Single-Color Barcoding for Multiplexed Hydrogel Bead-Based Immunoassays

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ABSTRACT: Current developments in precision medicine require the simultaneous detection of an increasing number of biomarkers in heterogeneous, complex solutions, such as blood samples. To meet this need, immunoassays on barcoded hydrogel beads have been proposed, although the encoding and decoding of these barcodes is usually complex and/or resource-intensive. Herein, an efficient method for the fabrication of barcoded, functionalized hydrogel beads is presented. The hydrogel beads are generated using droplet-based microfluidics in combination with photochemically induced C−H insertion reactions, allowing photo-crosslinking, (bio-)functionalization, and barcode integration to be performed in a single step. The generated functionalized beads carry single-color barcodes consisting of green-fluorescent particles of different sizes and concentrations, allowing simple and simultaneous readout with a standard plate reader. As a test example, the performance of barcoded hydrogel beads (3 × 3 matrix) functionalized with capture molecules of interest (e.g., antigens) is investigated for the detection of Lyme-disease-specific antibodies in patient sera. The described barcoding strategy for hydrogel beads does not interfere with the bioanalytical process and captivates by its simplicity and versatility, making it an attractive candidate for multiplex bioanalytical processes.

KEYWORDS: Hydrogel beads, C,H-insertion cross-linking, biofunctional particles, two-phase flow, immunoassay, barcoding

INTRODUCTION

The growing field of precision medicine aims to classify patients or diseases into subgroups that help in the selection of patient-specific treatment, leading to higher efficacy of therapy. Quantitative analysis of multiple biomarkers, whose presence correlates with the state of a disease, provides more disease-specific information than the analysis of a single biomarker.1,2 In this context, quantification of several biomarkers may be required to detect and characterize the disease3 and a variety of multiparametric new screening platforms have been developed. One example for such platforms is the generation of microarrays in which spots functionalized with capture molecules (e.g., antibodies4 or aptamers5) are printed onto planar surfaces and used to detect a target analyte. However, in such an approach the probe density on a 2D surface is limited due to steric hindrance and quenching.6 Another example is suspension assays (assays performed on suspended solid beads) which circumvent many of these limitations and benefit from fast reaction kinetics. To achieve higher probe densities, Meiring et al.7 proposed hydrogel-based beads as a substitute for solid, opaque beads. In this approach, probes are distributed throughout the hydrogel, resulting in a higher probe density when projecting the 3D volume onto a 2D image.8 In addition, because such beads are transparent, the shading effect of overlapping projections of beads during transmission read-out is less pronounced.

In recent years, various labeling strategies for differentiating mixed hydrogel beads in a multiplex assay have emerged, as for
example described by Roh et al.18 and Xu et al.19 Examples of the best-known strategies are optical encoding (incorporation of luminescent dyes), physical encoding (fabrication of differently shaped beads), and graphical encoding (fabrication of 2D-patterned beads). In optical encoding, the generation of optical barcodes can be achieved by incorporating quantum dots,10,11 lanthanoid nanophosphors,12 or fluorescent dyes.13 In physical encoding, differently shaped hydrogel beads can be produced in a photolithographic process using a microfluidic stop-flow14,15 or in a batch-wise-filled mold,7,16 while graphical encoding typically relies on the use of 2D-patterned beads. Such hydrogel beads can be prepared by combining microfluidic co-flow with a photolithographic process.17,18 In the strategies presented, the materials used range from polysiloxane or silica for solid beads13,17,19 to polyethylene glycol (PEG) or alginate for hydrogel beads.10,16,21

Although many different labeling strategies for hydrogel beads have been developed, no concept to date provides efficient encoding and decoding that could be used for low-cost, high-throughput production. Optical labeling strategies provide a simple encoding strategy, but require expensive readout devices such as flow cytometers for excitation and need a detection of multiple wavelengths. In addition, crosstalk between the fluorescent barcodes and the fluorescent dye bound as a result of the immunoassay must be considered.

Physical encoding strategies affect the performance of the assay by altering parameters such as the surface-to-volume ratio or diffusion properties within the hydrogel beads. In the case of graphical barcodes, readout is challenging due to the requirement of a proper bead alignment during imaging. In addition, the commonly used stop-flow process in these strategies limits the production rate of the beads since the droplet formation is paused during the photolithographic process. Another limitation is the use of photomasks, which reduces the adaptability of the encoding system. In summary, most approaches for the labeling of hydrogel beads need improvements in terms of complexity, efficiency, or adaptability.

