Biosensors and Bioelectronics 115 (2018) 97–103

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Multiplexed antibody detection from blood sera by immobilization of *in vitro* expressed antigens and label-free readout via imaging reflectometric interferometry (iRIf)

Julian Bender[a,e,1,3], Sabine Bognar[a,e,1], Maurizio Camagna[b,e,1,4], Julia A.M. Donauer[b,e,1], Julian W. Eble[b,e,1,5], Ramona Emig[b,e,1], Sabrina Fischer[b,e,1], Rabea Jesser[b,e,1,6], Luisa Keilholz[a,e,1], Daniel M.U. Kokotek[b,e,1], Julika Neumann[c,e,1], Simon Nicklaus[a,e,1], Ricardo R.Q.P.T. Oude Weernink[d,e,1,7], Lara G. Stühn[b,e,1,8], Nathalie Wössner[a,e,1], Stefan D. Krämer[b,f,2], Philipp Schwenk[b,g,h,2], Nicole Gensch[e,2], Günter Roth[b,e,f,*,2], Maximilian H. Ulbrich[c,e,i,*,2]

[a] *Faculty of Chemistry and Pharmacy, University of Freiburg, 79104 Freiburg, Germany*
[b] *Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany*
[c] *Faculty of Medicine, University of Freiburg, 79110 Freiburg, Germany*
[d] *Institute of Physics, University of Freiburg, 79104 Freiburg, Germany*
[e] *BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany*
[f] *ZBSA – Center for Biological Systems Analysis, University of Freiburg, 79104 Freiburg, Germany*
[g] *Institute of Biology II, Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany*
[h] *Spemann Graduate School of Biology and Medicine (SGBM), University of Freiburg, 79104 Freiburg, Germany*
[i] *Renal Division, Freiburg University Medical Center, 79106 Freiburg, Germany*

ARTICLE INFO

Keywords:
in vitro expression
Surface immobilization
Antibody detection
Imaging reflectometric interferometry (iRIf)

ABSTRACT

The detection of antibodies from blood sera is crucial for diagnostic purposes. Miniaturized protein assays in combination with microfluidic setups hold great potential by enabling automated handling and multiplexed analyses. Yet, the separate expression, purification, and storage of many individual proteins are time consuming and limit applicability. *In vitro* cell-free expression has been proposed as an alternative procedure for the generation of protein assays. We report the successful *in vitro* expression of different model proteins from DNA templates with an optimized expression mix. His_{10}-tagged proteins were specifically captured and immobilized on a Ni-NTA coated sensor surface directly from the *in vitro* expression mix. Finally, the specific binding of antibodies from rabbit-derived blood sera to the immobilized proteins was monitored by imaging reflectometric interferometry (iRIf). Antibodies in the blood sera could be identified by binding to the respective epitopes with minimal cross reactivity. The results show the potential of *in vitro* expression and label-free detection for binding assays in general and diagnostic purposes in specific.

1. Introduction

The accurate and timely treatment of patients is a key task of modern medicine and critically relies on a fast diagnosis. Accordingly, devices for diagnostics should ideally offer characteristics such as rapidity, simplicity, unambiguity, and compatibility with multiplexing

* Corresponding authors at: BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany.
E-mail addresses: guenter.roth@zbsa.uni-freiburg.de (G. Roth), max.ulbrich@bioss.uni-freiburg.de (M.H. Ulbrich).
[1] These authors contributed equally to this work.
[2] The last 5 authors (Krämer to Ulbrich) have an equal contribution (2) equivalent to senior author / project leaders.
[3] Present address: Interdisciplinary Research Center HALOmem, Martin Luther University Halle-Wittenberg, 06120 Halle, Germany
[4] Present address: Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
[5] Present address: Faculty of Medicine, University of Freiburg, 79110 Freiburg, Germany
[6] Present address: Epigenetics and Neurobiology Unit, European Molecular Biology Laboratory (EMBL), 00015 Monterotondo, Italy
[7] Present address: Institute of Theoretical Physics, FSU Jena, 07743 Jena, Germany
[8] Present address: ZBSA – Center for Biological Systems Analysis, University of Freiburg, 79104 Freiburg, Germany

https://doi.org/10.1016/j.bios.2018.05.022
Received 11 February 2018; Received in revised form 18 April 2018; Accepted 10 May 2018
Available online 12 May 2018
0956-5663/

