fluorescence image of the sample from an rbST-treated animal (Fig. 3(A.2)), the majority of microspheres shows a fluorescence signal, whereas in the sample from an untreated animal, only a few fluorescence signals are visible (Fig. 3(B.2)). The decision limit was determined by ROC curve analysis ("Validation" section, ESM Table S2 and Fig. S4.B) and was found to be at a microsphere count ratio of 0.1786 (ESM Table S2). Based on this decision limit, our tests revealed that 16 out of the 20 tested milk extracts (80 %) from rbST-treated animals were found suspicious for rbST treatment, whereas 19 out of the 20 tested milk extracts (95 %) from untreated cows were found negative. These results yielded a total accuracy of 87.5 %. Of course, the 80 % truly positive screening rate of the cellphone is not good enough for official testing yet; on the other hand, it is quite remarkable that such a simple low-cost device demonstrates such a rate already now.

The laboratory-based FCIA reference method was used to test the same 20 milk samples from untreated animals followed by the 20 milk samples from rbST-treated animals. The decision limit (calculated as outlined in the "Validation" section) was found to be at 198.1 MFI, and based on this decision limit (ESM Fig. S4.D and Table S2), all tested milk extracts from rbST-treated cows were found suspicious for rbST treatment (100 %) and 19 milk sample extracts from untreated animals were found truly negative (95 %). Therefore, an accuracy of 97.5 % was obtained using the lab-based FCIA method.

Planar imaging array assay

Development of the planar imaging array assay

For the planar imaging array-based detection method, the same sample preparation protocol of the cellphone-based approach (with a single step of sample dilution) was used. The assay procedures were adopted from the FCIA reference method (Figs. 2c, S2, and "Planar imaging array detection platform" section).

Results of milk sample extracts analyzed with the planar imaging array instrument

When the 20 milk samples from untreated animals were analyzed using the planar imaging array instrument, a decision limit of 26.5 MFI was calculated as described in the "Validation" section (ESM Fig. S4.C and Table S2). Furthermore, the variability in between the negative samples was quite low (4.3 % CV). Of the 20 analyzed milk sample extracts from rbST-treated animals, 16 (80 %) were correctly identified as being suspicious for rbST treatment with the planar imaging array platform, whereas 19 milk sample extracts from untreated animals were identified as negative (95 %). These results led to an accuracy of 87.5 %, which is the same as the cellphone results. The individual results of each milk sample in correlation to the results of the reference flow cytometer method are depicted in Fig. 4(C).

Discussion

We presented the development and initial real-life applicability testing of a field-portable cellphone-based detection platform for the analysis of rbST biomarker in milk extracts. Other cellphone-based detection platforms were developed before for several different applications [2–5, 10, 13–25]. The here presented platform, however, combines for the first time microsphere immunofluorescence detection from real sample extracts using a cellphone for fluorescence and dark-field imaging. The novel cellphone attachment described here is designed in a way that it integrates both, dark-field and fluorescence imaging, in one attachment. Only the combination of the two imaging techniques provides the possibility to obtain the microsphere count ratio. Dark-field imaging was enabled by the use of white LEDs and fluorescence imaging was performed by the use of the UV LEDs. Switching between the two light sources was possible without moving the sample and therewith, it was ensured that in both light modes the same field of view was analyzed.

Table 1 Performance of the cellphone assay for rbST biomarker detection in cows' milk versus alternative approaches

	Cellphone detection	Planar imaging array platform	Flow cytometer reference method
True-positive rate (%)	80	80	100
True-negative rate (%)	95	95	95
Accuracy (%)	87.5	87.5	97.5
Assay time (min)	160	100	105
Reading time (min per sample)	5	1	1
Portability	Portable	Transportable	Not transportable
Infield applicability	No external power supply needed	Operation requires external power supply	Not applicable in field
Multiplexing capability	Requires interchangeable filters	Multiplex ready	Multiplex ready
Wireless connectivity	Yes	No	No

When testing 20 milk extracts derived from rbST-treated cows, our cellphone-based detection platform performed equally well compared to the planar imaging array (Table 1). Currently, the assay time is substantial, and therefore, future studies should focus on simplified on-site sample preparation and shorter incubation times. For example, as a first step, in-field sample extraction could be performed by simply diluting the milk sample and removing fat micelles by quick syringe filtration. Second, the QD detection antibody may be prepared as a single reagent, thereby omitting the 30-min incubation step for streptavidin-QD in ESM Fig. S2. Third, co-incubating all assay reagents simultaneously may reduce the several individual incubation steps down to only one. Fourth, the employment of a microfluidic chip comprising all assay reagents prepared would facilitate the entire assay procedure. One should be aware that with the cellphone-based approach, the results can be obtained at the site where the sample was taken, i.e., transporting the samples to a specialized laboratory is no longer required. When a suspicious sample is identified during the on-site screening process, further samples, for instance blood samples, can also be taken for subsequent laboratory-based analysis (ESM Fig. S1). In that case, the multiple serum protein biomarker screening test previously developed [8] and direct confirmatory analysis of rbST itself using LC-MS/MS [26] can be applied.

When benchmarking the anti-rbST platforms available so far, the cellphone-based detection device is the only option for on-farm analysis of tank milk by inspection services (or truck milk at the dairy gate). The planar imaging array platform is, compared to the flow cytometer instrument, transportable, but its dimensions (16.5×60×43 cm) and its weight of 17.5 kg (the necessary operating computer not included) do not favor infield use. Note that, as highly desired, the cellphone-based detection platform combines not only a camera and image analysis tool, but it also allows data storage and wireless transmission via a mobile network to food quality and safety officers, central inspection agencies, control laboratories, or industrial QA/QC decision makers.

Multiplexing can be easily achieved by the planar imaging array platform and the flow cytometer reference method, which are designed especially for color-encoded microsphere ligand binding assays. For the cellphone-based detection device, multiplexing may be achieved easily by using either different fluorescent labels for additional analytes or different color-encoded or size-encoded microspheres in the microsphere counting process. In both cases, the cellphone attachment needs LEDs of different wavelengths and exchangeable optical filters as demonstrated recently by Zhu et al. [5]. Alternatively, using microspheres of a different size would only require adjustments in the GotMilk application in order to discriminate bigger and smaller particles in the fluorescence and dark-field images.

In conclusion, we demonstrated the development and real-life applicability of a novel cellphone-based detection platform for the analysis of rbST biomarker in milk as a prescreening method for the detection of rbST abuse in dairy cattle. Applying this cellphone-based on-site prescreening in future contaminant and residue monitoring can limit the number of samples to be processed already at an early stage. Therewith, the administration and transport of an extensive number of compliant samples can be avoided, and the entire monitoring process can become much more efficient and economical (ESM Fig. S1). The cellphone platform's small dimensions, light weight, and cost-effectiveness make it highly desirable for field testing even in farm settings.

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