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Read-out concepts for multiplexed bead-based fluorescence immunoassays on centrifugal microfluidic platforms

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Abstract

We present novel concepts to process and read out multiplexed, bead-based fluorescence immunoassays. At the start of the read-out process, a statistically arranged monolayer of color-encoded beads is aggregated in a detection chamber. Each bead is first identified by incorporated color tags which are either dyes or luminescing quantum dots (QDs). Subsequently, the reaction-specific fluorescence signal is quantified. The read-out process is accelerated by an in-house-developed image-processing algorithm. The optical read-out device consists of standard components, e.g. a color CCD-camera as detection unit, an LED as light source, optical filters, and a drive to spin the polymer disk. The liquid handling along the complete assay protocol is realized on a centrifugal lab-on-a-disk platform. We successfully demonstrate the performance of this device by the implementation of a hepatitis A and a tetanus assay.

Keywords: FIA; Multiplexed; Beads; Quantum dots; Centrifugal microfluidics; Rotation

1. Introduction

The strong trend towards decentralized point-of-care technologies in medical diagnostics has stimulated the development of miniaturized "lab-on-a-chip" systems [1–4]. These labs-on-a-chip feature a set of basic unit operations such as sample injection, separation, metering, mixing, and reacting to integrate and thus automate full diagnostic test protocols on a credit card-sized microfluidic substrate. Many complex tasks have already been realized, among them simultaneous DNA amplification and detection [5], HIV immunoassays [6], fully integrated single-cell processing [7], and combinatorial chemistry [8]. Apart from the process integration, the major benefits of lab-on-a-chip technologies are the minute consumption of sample and reagents, short times-to-result, as well as their amenability for multiplexing and parallelization.

We here consider "lab-on-a-disk" technologies which utilize centrifugal forces for the transport of fluids. These "labs-on-a-disk" have been investigated by many groups [9–16], and several commercial products have been launched to the market [17–20]. We here for the first time investigate multiplexed immunoassays carried out on a microfluidic "lab-on-a-disk." Our modular centrifugal platform [21,22] is constituted by a passive microstructured disk as a disposable part and by a reusable centrifuge and detection unit. Our novel read-out technique enables a multiplexed screening of a set of fluorescence immunoassays within a single channel and for several channels in parallel.

This paper is structured in the following way. We first describe the principle of a multiplexed assay. Next, all experimental components are outlined, followed by a discussion of the implemented read-out strategies. Finally, we present the results of two immunoassays and conclude.

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2. Fluorescent immunoassays

One of the prominent methods for detecting concentrations of antigens or antibodies in blood samples are the fluorescence immunoassays (FIAs). In the common sandwich FIA, a target protein within the sample first specifically binds to capture proteins which are immobilized on a solid phase, e.g. a well plate surface or, in our case, a polymer bead. In the next step, unspecifically bound molecules are removed by a wash buffer. The captured target proteins are then exposed to a specific secondary detection antibody. At the end of the liquid handling procedure, unspecifically bound detection antibodies are flushed out by another washing step. The resulting fluorescence intensity hence provides a specific measure for the concentration of the target molecules in the sample.

In order to enhance the sensitivity of the detection setup while still meeting the economic demands of a point-of-care device, our detection antibodies are tagged with signal amplifying labels which are provided in a constant concentration. These so-called FluoSpheres® are yellow–green fluorescent polystyrene microspheres with a diameter of $d_{Fluo} = 200$ nm containing roughly 110,000 fluorescein equivalents [23]. Apart from a signal amplification by about two orders of magnitude compared to standard fluorochrome molecules, these FluoSpheres® exhibit a strong resistance against photobleaching.

3. Bead-based multiplexing

In multiplexed FIAs, different types of target proteins in a given sample are detected simultaneously in a single fluidic channel. We use color-encoded beads to represent different capture protein coatings (Fig. 1). In contrast to coatings immobilized on the channel wall, the polymer beads can easily be functionalized with the capture protein in off-chip preparative steps and then loaded into the microfluidic chip after physicochemically harsh sealing and surface functionalization procedures. To permit simple adhesive coupling of the capture probes on the bead surface and to directly reference to experiments conducted in common microtiter plates, we use polystyrene (PS) beads.