(Yager et al., 2006). Common standard laboratory techniques for detecting antigen-antibody binding events are enzyme-linked immunosorbent assays (ELISA) or lateral flow tests (Chin et al., 2012). An emerging approach to enable rapid detection of specific analytes are lab-on-a-chip (LOC) systems (Mark et al., 2010) combined with microfluidics in the context of point-of-care (POC) diagnostics (Chin et al., 2012; Gervais et al., 2011). Most recently, many companies have been working on microfluidics-based POC systems, comprising applications for blood chemistry, toxicology screenings or pathogen identification, amongst others (Chin et al., 2012). Driven by the constant improvement of microfluidic technologies, LOC applications are meeting more and more complex demands (Lafleur et al., 2016). The parallel screening for multiple analytes as well as the miniaturization and automatization is desirable for the development of new assays to detect protein-binding events, e.g. the binding of antibodies present in a patient's serum sample to disease-specific antigens.

Suitable tools for these requirements are protein microarrays that allow arranging up to thousands of proteins in a predesigned layout (Zhu and Qian, 2012). Major challenges of protein microarrays are the elaborate synthesis ensuring proper protein folding and functionality as well as the prevention of degradation during storage (He et al., 2008; Mark et al., 2010). A suitable tool to overcome these restrictions are *in vitro* expression systems derived from prokaryotic (Nirenberg and Matthaei, 1961) or eukaryotic cells (Bank and Marks, 1966). Such cell-free expression lysates allow on-demand protein synthesis from durable and easily storable DNA templates. Meanwhile, several methods have been developed to generate protein microarrays from DNA arrays on demand, thereby avoiding laborious purification of proteins (Bernhard and Tozawa, 2013; Carlson et al., 2014; Chatterjee et al., 2008; He et al., 2008; Kilb et al., 2014; Ramachandran et al., 2004). Moreover, the application of *in vitro* expression systems greatly reduces the required reaction volume compared to standard protein purification methods (Kigawa et al., 1999). Additionally, microarray technologies only need small amounts of samples for reliable test results and allow investigating many analytes at once (He and Taussig, 2001; Zhu and Snyder, 2003). Another advantage of LOC systems are novel detection and handling methods.

Label-free optical detection systems, which are able to detect binding events of analytes, offer real-time readouts, bypass substrate development steps required for ELISA and avoid artefacts from fluorescent or enzymatically coupled labels (Daaboul et al., 2011; Kigawa et al., 1999). These systems are based on detecting changes in the refractive index at the surface of a sensor as a result of an antibody binding to its antigen. Different physical principles have been employed, such as surface plasmon resonance (Homola, 2003), reflectometric interference spectroscopy (Fechner et al., 2009; Pröll et al., 2005) or 1-λ imaging reflectometric interferometry (iRIf) (Burger et al., 2016; Fan et al., 2008). Using label-free methods, *Salmonella* (Ewald et al., 2013), tuberculosis (Nagel et al., 2008), influenza (Schwarz et al., 2010) or vaccinia virus (Proll et al., 2014) infection-related molecules were successfully identified by specific binding of antibodies.