An example where a multiparametric assay is required is the detection of Lyme disease, an infection caused by bacteria of the genus Borrelia and transmitted by ticks. One possible assay is based on the quantification of human immune globulins (IgG) against the outer surface protein C (OspC) and the variable minor proteinlike sequence E1 (VlsE1). Enzyme-linked immunosorbent assays (ELISA) in microwells represent a commonly used platform in clinical diagnostics for the detection of such proteins in patient samples. Characteristics of these assays include highly selective binding of the capture antibody to its antigen and high sensitivity of an enzymatic readout.22 A multiplexed analysis based on this technique has been developed in the form of protein microarrays.23,24

However, drawbacks of this technology include a diffusion-limited binding process during sample incubation and low flexibility in the probe configuration for patient-specific assays.25

To address these issues, we present a straightforward strategy to incorporate optical barcodes into biofunctionalized hydrogel beads for simple and efficient encoding and decoding. The hydrogel bead formation, optical barcode incorporation, and biofunctionalization are performed in a continuous, one-step process. For barcoding, two green-fluorescent particles that differ in size are homogeneously distributed in the hydrogel beads. This allows a low-cost readout with two fluorescence channels as present in conventional plate readers. One channel is used to decode the barcodes, the other to quantify the intensity of the fluorescence immunoassay. The suitability of the system as a bioanalytical platform is demonstrated by the multiplexed analysis of patient sera for Lyme-disease-specific biomarkers.

### MATERIALS AND METHODS

#### Polymer Synthesis.

The copolymer P(DMAA-co-5% MABP-co-2.5% SSNa) was synthesized according to Rendl et al.26 by free radical polymerization of N,N-dimethylacrylamide (DMAA), methacryloyloxybenzophenone (MABP), and Na4-styrenesulfonate (SSNa), using the initiator 4,4′-azoisobutyronitrile (AIBN). Its molecular weight $M_w$ was 285 kg/mol with a polydispersity index (PDI) of 2.9 and a MABP content of 4.6%.

#### 3 x 3 Barcoding.

Green-fluorescent PMMA particles (PolyAn GmbH, PolyAn Pink 20, Art. 10670009, $d \approx 10 \, \text{μm}$) and green-fluorescent melamine particles (Microparticle GmbH, MF-FluoGrün-0.5, $d \approx 450 \, \text{nm}$) were used for the micro- and nanoscale barcodes, respectively. The copolymer was dissolved in DI water (150 mg/mL) and mixed with the fluorescent particles to obtain nine solutions with all combinations of $[0; 1; 2] \, \text{mg/mL}$ of the melamine and $[0; 0.4; 0.8] \, \text{mg/mL}$ of the PMMA particles.

Similar to the previous work by Schönberg et al.27 two syringes (Hamilton Bonaduz AG, Gastight Syringe, 500 μL and Setonic GmbH, Glass Syringe, 1000 μL) were filled with fluorinated oil (SM, Fluoriniert FC-3283) and connected to a syringe pump (Cetoni GmbH, neMESYS 290N). The polymer solutions were aspirated by the syringe pump into an FEP tube (ProLiquid GmbH, Fluorinert FEP, ID: 0.25 mm). One microliter of fluorinated oil was used in between the different solutions as a spacer to prevent mixing. A T-junction (VICI AG International, CTFE TEE connector) was connected to the tubes exiting the syringes. A tube exiting the T-junction was coiled inside a UV-chamber.

Flow rates of 1.3 and 6.5 μL/min for the dispersed and continuous phase, respectively, as well as an irradiation time of 30 min at 365 nm (vilber GmbH, high performance xenon lamp) were selected for the hydrogel bead production. The cross-linked beads were collected in a 96-well microplate placed on a ThermoCell mixing block and washed with DI water (3 × 100 μL, 3 min, 500 rpm). The fluorescent labels were read out on a fluorescence plate reader (Sensovation, SensoSpot).