Bead-based assays are commonly read out sequentially by a flow-cytometer principle [24]. However, this well-established technique is unfortunately not amenable to our centrifugal platform as the image capture rate would be very tedious to synchronize with the frequency of beads passing a stationary detector. This is because the (maximum) image capture rate at a fixed azimuthal position is given by the frequency of rotation while the frequency of beads passing the detector follows a (usually shorter) time scale governed by the centrifugally induced hydrodynamic flow rate where the frequency of rotation is only one of many impact factor (geometry, liquid density, viscosity).

Instead of the flow-cytometer principle, we aggregate the bead ensemble into a monolayer to guarantee free optical accessibility for each bead [25,26]. This monolayer alignment is enforced by flushing a suspension of the beads into the chamber displaying a height which only slightly exceeds the diameter of the monodisperse beads. Geometrical barriers at the outlets of the detection chamber retain the beads. Once the bead monolayer has formed in the detection chamber, a standard FIA protocol is run and read out at rest.

In order to realize multiplexing, i.e. probing for different targets in the same fluidic channel, a color labeling of the beads must be implemented. The number of such spectrally distinguishable "color IDs" must at least match the number of different target proteins to be scanned in the FIA. The use of the powerful two-dimensional fluorescence intensity encoding offered by Luminex is not feasible as their beads exhibit a diameter of 5.6 μm, only [27]. This diameter falls much too short off the minimum height at which the shallow detection chamber where the monolayer forms can still be manufactured by common prototyping techniques. We therefore have to pursue alternative

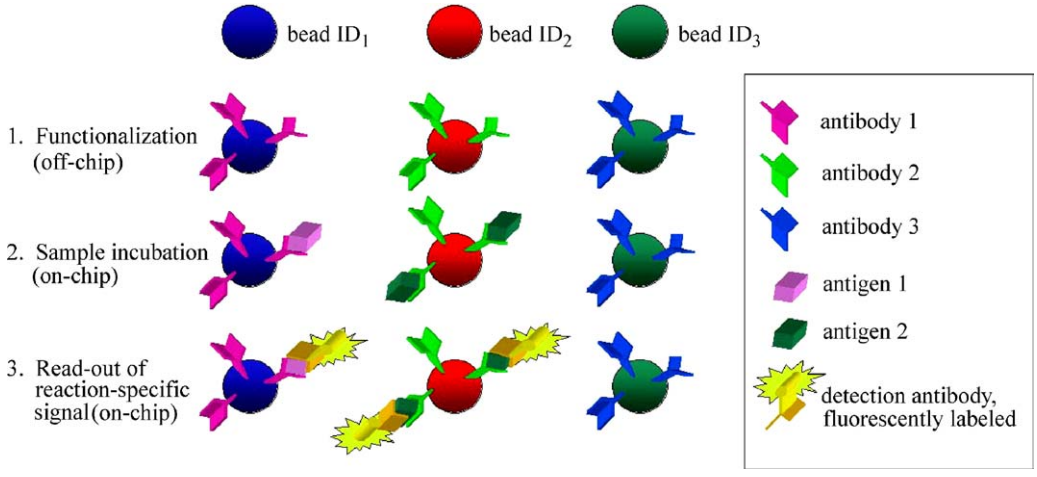

Fig. 1. Principle of a threefold multiplexed fluorescence immunoassay based on optically encoded beads (ID_1, ID_2, ID_3). According to the FIA concept, beads are first functionalized according to their color tag (the bead ID) with distinct capture proteins, mixed, and then loaded into the disk-based detection chamber. Next, the serum containing the target antigens is transported into the detection chamber and the specific antigen–antibody complex is formed. After washing, the detection antibody is coupled to these antigen–antibody complexes and the reaction-specific fluorescence signal is detected.