In this study, we combined protein microarray generation with *in vitro* expression and label-free iRIf detection of antibodies from blood sera (Fig. 1) (Burger et al., 2016). A first proof of concept of the system was developed during the iGEM (international Genetically Engineered Machine) competition 2015 (detailed lab journals, scripts and results are accessible at http://2015.igem.org/Team:Freiburg) and completed with the present study. Ideally, antigen expression from DNA and subsequent detection of antibodies present in a patient's serum sample would be carried out in one single microfluidic chamber. However, to reduce complexity, here we expressed DNA templates *in vitro* in separate vials. We chose the well-characterized green fluorescent protein (GFP) and the red fluorescent protein mCherry (mCh) to develop and optimize an *E. coli*-based *in vitro* expression system. His_{10}-tagged proteins were then specifically immobilized on a nickel nitrilotriacetic acid (Ni-NTA) functionalized glass slide, which prevents non-specific binding of other components from the *in vitro* expression lysate. Finally, diluted sera of rabbits immunized to GFP or mCh were applied to the glass slides via microfluidics. The binding of antibodies recognizing GFP and mCh from sera to their corresponding immobilized antigens on the glass slides was monitored via iRIf (Suppl. Fig. 1). Our results illustrate the possibility of combining *in vitro* expression systems with label-free microfluidic readouts, and emphasize the potential of on-site produced protein microarrays for multiplexed analysis in diagnostic applications.

2. Material and methods

For full protocols, see Supplementary information.

2.1. Reagents

iRIf glass slides with buried Ta_2O_5 and SiO_2 layers were kindly provided by Biametrics (Tübingen, Germany). Anti-GFP serum (rabbit) and negative serum (rabbit, chalcone synthase) were kindly provided by Dr. Thomas Kretsch and Dr. Stefan Kircher, University of Freiburg. Other antibodies and sera are commercially available (anti-mCh serum: Abcam ab218032; Anti-GFP antibody: Rockland 600–106-215; anti-mCh antibody: Abcam ab167453; HRP-coupled anti-rabbit and anti-goat: SantaCruz, sc-2030, sc-2768).

2.2. In vivo protein expression and purification

The *in vivo* expression was realized in a standard approach. The pET constructs were transformed into BL21(DE3) cells for protein expression. After culturing, the cells were lysed and the proteins were purified via the His-Tag with Ni-NTA coated beads. For a subset of purified proteins, the His-Tag was removed by TEV cleavage.

2.3. In vitro protein expression

A prokaryotic *in vitro* transcription/translation system based on *Escherichia coli* was established. BL21(DE3) cells expressing rare codon tRNA and T7 RNA polymerase were lysed, the ribosomal fraction was isolated via centrifugation, supplemented with energy buffer and amino acids, and stored in aliquots at −80 °C.

To set up an *in vitro* transcription and translation reaction, 45% (v/v) processed lysate was mixed with reagents for energy regeneration, transcription and translation. The reaction was started by adding plasmid DNA, followed by incubation at 37 °C for 1–3 h. We used 50 or 100 µl reactions in tubes or flat black 96-well plates to measure fluorescent protein abundance.

2.4. Surface chemistry

For Ni-NTA functionalization of surfaces an universal protocol to bind amino-moieties to surfaces with 1,4-phenylene diisothiocyanate (PDITC) (adapted by Hoffmann et al., 2012) was used as first step. In a second step, slides were incubated with aminobutyl-NTA (AB-NTA) to introduce the Ni-NTA moiety. Directly before the experiment surfaces were blocked with BSA, loaded with a $NiSO_4$ solution, and washed.

2.5. Protein immobilization/capturing

Purified proteins or expression lysates were spotted onto functionalized slides using a PDMS spotting mask, incubated at 4 °C for at least 4 h, and blocked with BSA. The spotting mask was removed, and the slides were directly transferred to slide holders, immersed in BSA for blocking, washed with PBS and imidazole, and blocked again with BSA.