Table 1. End Concentration of Each Reagent in the Prepared Hydrogel Beads Used for the Detection of Borrelia-Specific Antibodies

| Test | OspC | VlsE1 | Negative control | Positive control |
|------|------|------|-----------------|-----------------|
| test 1 OspC | 120 mg/mL | 120 mg/mL | 120 mg/mL | 120 mg/mL |
| rabbit anti-goat IgG | 0.6 mg/mL | 0.6 mg/mL | 0.54 mg/mL | 0.9 mg/mL |
| OspC Bb | 0.6 mg/mL | 0.6 mg/mL | 0.4 mg/mL | 0.4 mg/mL |
| OspC Bs | 0.9 mg/mL | 0.9 mg/mL | 0.9 mg/mL | 0.9 mg/mL |
| VlsE1 Bb | 0.9 mg/mL | 0.9 mg/mL | 0.9 mg/mL | 0.9 mg/mL |
| melamine particles | 0.4 mg/mL | 0.4 mg/mL | 0.4 mg/mL | 0.4 mg/mL |

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Microarray Analyzer) in the green channel at an exposure time of 50 ms.

**Immunoblot.** The assay protocol used in this work, including optimization of antigen, serum, and detection antibody concentrations was adapted from Fosso Tene et al., where the detection of borrelia-specific IgG/ IgM in patient sera was performed using porous hydrogels containing the same capture proteins used in this work. Those capture proteins are OspC Bb and OspC Bb (DIARECT GmbH), VlsE1 Bb (DIARECT GmbH), and Rabbit Anti-Goat IgG (Dianova GmbH, Art. 305-005-045).

For the borrelia assay, four typical test beads were generated. They were prepared from the following solutions (Table 1): (1) a test solution containing the copolymer, a mixture of OspC Bb and OspC Bb and PMMA particles; (2) a test solution containing the copolymer, VlsE1 Bb and melamine particles; (3) the negative control containing the copolymer only; (4) the positive control in which the copolymer was mixed with Rabbit Anti-Goat IgG, PMMA, and melamine particles.

The generation of the beads was conducted as follows: the test solutions, separated by 1 μL of fluorescent oil, were aspirated into an FEP tube. The droplets were produced in a T-junction with a flow rate of 1.6 and 8 μL/min for the dispersed and continuous phase, respectively, and cross-linked for 30 min at 365 nm. The hydrogel beads were collected in a 96-well microplate, placed on a mixing block, and washed as described above. The mixing block was kept at 500 rpm for all further steps.

To perform the assays, the prepared hydrogel beads were washed three times with 100 μL of a PBS solution containing 0.1 wt % BSA (Sigma, Albumin Fraktion V, Art. 0163.2) for 10 min and then incubated for 60 min with 100 μL of the serum to be analyzed which was diluted 1:50 in PBS-0.1% BSA. After incubation with serum, the beads were washed again three times with 100 μL of PBS-0.1% BSA solution for 5 min each washing. Thereafter, they were incubated for 30 min with 100 μL of a solution containing 2 μg/mL Goat Anti-Human IgG DyLight 650 (Invitrogen, Art. SAS-10137) dissolved in PBS-0.1% BSA. In a final wash step, the beads were incubated three times with 100 μL of a PBS solution containing 0.1 wt % Tween 20 for 5 min. For the analysis, the beads were transferred to wells containing 100 μL of DI water and incubated for 30 min before the read out using the fluorescence plate reader in the red and green channels with an exposure time of 50 ms.

The sera (PS1623, PS1646, PS1670, PS1675, and PS1922) from infected patients and (BSP8052, BSP8056, BSP8079, BSP8094, and BSP8106) from healthy patients used in this work were provided and characterized by DIARECT GmbH, using either a commercially available line immunosay (Mikrogen recomLINE Borrelia IgG/ IgM) or a chemiluminescence immunosay kit (LIAISON LymeDetect), as summarized in Table 2.

**Fluorescence Measurements and Validation of Assay Results.** The obtained fluorescence images were analyzed using ImageJ. For each hydrogel bead, the microscale barcode was evaluated by manually counting the number of green-fluorescent spots. The pixels of these spots were then set to a non-numeric value (NaN) by a threshold filter to exclude them from the further analysis. The median green-fluorescence intensity of the remaining pixels, corresponding to the nanoscale barcode, was then measured. Both resulting quantities were normalized to the average value of all hydrogel beads with the second-lowest concentration of the respective type to obtain a simple translation of the visual representation to the numeric value of the respective barcode.