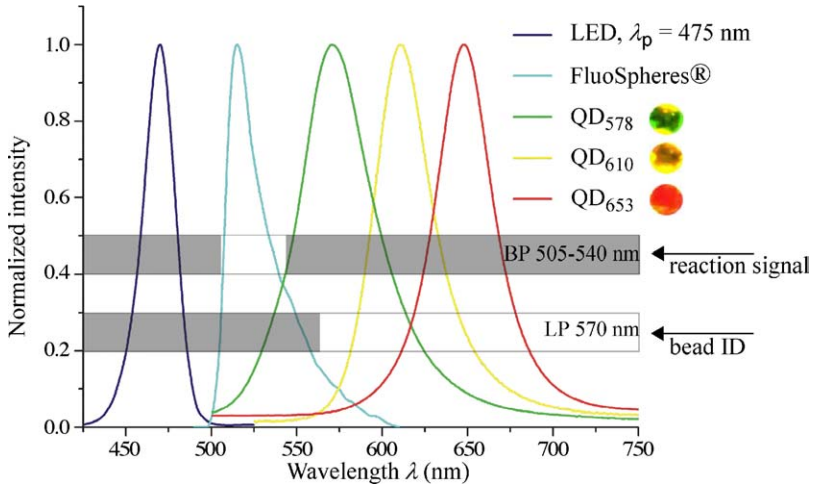

Fig. 2. Spectral distribution of the excitation source (λ_p = 475 nm), the fluorescent label (FluoSpheres®), three types of QD-Beads (QD 578 nm, QD 610 nm, and QD 653 nm), and the applied filters (BP 505–540 nm and LP 570 nm).

concepts for the color-encoding of the beads. In this paper, we investigate dyes and quantum dots (QDs).

3.1. Dyes as bead-tag

Dyed beads are fabricated in a layer-by-layer polymerization process of a styrene-dye suspension [28]. A high reproducibility in color and size ($d_{bead} = 150 \pm 4.2\,\mu m$) is achieved which is essential for monolayer-based spectral multiplexing. The dyed beads are illuminated by a simple "white" light source. Upon LED excitation in the second step of the FIA, they display a low unspecific fluorescence background. Based on our experience, we estimate the number of dyes which are optically distinguishable by our setup, i.e. the maximum number of FIAs that can be multiplexed, to about 15. However, this number cannot be experimentally verified at present, as the purchase of 15 batches with different colors from our present supplier is excessively expensive.

3.2. Quantum dots as bead-tag

In an alternative approach, we use QDs for the color identification of beads. QDs are known to excel with their facile broadband excitation, their spectrally sharp emission, and their stability against photobleaching [29,30]. The spectral range accessible with our CdSe quantum dots with a diameter of roughly 5 nm extends between 520 and 670 nm [31,32]. To incorporate the tags, colorless polystyrene beads are swollen in an organic solvent (butanol) suspending QDs of a given color.

In a FIA using QD-labeled luminescing beads, spectral regimes have to be reserved for the excitation source and the fluorescence signal. These blocked regimes restrict the QD emission wavelength for the color ID to the yellow to red spectral range (570–670 nm) which is selected by a long-pass filter [33]. Considering the typical 30 nm emission linewidths of our QDs, the level of multiplexing is limited to about five, only.

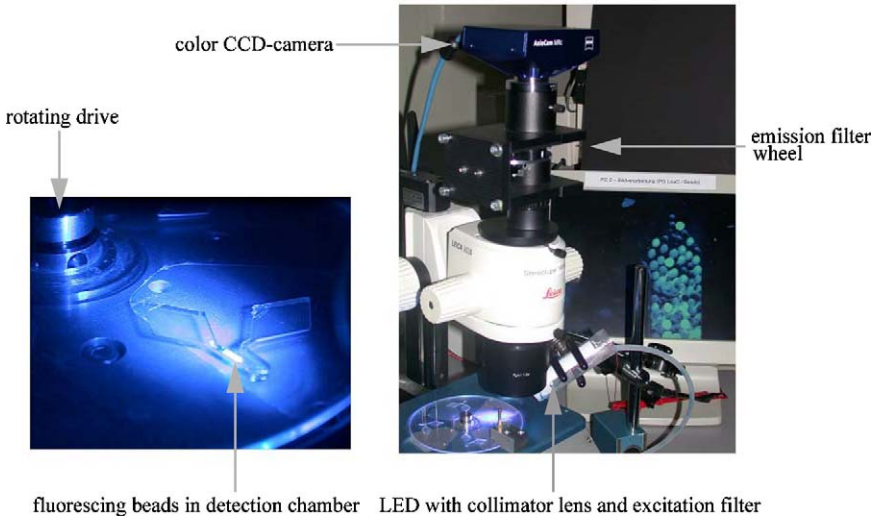

Fig. 3. Laboratory setup for the multiplexed assay. Fluorescing beads are aggregated in the detection chamber of the disk on the rotating drive. The beads are excited with a blue LED which features a collimator lens and an excitation-filter. The emitted fluorescence intensity is detected with a color CCD-camera after it passes the emission filter fixed in the emission filter wheel.