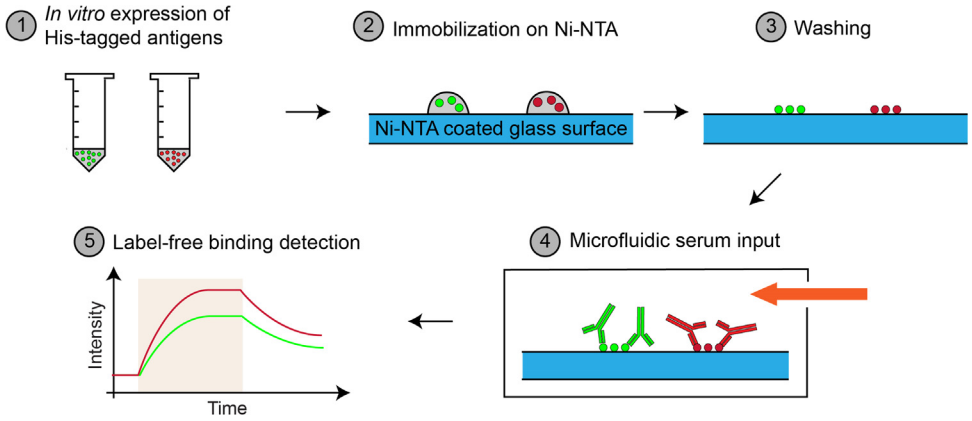

Fig. 1. Schematic illustration of the workflow. (1) Antigens (GFP-His_{10} and mCh-His_{10}) were expressed *in vitro*. (2) The *in vitro* expression mix containing the antigens was applied to a Ni-NTA coated glass surface. (3) After washing, only the His_{10}-tagged proteins remain at the surface because of specific binding. (4) The slides were flushed with sera containing antibodies via a microfluidic system. (5) Binding events were detected label-free by imaging reflectometric interferometry (iRIf).

2.6. Fluorescence measurements

For monitoring *in vitro* expression, reactions were set up in black 96-well plates, and GFP or mCh fluorescence was monitored in a microplate reader. The spot fluorescence on slides was measured by a Nikon AZ100 microscope with a Plan Apo 0.5× objective lens.

2.7. Imaging reflectometric interferometry (iRIf)

We used the published device from the research group of G. Roth (ZBSA, University Tübingen) (Burger et al., 2016) for label-free real-time imaging of binding events, automated data acquisition, image generation and binding kinetics. Prior to serum and antibody measurements, slides were washed, blocked with BSA and washed with PBS for equilibration. Binding kinetic analysis was performed by marking spots of interest and an according local background. For each spot, a quotient value was calculated during the measurement (for description of detection principle, device, mathematics and calculation routine, see Burger et al., 2016).

2.8. ELISA

To determine antibody titers in sera, standard ELISA was performed using purified antigens (GFP or mCh) to capture respective antibodies from the sera.

3. Results & discussion

3.1. In vitro expression is a suitable tool for fast and multiplexed protein expression

First, we searched for an *in vitro* expression system suitable for different DNA templates. Many protocols and commercial products with proprietary formulation are available. With the aim of using basic compounds that are easily available, we developed an *in vitro* expression mix based on *E. coli* lysate and optimized it for high yield.

To assess the performance of the mix via fluorescence, we expressed GFP-His_{10} and mCh-His_{10} (Fig. 2). First signals were visible for GFP after 10 min and 70 min for mCherry. A maximum signal peak was reached after 80 min (GFP) and 300 min (mCherry). We assume photobleaching effects to cause the slight drop of signal after the peak. The signal delay between GFP and the slower mCherry may be due to slower folding and activation (Kelkar et al., 2012). Our finding is consistent with published data stating 3 min maturation time for GFP and 57 min for mCh in *in vitro* systems at 37 °C (MacDonald et al., 2012). Therefore the mCh signal still increases at 4 °C. *In vitro* expression yielded protein levels between 50 and 100 µg/ml for GFP and 12.5–25 µg/ml for mCh, as determined by fluorescence in comparison to protein standards (Suppl. Fig. 2). The presence and accumulation of fluorescent GFP and mCh demonstrate the suitability of the *in vitro* expression system for multiplexed expression of proteins.

3.2. Specific capture of in vitro expressed, His_{10}-tagged proteins on Ni-NTA functionalized glass slides

Next, we wanted to generate a protein microarray assembled from the *in vitro* expressed proteins of interest by capture from the *in vitro* expression mix and immobilization on a surface. Commonly used PDITC surfaces bind all molecules carrying an amino group and therefore are not suited for specific capture of tagged proteins from *in vitro* expression systems (Hoffmann et al., 2012). We chose the His-tag / Ni-NTA ligand system, which exploits the high affinity between nitrogen atoms of adjacent histidine residues of a genetically encodable poly-histidine tag and the free coordination sites of Nickel ions which are immobilized on nitrilotriacetic acid (Beers and Callis, 1993).