For the evaluation of the multiplex immunobsoasys, the mean red-fluorescence intensity of each hydrogel bead (I) for each serum was measured and the net fluorescence intensity (I)net of each test bead (OspC or VlsE1) was calculated using eq 1

$$T_{net} = \text{mean}(T_{test} - T_{sc})$$

(1)

Here, (Ttest) and (Tsc) represent the measured fluorescence intensities for a negative control bead and test bead, respectively.

To determine whether a test bead is positive or negative for an incubated serum, the threshold value T for each test bead was calculated by analyzing (as described above) the five sera from healthy patients using eq 2

$$T_{test} = (I)_{net} + 3 \sigma_{I_{net}}$$

(2)

Here, (I)net and σI_{net} represent the average and standard deviation, respectively, of the measured net fluorescence intensity for each test bead.

**RESULTS AND DISCUSSION**

**Concept for a Simple Single-Color Barcoding.** Barcodes are used to represent product information in a machine-readable layout. They come in a variety of forms, most notably 1D barcodes such as the Universal Product Code (UPC) or 2D barcodes such as the QR code. Generally, the code consists of an optical structure (visual representation) that can be decoded into a number. With the help of lookup tables, this number can be matched with a product.

In this work, the visual representation of the barcode (shown in Figure 1a) is based on two differently sized green fluorescent particles, covalently immobilized in a hydrogel network. The incorporation of 450 nm-sized melamine particles leads to a homogeneous staining of the hydrogel matrix, since the particles are smaller than the optical resolution of the used readout equipment. Ten micrometer-sized PMMA particles, on the other hand, are visible as countable fluorescent spots within the hydrogel beads. The concentration of these particles correlates with the fluorescence intensity of the hydrogel particles.

![Figure 1](https://example.com/1.png)

**Figure 1.** Schematic principle of the barcode allocation table. (a) The barcodes are visually represented by the staining of hydrogel beads with a mixture of nano- and microscale fluorescent particles (homogeneous staining in different intensities and visible spots in different quantities within the elongated beads, respectively). (b) The normalized values for the fluorescence intensity and the number of particles per hydrogel bead can be translated into a numerical code C_{XY} which can be assigned to a particular capture molecule, such as an antibody, immobilized in the respective bead.

Table 2. Reactivity of Sera from Infected Patients in Case of IgG-Detection

| Serum      | OspC Bb and Bb results | VlsE1 Bb results |
|------------|------------------------|-----------------|
| PS1623     | negative               | negative        |
| PS1646     | positive               | positive        |
| PS1670     | negative               | positive        |
| PS1675     | negative               | positive        |
| PS1922     | positive               | positive        |

*The sera were tested by DIARECT GmbH using commercially available reference tests.*
Droplets are generated by shearing aqueous solution by a fluorinated oil in a T-junction, a process comprehensively summarized by Christoffer et al. and Nunes et al. Here, the aqueous solution consists of a copolymer for the formation of the network, capture proteins for the immunoassay, and the fluorescent particles for barcoding, all mixed together. The copolymer is based on dimethylacrylamide (DMAA) as the hydrophilic backbone, sodium styrene sulfonate (SSNa) to improve the water solubility of the polymer at concentrations above 300 mg/mL, and methacryloyloxy-benzophenone (MABP) as a photo-reactive cross-linker.

In a single irradiation step, the hydrogel network is formed, biofunctionalized with the capture proteins, and labeled with fluorescent particles by a photoinduced C,H-insertion reaction (CHic) described in detail by Prucker et al. In short, UV-irradiation (365 nm) generates a biradical on the benzophenone that can abstract a hydrogen atom from a neighboring C,H-group. Through recombination, a covalent C,C-bond is formed, leading to the linkage of two polymer chains and ultimately to the formation of a network when the percolation point is reached. Simultaneously, C,H-groups-containing biomolecules and C,H-groups-containing fluorescent particles become covalently attached to the network (see Figure 2, CHic-reaction). Subsequently, the hydrogel beads are collected in a microplate. Each type of bead can carry capture proteins for a specific target which are later identified by the barcode. Several types of hydrogel beads are then combined in one well for a multiplexed analysis.