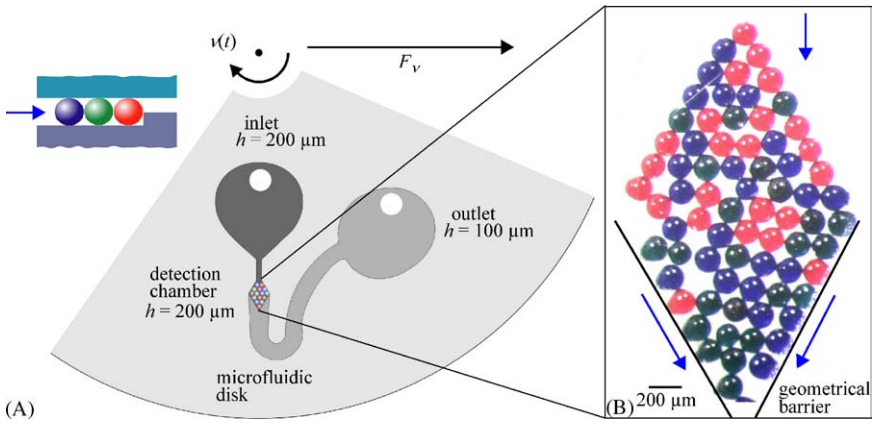

Fig. 4. (A) The chip which follows the format of a compact disk (CD) features only passive microfluidic elements: an inlet chamber ($h = 200$ µm), a detection chamber ($h = 200$ µm), and an outlet drain ($h = 100$ µm). Flow rates and incubation times are controlled by a centrifugal frequency protocol $\nu(t)$. (B) In a preparative step of the sealed disk, the beads are aggregated in form of a periodic monolayer at a geometrical barrier.

A band pass filter [34] transmitting the fluorescence line of the detection antibody is utilized to quantify the specific reactions of the target molecules with the capture probes in a FIA. This band pass is mainly necessary to suppress spectral cross-talk of the QD luminescence which would falsify the assay results. The spectral composition exhibiting the short-wavelength excitation source, the FluoSphere® emission, and the emission of three types of QD-beads as well as an overlay representing the 570 nm long-pass and the 505–540 nm band pass filters are depicted in Fig. 2.

3.3. Excitation source

Aiming at cost-efficient POC devices, there are two options for excitation sources: a laser diode and an LED. To comply with the detection scheme of our multiplexed FIA, the exci-