In the following step, we aimed to show the specific and exclusive binding of His_{10}-tagged proteins to Ni-NTA coated glass slides as well as their direct capturing from the *in vitro* expression mix. To this end, we compared the binding of His_{10}-tagged proteins expressed in *E. coli* and purified by immobilized metal ion affinity chromatography with that of the crude, *in vitro* expressed proteins to PDITC and Ni-NTA surfaces. *In vivo* expressed GFP-His_{10} and mCh-His_{10} were purified using Ni-NTA beads. Subsequently, for a subset the His-tag was removed by TEV cleavage. *In vitro* expression of GFP-His_{10} and mCh-His_{10} was performed with the *in vitro* expression mix as in the previous section. Purified GFP-His_{10}, mCh-His_{10}, GFP and mCh as well as non-purified, *in vitro* expressed GFP-His_{10} and mCh-His_{10} were spotted on glass slides with a PDITC or Ni-NTA surface and incubated for 4 h at 4 °C. After washing, we assessed the presence of bound GFP or mCh proteins on PDITC and Ni-NTA surfaces via fluorescence (Fig. 3).

Both, GFP and mCh, bound to the PDITC surface independent of their His_{10}-tag's presence (Fig. 3A). In contrast, we did not detect significant binding of the fluorescent proteins from the *in vitro* expression mix to the PDITC surface, likely because they had to compete for binding with the large excess of untagged proteins from the *in vitro* expression mix, highlighting the need for a specific capturing surface. On the Ni-NTA surface, purified as well as *in vitro* expressed His_{10}-tagged proteins could be detected. However, no purified GFP or mCh without His-tag could be observed, since the Ni-NTA surface specifically captures His_{10}-tagged proteins only (Fig. 3B). Most importantly, the *in vitro* expressed GFP-His_{10} and mCh-His_{10} proteins bound to the Ni-NTA surface despite their low quantity in the expression mix.

We demonstrate that the application of Ni-NTA as the top layer of the sensor surface allows specific immobilization of target proteins from a complex mixture, thereby avoiding the need for conventional purification. We conclude that the combination of *in vitro* expression and specific immobilization is a suitable tool for the fast generation of protein microarrays.

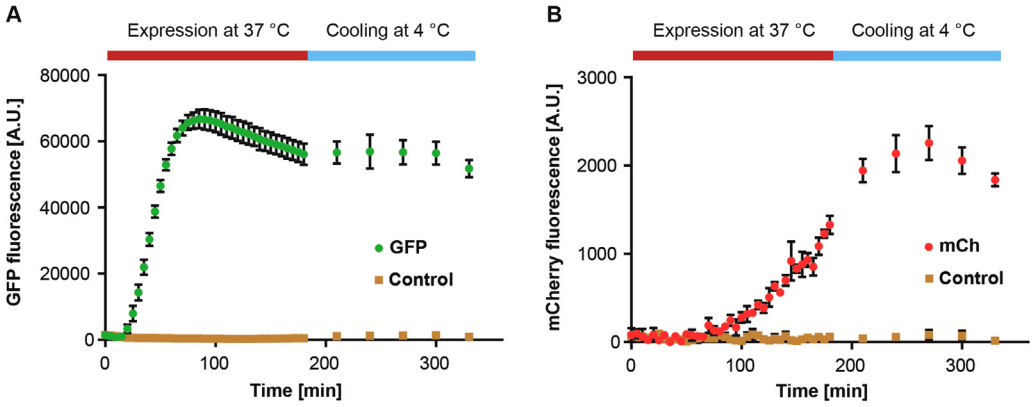

Fig. 2. Protein expression with an optimized *E. coli*-based *in vitro* expression mix. Fluorescence was recorded with a microplate reader from 96-well plates (n = 3, error bars are s.d.). (A) GFP fluorescence peaked at 80 min and then decreased due to photobleaching. During the cooling phase, virtually no photobleaching occurred due to the longer intervals between sampling points. (B) mCh fluorescence appeared much later than GFP due to longer maturation time, and peaked at 270 min. As negative control *in vitro* transcription and translation mix without template DNA was used.