This process offers several advantages over the current production of commercially available state-of-the-art assays. The use of hydrogel beads in general, as opposed to solid beads, offers the advantage that the whole volume of the beads can be used to capture the target molecules. Transparent 3D beads in which the probes are accessible in the whole bead volume allow a higher probe density than opaque beads, where only surface molecules facing the readout optics contribute to the assay signal. In addition, beads whose images overlap in the z-axis do not obscure each other, so that these beads can also be used for the analysis.

The method for preparing hydrogel beads presented herein offers the particular advantage that all reactions, that is, cross-linking, immobilization of biomolecules, and incorporation of barcodes, are performed in a single irradiation step. The preparation of each solution could be automated by a suitable fluidic system. In addition, no potentially toxic low-molecular-weight substances, such as monomers or added cross-linkers, are used in the reaction. This has the advantage that no unreacted components can leach out after network formation, which would affect the performance of the assay after prolonged storage.

Fabrication of a 3 × 3 Barcoding Matrix. For a reliable identification, the maximum concentration of the nanoscale particles was chosen such that the resulting mean intensity of the nanoscale barcode was less than half the intensity of the individual microscale particles. Therefore, the upper limit for the nanoscale particles was set at 2 mg/mL. The maximum load of microscale particles was defined as 100 particles per hydrogel bead, which corresponds to a particle concentration of 1.2 mg/mL in the polymer solution, since higher concentrations would lead to an overlap of the individual particles and thus an inaccurate readout. Three concentrations of the green-fluorescent melamine particles [0, 1, 2] mg/mL and the green-fluorescent PMMA particles [0, 0.6, 1.2] mg/mL were selected for the nano- and microscale barcode, respectively.

Images of the barcodes with two sizes of green-fluorescent particles incorporated into the network of P(DMAA-co-4.6% MABP-co-2.5% SSNa) are shown in Figure 3a. Both fluorescent particles are homogeneously distributed throughout the volume of the hydrogel without a significant formation of agglomerations. Furthermore, because of the immobilization via covalent bonds and physical entrapment, no leaching-out of particles was observed. The microscale barcode stands out...
Figure 3. Analysis of the $3 \times 3$ green-fluorescent barcoding matrix. (a) Stitched green-fluorescent image of hydrogel beads carrying all combinations of the nano- and microscale particles at concentrations of $[0, 0.6, 1.2]$ and $[0, 1, 2]$ mg/mL, respectively. (b) Statistical analysis of the barcoding matrix and assignment of the respective numerical code $C_{XY}$. Error bars represent standard errors ($n = 24$).

clearly from the background of the nanoscale barcode ($C_{X1}$ and $C_{X2}$), resulting in a clear readout and a simplified counting of the bright fluorescent spots.

Figure 3b shows the statistical analysis for an average of 24 hydrogel beads per barcode. The nanoscale barcode can be incorporated with high accuracy, resulting in low standard deviations (the error bars for the six codes $C_{0Y}$ and $C_{2Y}$ are hidden by the symbol in the graph). In the case of the microscale barcodes, the three particle concentrations can be separated by more than three standard deviations. The high precision (difference between the mean measured value and the target values of 0, 1, and 2) allows a reproducible production of the individual barcodes. On the basis of the statistical analysis, the visual representation of the barcodes can be directly translated into the numerical code ($C_{XY}$) by using the normalized values.

The combination of the photoreactive copolymer and the differently sized, fluorescent particles allows a simple customization of the barcodes by mixing the particles in the desired ratio. The production of the functionalized hydrogel beads is carried out in a continuous process and the analysis can be performed with a single image from a commercially available plate reader and is independent of the orientation of the hydrogel beads during readout.

**Multiplexed Immunoassay on Biofunctional Hydrogels.** After establishing the model reactions in a $3 \times 3$ matrix, the suitability of the presented system for serological assays is demonstrated by detecting antibodies specifically present in human serum in the case of Lyme disease. For this purpose, a green-fluorescence image of the hydrogel beads is acquired to read out the barcodes and thus identify the immunoassays carried out on each bead. A red-fluorescence image of the same beads is then used to quantify the intensity of the immunoassay. Since four different assays are carried out in parallel, a $2 \times 2$ barcoding matrix was created. This simplifies the decoding of the barcodes to a binary decision based on the presence or absence of the micro- and nanoscale particles, as shown in Figure 4.