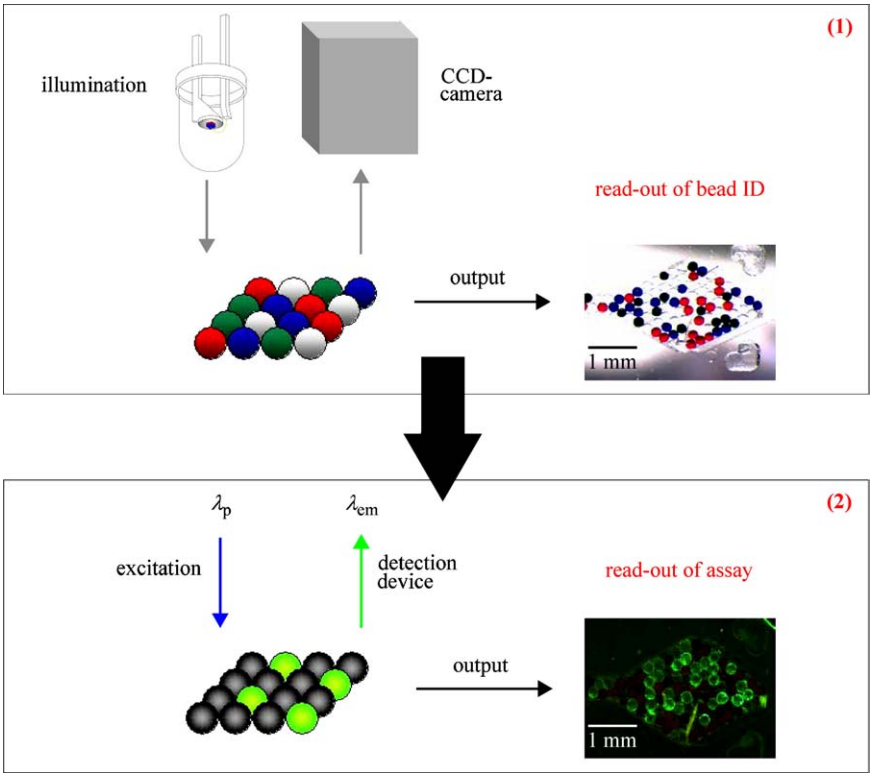

Fig. 5. Schematic overview of the read-out procedure of the multiplexed assay. In a first step, the spectral information of the tagged beads is acquired with a CCD-camera while being illuminated to identify the optical tag. In a second step, the solid phase is excited by LED-light with peak wavelength λ_p and the fluorescence intensity is measured to determine the assay result for each optical tag. As a consequence, only zones of captured antigens emit a fluorescence signal with emission wavelength λ_{em}. Results of this two-step procedure with the use of dyes as bead-tag are displayed for each step.

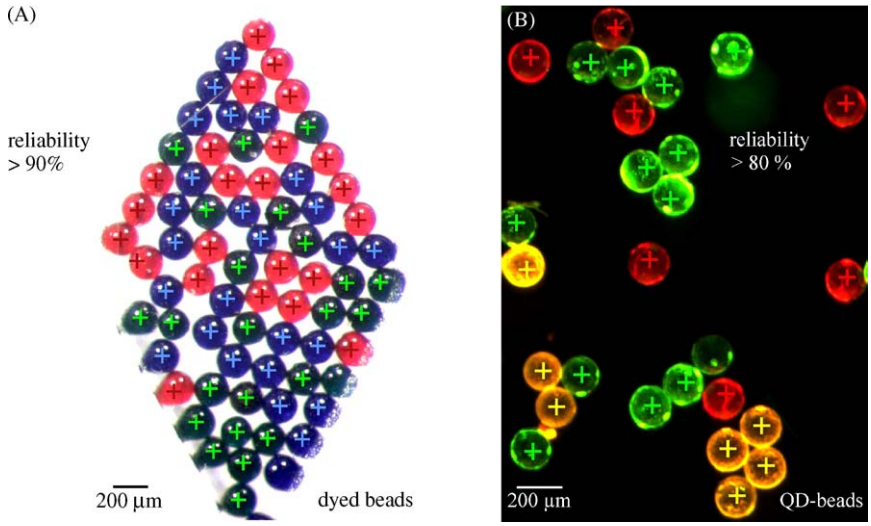

Fig. 6. Results of the image-processing routine for the evaluation of the bead ID. The localized bead centers are marked by a cross, where the assigned IDs are denoted by their color. (A) For the dyed beads, a reliability of higher than 90% can be achieved. (B) For the QD-beads, due to the inhomogeneity of the luminescence color over the bead surface, the reliability is reduced (slightly above 80%).

tation source must be located as far as possible towards the short-wavelength side of the UV–vis regime (Fig. 2). However, laser diodes are presently only available in the red domain at a reasonable price. We therefore selected a high-power LED [35] for exciting the fluorescent labels as well as the quantum dots by a blue 475 nm line.

3.4. Detector

We have evaluated two technological options which both have proven to be feasible for color identification and fluorescence read-out of the multiplexed FIA, a color CCD-camera [36] and a spectrophotometer [37]. However, the sequential bead-to-bead scan of the two-dimensional monolayer with the fiber tip of the spectrophotometer leads to extensive measurement times (about 10 s per single bead are required to generate sufficient signal). We therefore chose the color CCD-camera which acquires a picture of all beads in the detection chamber in parallel, i.e. in a single shot. As expostition time, 1–10 s are required to register sufficient signal.