3.3. Simultaneous label-free detection of antibodies from rabbit-derived sera by iRIf

Next, we aimed to demonstrate the applicability of the on-demand microarray for antibody screening. As a proof of principle, we used the Ni-NTA functionalized glass slides with bound GFP-His_{10} and mCh-His_{10} to detect anti-GFP and anti-mCh antibodies from diluted blood sera of rabbits that were immunized with GFP and mCh, respectively. We measured the time course of antibody binding to the microarray by iRIf. The measuring principle via iRIf is illustrated schematically in Supplementary Fig. 1.

Ni-NTA functionalized glass slides from the previous experiment were used with the identical spotting pattern (Fig. 4A). The slides were flushed successively with an anti-chalcone synthase (CHS) serum, which served as negative control, anti-GFP serum, anti-mCh serum and an anti-rabbit antibody solution for 10 min each, while continuously recording the iRIf image to monitor the amount of protein bound to the surface. Between serum applications, the slide was flushed with PBS for 10 min each time.

When flushing with anti-CHS serum, no binding events were detected (Fig. 4B). However, when flushing with anti-GFP serum, binding was detected exclusively at GFP-His_{10} spots (Fig. 4C). When flushing with anti-mCh serum, binding events were detected exclusively at mCh-His_{10} spots (Fig. 4D). To verify that the binding events were indeed caused by the binding of rabbit-derived antibodies (all three sera were derived from rabbit), the slides were finally flushed with an anti-rabbit antibody solution. During the last flush, binding events were detected at positions where GFP and mCh antibodies had been detected in the previous flushes (Fig. 4E).

Notably, the maximum reflectivity change caused by the binding of anti-mCh was > 10 fold greater (approx. 2.2% maximum change) than the maximum relative signal change caused by the binding of anti-GFP antibodies (approx. 0.15% maximum change) (Fig. 4F). Furthermore, the maximum reflectivity change caused by anti-rabbit antibodies differs between spots where GFP and mCh antibodies have been detected (further change of reflectivity of approx. 0.2% for GFP spots and 0.6% for mCh spots). The quantitative change in reflectivity was consistent between independent experiments (Fig. 4G, Suppl. Table 1).

The amount of reflectivity change mainly depends on the titer of the antibody, the binding affinity (K_D) of the antibody to its corresponding antigen, and the accessibility of the antigen after immobilization on the surface. Anti-GFP/anti-mCh antibody-titers of sera were determined by ELISA (Suppl. Fig. 3). For anti-GFP serum, a concentration of 0.32 ± 0.05 mg/ml (s.d., n = 6) antibody equivalents was determined, for anti-mCh serum an antibody titer of 3.3 ± 0.7 mg/ml (n = 5). We assume that the difference in signal shifts for anti-GFP and anti-mCh