Figure 4. Allocation table for the analysis of patient sera for Lyme disease. The two sizes of fluorescent particles form the code $C_{XY}$, which is used to identify the assay carried out on the respective hydrogel bead. VlsE1 Bb, OspC Bb and Bs, and Rabbit Anti-Goat IgG are covalently incorporated into the hydrogels to capture the corresponding antibodies. The target molecules, if present, are detected by fluorescently labeled Goat Anti-Human IgG.

Four bead types (all consisting of the copolymer) were prepared for the analysis of patient sera in a serological immunoassay format: the negative control, the positive control, and two test beads. One test bead contained the *Borrelia burgdorferi* (Bb) and *Borrelia spielmanii* (Bs) outer surface protein C (OspC) and microscale fluorescent particles, while the other test bead contained the variable major proteinlike sequence E1 (VlsE1) Bb and nanoscale fluorescent particles. The positive control contained a Rabbit Anti-Goat IgG and both fluorescent particle types, while the negative control contained only the copolymer. The first assay step was to incubate the beads (all four types present in the wells of a microplate) with serum, where humoral antibodies can specifically bind to the presented proteins. In a further incubation step, the antibodies to be detected (if bound to OspC or VlsE1) are recognized by a fluorescently labeled detection antibody (Goat Anti-Human IgG DyLight 650). In the positive control, the capture antibody binds to the detection antibody. It is required to demonstrate that the capture antibody is in a native state. Denaturation of the capture antibody due to damage during storage or any of the process steps would negatively affect its functionality, making it unable to bind the detection antibody. In the case of the negative control, the detection antibody can only adsorb to the hydrogel network without specific binding. This fluorescence...
intensity represents the background intensity of the assay and is therefore subtracted from the signal obtained with the other beads.

A green-fluorescence image and a red-fluorescence image of one well are shown in Figure 5a,b, respectively. The outlines of the hydrogel beads without green-fluorescent particles are traced by a dashed line in Figure 5a for better visibility. After incubation of the hydrogel beads in suspension, the beads sediment and assemble next to each other, allowing up to 24 beads per well to be analyzed without overlap. Movement of the beads between taking the green and red images is negligible when the two images are acquired within a short time. Therefore, the image taken in the green channel, containing the barcodes, was successfully used to identify the immunoassays in the red channel.

In Figure 5a, it is noticeable that the red fluorescent dye (DyLight 650) has a spectral bleed through to the green channel, which falsely increases the intensity of the green-fluorescence of the nanoscale barcode. This effect is particularly pronounced with highly positive sera, as is the case with the detection of anti-OspC in the example shown. However, this constant effect, which accounts for about 1.4% of the red-fluorescence intensity, can be subtracted from the individual hydrogel beads in the green channel. On the other hand, the emission spectrum and the concentration of the green-fluorescent particles were selected so that the bleed through into the red (immunoassay) channel can be neglected.

Figure 5c summarizes the fluorescence intensities of the four different assays for one well of the positively tested patient serum PS1922 and the negative blood donor sample BSP8056. In the case of the patient serum, the intensities of the two serological assays (test for anti-OspC and test for anti-VlsE1) are strongly increased compared to the intensity of the negative control. No significant increase in the intensity of the test for anti-OspC and anti-VlsE1 was observed in the blood donor sample compared to the negative control. These results indicate that the capture proteins and the detection antibodies are able to bind specifically to the humoral antibodies present in the patient serum but not to other proteins present in the serum. The small standard deviation and the low intensity of the negative control allow the reliable detection of the increased signal of the test assays.

A low concentration of capture antibody (0.3 mg/mL) was chosen for the positive control compared to the concentrations of VlsE1 (0.56 mg/mL) and OspC (two times 0.6 mg/mL). This was done so to reduce competition for the binding sites of the detection antibody between the test assays and the positive control. This decision leads to results where the test assays can have a higher intensity than the positive control. Nevertheless, in all cases the intensity of the positive control was significantly higher than the intensity of the negative control, as confirmed by a paired sample $t$ test with 10 sera in 3 replicas each ($p < 0.001$). In these experiments, the positive control indicates that the capture antibodies binding to the Fc-region of the detection antibody maintained their native state during hydrogel fabrication and storage.