The entire setup comprising the LED as fluorescence excitation source, optical emission filters and the color CCD-camera mounted in a top view position is depicted in Fig. 3.

3.5. Microfluidic disk

Our system is set up in a modular fashion of a reusable detection unit and a centrifuge drive which receives a disposable polymer disk in the format of a standard compact disk (CD). The disk features a set of passive microfluidic structures, i.e. an inlet reservoir, the detection chamber, a geometrical barrier for the bead aggregation, and an outlet drain (Fig. 4). The sample as well as the reagents and buffers are dispensed into the inlet reservoir with a standard pipette and incubated under constant flow. The flow control for the standard steps of a FIA is achieved by the interplay of designated channel geometries and hydrophilic/hydrophobic surface patterning with the centrifugal force which is varied in time by a distinguished frequency protocol.

Due to contact-free transmission of force and the lack of pressure-tight interfaces, the disk can easily be exchanged in a similar way as a compact disk on a conventional CD player. This facilitates handling by the user as the disposable disks hosts all parts getting in touch with liquids. The passive disk modules without moving parts are fabricated by CNC micromachining [38]. Similar disks have also been replicated by hot embossing or injection molding which is cost-efficient at high production numbers.

4. Read-out strategies

The read out of the multiplexed FIA is based on the two successive color images, the first for the determination of the bead ID, second for the read out of the assay result. The read-out procedure for using dyes as bead-tag is depicted in Fig. 5.

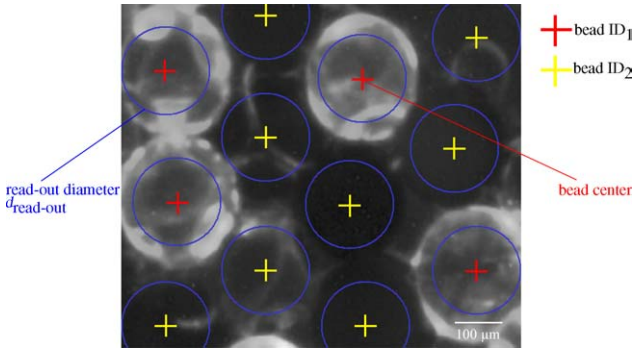

Fig. 7. Evaluation of the fluorescence signal. The assay result for each bead ID is measured by averaging the fluorescence signal within a circle of $d_{\text{read-out}} < d_{\text{bead}}$ around the bead center for all beads of a certain ID.

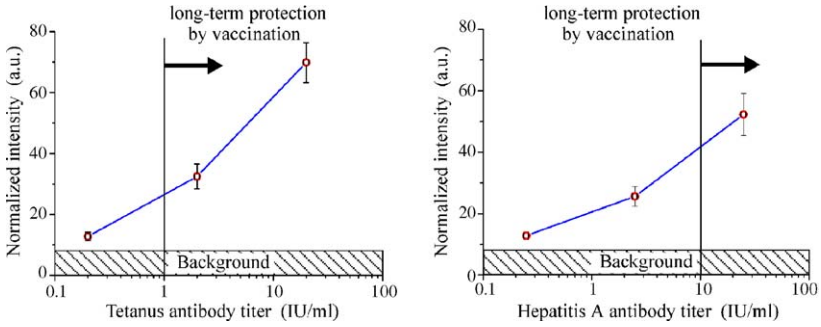

Fig. 8. Results of the hepatitis A and the tetanus assay. The curves clearly allow to determine the state of long-term protection by tetanus (CV = 11.1%, LOD = 158 mIU/ml) and hepatitis A (CV = 11%, LOD = 215 mIU/ml) vaccination (limit at 1 and 10 IU/ml, respectively [41,42]).

The software-automated read out of the multiplexed assay is enabled by image-processing algorithms which locate and color-identify each bead in the first picture and subsequently quantify the accumulated fluorescence intensity of each bead in the second picture.