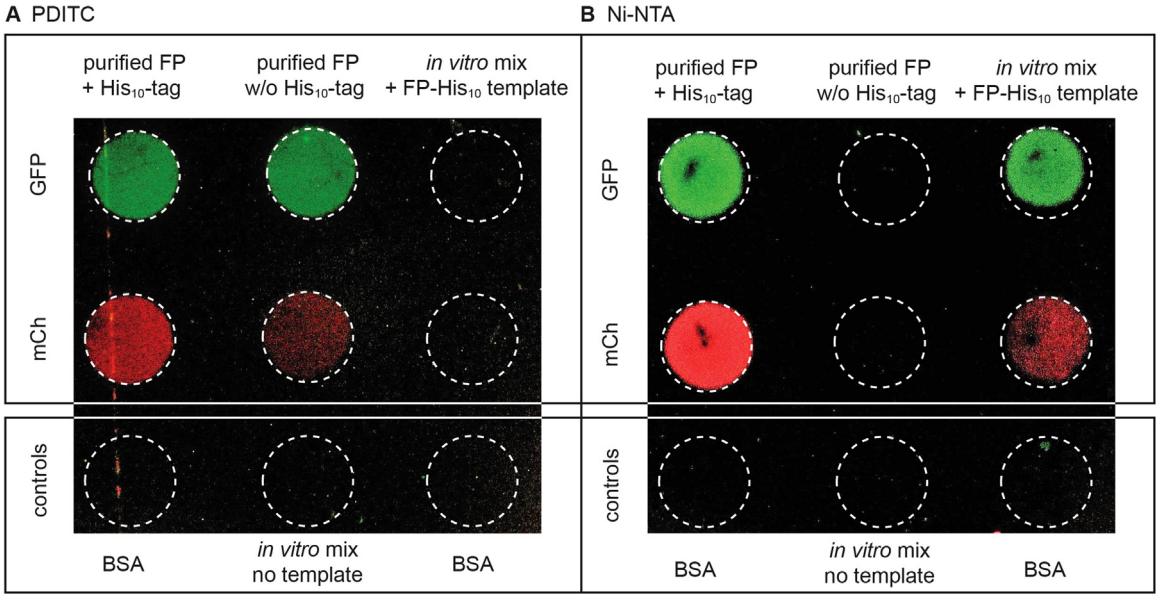

Fig. 3. Specific capture of His_{10}-tagged proteins by Ni-NTA coated glass slides. The fluorescence of GFP and mCh immobilized on functionalized glass slides was imaged by wide-field microscopy. (A) On PDITC, fluorescence was only detected for purified GFP and mCh, independent of the presence of the His_{10}-tag, but not for the fluorescent proteins (FPs) from the *in vitro* mix. (B) On Ni-NTA, fluorescence was detected only for His_{10}-tagged GFP and mCh, regardless of whether they were purified or still in the *in vitro* mix, whereas no binding was detected for the FPs without His_{10}-tag.