The multiplexing of four assays in a single well has several advantages. First, the required volume of sera and reagents is reduced by a factor of 4 compared to singleplex assays. Second, since all process steps are carried out simultaneously, all parameters such as temperature, reagent concentrations, volume of incubated serum, or incubation time are identical for all beads in one well. This leads to an ideal comparability between the resulting intensities of the control and test assays.

Analysis of Patient Sera. To establish a threshold intensity for characterizing patient samples as “positive” or “negative” for antibodies against OspC or VlsE1, five negative blood donor sera were analyzed. The negative control, the test for anti-OspC, and the test for anti-VlsE1 were each carried out on an average of five beads per well, in a replicate of three wells for each of the five blood donor sera. Intensity thresholds, plotted as dashed lines in Figure 6, were then calculated as the mean background-corrected intensity plus three standard deviations ($n = 45$) of each assay according to eq 2 in Material and Methods.

The mean background-corrected intensities of hydrogel beads testing for antibodies against OspC and VlsE1 were compared to the previously established threshold in Figure 6a,b, respectively. Each patient serum was measured in triplicate with an average of five hydrogel beads for each assay type and replicate. The error bars in Figure 6 represent the standard error. Following similar assay formats, an intensity value above or equal to the threshold value represents a positive test for the respective biomarker, a sample slightly below the threshold is marked as marginal, and a signal clearly below the threshold is considered negative for the biomarker.
In Figure 6a, two of the patient sera (PS1646 and PS1922) were measured positive for antibodies against the protein OspC, while the other three were clearly below the threshold and thus negative for OspC. When tested for antibodies against the protein VlsE1, all but one serum (PS1623) showed an intensity above the threshold and thus tested positive for the biomarker (Figure 6b). With an intensity value of 2281, the test for anti-VlsE1 on the sample PS1670 is above the threshold value of 2215 and is thus also considered positive. The serum PS1623 is clearly below the threshold. Thus, all sera are in agreement with the external characterization by reference tests, stated in parentheses under the respective sample number.

### CONCLUSION AND OUTLOOK

Photoinduced hydrogel formation in a two-phase flow is a versatile way to produce barcoded and biofunctionalized hydrogel beads for multiplexed immunoassays in a single-step reaction. The incorporation of the photoreactive cross-linker as a side group of the copolymer allows the formation of hydrogel particles through C,H-insertion without the use of low-molecular weight cross-linkers or monomers that could contaminate the beads and leach out during storage. Such material loss and contamination of the storage solution would pose an additional challenge for quality control. In addition, the issue of immiscibility between hydrophilic proteins and hydrophobic cross-linkers is circumvented.

The visual representation of the barcodes is based on the covalent incorporation of off-the-shelf particles during the hydrogel network formation. In this way, a 2D matrix of individual barcodes is generated that can be decoded with a single fluorescence image, independent of the orientation of the hydrogel beads during readout.

In addition to its efficient fabrication and readout, the presented approach for barcoding hydrogel beads also allows for a variety of simple adaptations to increase the number of different barcodes. The main requirement for the fluorescent particles is that they form at least a temporarily stable dispersion in the aqueous polymer solution used in the two-phase-flow microfluidics. The use of differently shaped green-fluorescent particles, such as rods, would further increase the dimensionality of the barcoding system.

The production of a larger number of graphically encoded labels, in contrast, would require sufficient optical resolution of the photolithographic setup to produce small-scale features. On the other hand, reading out larger numbers of fluorescent labels with a multicolor optical barcode would be challenging as deconvolution of overlapping spectra is required. This strongly limits the information encoding process as with increasing number of labels the read-out becomes more and more challenging. In the approach presented herein, the complexity of fabrication and readout does not scale with the number of total barcodes.

The presented concept for the generation of barcoded hydrogel beads is well suited for a scale-up to an industrial process since the beads are produced in a continuous process that allows for faster production than a stop-flow or batch process. In addition, the fluidic setup is simple, making a scale-up through parallelization rather simple. An efficient and cost-effective analysis of the barcodes is possible for both high-throughput and benchtop experiments, because a single fluorescence image of the rotationally symmetric hydrogel beads obtained by a standard plate reader is sufficient.

Overall, the presented process is a simple and robust way to encode information into hydrogel beads. The labels and the attached capture molecules can be tailored to the specific needs with little effort. The encoded information of the barcodes can be read out in an efficient and cost-effective way using a single fluorescence image.

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