In more detail, the first algorithm is based on the Hough transform [39]. The image processing starts by evaluating the Euclidean distance between the color of the current pixel in the read-out image and a reference color (the bead ID) in RGB space. This distance is compared to a threshold and, if accepted, the current image position is transformed to the Hough space of the bead centers for a given bead radius. Here, each pixel-value within the bead radius of the current pixel is incremented by one. The bead centers are then pinpointed by finding the maxima in Hough space. This procedure is subsequently repeated for the other bead IDs. Additional robustness is achieved by constraining the distance of neighboring beads to distances greater than the (known) bead radius. The programmed routine features a processing time of less than 15 s in combination with a reliability of higher than 80% (Fig. 6).

For the dyed beads, the algorithm achieves a combined success rate for the bead center localization (weighted by 1/3) and bead ID recognition (weighted by 2/3) of higher than 90%. Due to the inhomogenous distribution of the luminescence intensity over the bead surface for the QD-labeled beads, the Euclidean distance of an increased number of pixels for a certain bead ID falls short of or exceeds the threshold. Thus, the combined success rate for the QD-labeled beads is slightly lower (more than 80%).

With the second algorithm, the fluorescence intensity of each bead is determined (Fig. 7). Due to the spatial overlap of the fluorescence signal of two adjacent beads, the software cannot correctly assign the intensity in the bead boundary area. Thus, the intensity of all pixels within a circle of $d_{\text{read-out}} < d_{\text{bead}}$ (e.g., $d_{\text{bead}}/d_{\text{read-out}} = 1.2$) is accumulated and divided through the number of pixels. Subsequently, the values for all beads of a certain ID are averaged to obtain the mean fluorescence intensity for the respective assay. This method avoids measurement artifacts like trapped air bubbles or unspecific adsorption of FluoSpheres® between two adjacent beads which could possibly interfere with the quantitative evaluation, thus lowering the margin of error.

5. Experimental results

To demonstrate the performance of disk-based FIAs with the QD-encoded beads, dilution series are run. They start from standard human serum (Paul Ehrlich Institute, [40]) containing a calibrated concentration of antibodies (hepatitis A or tetanus) with 20 μl of sample while a constant concentration of detection antibodies and FluoSpheres® is applied (20 μl, 1:100 dilution of stock solution). For each incubation step, a mean flow rate of 113 nl/s is selected equaling an incubation time of 180 s whereas each washing step takes 20 s, only. The measured curves (Fig. 8) clearly allow to distinguish the state of long-term protection by tetanus or hepatitis A vaccination (1 or 10 IU/ml, respectively [41,42]) and feature a CV of 11% with an LOD of 215 mIU/ml for the hepatitis A assay and a CV of 11.1% with an LOD of 158 mIU/ml for the tetanus assay. The background level for both assays was determined with buffer solution as sample under identical processing steps. Using serum containing no antigen-directed antibodies as sample showed levels which were similar to the levels obtained with buffer indicating no or only marginal unspecific adsorption.

6. Summary and conclusion

We for the first time demonstrated the feasibility of color-multiplexed fluorescence immunoassays on a centrifugal microfluidic platform. Color-encoding is implemented by incorporating quantum dots or dyes into the beads while FluoSpheres® are tagged to the detection antibodies. The large signal amplification by the FluoSpheres® allows to significantly reduce the cost of the detection unit.

As common flow-cytometry principles cannot be applied to centrifugally propelled flows with a stationary detector, we aggregated the color-encoded beads in a monolayer within a disk-based detection chamber, thus allowing a parallel read out with a color CCD-camera. An image-processing software has been developed to implement automated localization, color identification, and fluorescent detection of color-multiplexed FIAs. As a proof of principle, we successfully demonstrated a hepatitis A and a tetanus assays on our microfluidic lab-on-a-disk platform.

The major benefits of the presented technology are the full process integration including an automated read out as well as

its the rugged modular setup composed of conventional optical components, a standard centrifuge drive, and a disposable polymer disk. We believe that these features are key to meet the technical and economic demands of diagnostic point-of-care applications.

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Biographies

Lutz Riegger was born 1978 in Freiburg, Germany. He studied Microsystem Technology at the University of Freiburg till 2004. Currently, he is a PhD candidate in the department of MEMS applications at the Institute of Microsystem Technology (IMTEK) in Freiburg. His research interests are the development of lab-on-a-disk systems for medical point-of-care diagnostics.

Markus Grumann was born in 1967 in Karlsruhe, Germany. He studied physics at the University of Freiburg and Ulm from 1989 to 1995. After that, he was working at Carl Zeiss AG in Goettingen as a service trainer for laser scanning microscopes. At 2001, he accepted a job as the director of the Muellheim production facility of Hoya-Lens GmbH. Since 2002 he is a PhD candidate in the department of MEMS applications at the Institute

of Microsystem Technology (IMTEK) in Freiburg. Currently he is working in the Bio-Disk project which aims at the development of a microfluidic platform for blood analysis.

Thomas Nann was born 1968 in Stuttgart (Germany). He studied chemistry at the Universities of Stuttgart and Freiburg. He received his diploma 1994 from the University of Freiburg. During his PhD studies he worked on digital simulation of kinetic processes at microelectrodes and the connected mechanisms (Mentor: Prof. Dr. J. Heinze). Presently, he is lecturer at the Chair for Sensors at the Institute for Microsystem Technology of the University of Freiburg and leader of the Nanoscience group at the Freiburg Materials Research Center (FMF). His scientific interests are particularly on the preparation, derivatization and application of nanocrystals for electrooptics and bioanalytics.

Jürgen Riegler was born in 1973 in Germany. He studied Chemistry at the University of Freiburg and at the Free University Berlin. He passed his Diploma Degree at the Free University Berlin in late 2000. During his Diploma thesis he worked on the stereospecific synthesis of natural products in the group of Prof. Dr. H. U. Reissig. After several months working in the field of theoretical chemistry in the group of Prof. Dr. J. Manz at the Free University he joined the group of PD T. Nann at Material Research Center Freiburg for his PhD. Actually, J. Riegler is interested in the synthesis and the properties of luminescent nanocrystals to bring them into applications for bioanalytics and diagnostics.

Oliver Ehlert studied chemistry at the University of Freiburg, Germany. His diploma thesis was about viscosity dependent electron transfer reactions in bacterial reaction centers of "Rhodobacter sphaeroides". His scientific interests are the synthesis, characterization (X-ray diffractometry, UV–vis/NIR and photoluminescence spectroscopy, transmission-electron-microscopy, energy-dispersive element-analysis, dynamic light scattering), and possible applications of rare-earth-doped nanocrystals.

Lars Pastewka was born in Marl, Germany, in 1979. In 2003 he received a Master of Science in physics while being a Fulbright scholar at North Carolina State University in Raleigh, NC, USA. Right now he is about to complete his Master of Science in MEMS engineering at the Albert-Ludwig University in Freiburg, Germany. During his studies he worked on pattern recognition techniques, ab initio simulations and multiscale modeling.

Thilo Brenner was born 1977 in Dortmund, Germany. He studied Microsystem Technology at the University of Freiburg till 2002. Currently, he is working towards his PhD in the department of MEMS applications at the Institute of Microsystem Technology (IMTEK) at the University of Freiburg. The focus of his work is the development of a centrifugal microfluidic platform for medical diagnostics.

Roland Zengerle was born in 1965 and studied physics at the Technical University of Munich, Germany. From 1990 till 1995 he was a research engineer in the group of microactuators at the Fraunhofer-Institute of Solid State Technology in Munich (today: FhG-IMS). Dr. Zengerle received his PhD degree with the development of an electrostatically driven micropump. Dr. Zengerle moved in 1995 to the Institute of Micro- and Information Technology (HSG-IMIT) in Villingen-Schwenningen, Germany. He was responsible for the microfluidics department. Since 1999 he is the head of the Laboratory of MEMS-Applications at the University of Freiburg, Germany. This Laboratory is a foundation of industry in order to stimulate the cooperation of industry and university.

Jens Ducrée was born in the late 1960s in Essen, Germany. After that he studied physics at the University of Heidelberg and Münster. With two intermezzos in Uwe Thumm's group at the Kansas State University he finished his PhD in 1999 in Prof. Andrae's group. Currently he works as a scientific assistant to the chair of MEMS applications at the Institute for Microsystem Technology (IMTEK) at the University of Freiburg in the field of microfluidics.