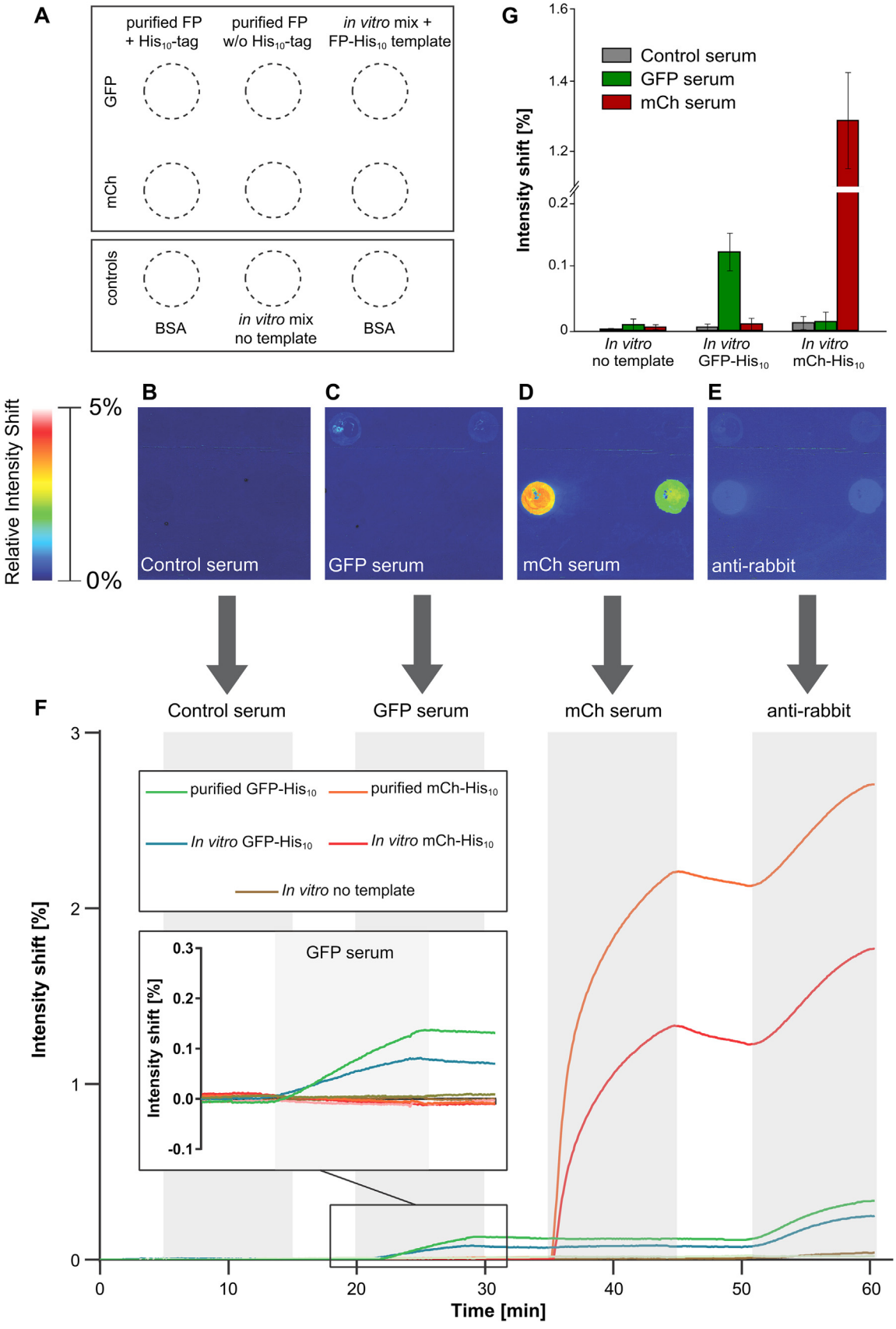

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Fig. 4. Detection of different antibodies from sera by iRIf. A protein microarray on a Ni-NTA sensor surface was sequentially flushed with rabbit-derived sera (CHS [negative control], GFP and mCh immunized) and anti-rabbit antibody solution. Binding events on the surface of the microarray were detected by iRIf. (A) *(Left)* Spotting pattern of the protein microarray on slides coated with Ni-NTA. *(Below)* Color scale for intensity shifts in the quotient pictures (B-E). (B) No binding was detected when CHS control serum was flushed. (C) Next, serum from rabbit immunized to GFP was flushed. Binding occurred at spots with purified GFP-His_{10} and GFP-His_{10} from the *in vitro* mix. (D) Serum from rabbit immunized to mCh was flushed. Binding occurred at spots with purified mCh-His_{10} and mCh-His_{10} from the *in vitro* mix. (E) Finally, anti-rabbit antibody solution was flushed. Binding occurred at all spots where antibodies from the rabbit sera had bound to GFP and mCh. (F) Relative intensity shift over time for all spots with His_{10}-tagged proteins and control in a representative experiment. The signal at spots with mCh-His_{10} was larger than that from GFP-His_{10} by a factor of > 10, likely because of higher antibody concentrations in the sera, as suggested by ELISA tests of the sera. (G) Maximum relative intensity shift for all spots with *in vitro* expressed His_{10}-tagged proteins and control after flushing the respective sera. n = 3, error bars represent s.d.

binding was mainly caused by differences in antibody titers. Further support for this explanation is provided by the higher signal increase at anti-mCh spots (0.6%) than at anti-GFP spots (0.2%) during the flush with anti-rabbit antibodies.

Ewald et al. (2015) showed the antibody-titer dependent signal shift for iRIf measurements exemplary by anti-*Salmonella* antibodies binding to their corresponding antigens. To assess the sensitivity of our assay and the correlation between signal strength and antibody titers, we used purified antibodies at different concentrations down to the detection limit (Suppl. Fig. 4). For both antibodies, the minimum detectable concentration was 0.3 µg/ml, and 0.1 µg/ml could not be discriminated from background. The different signal shifts we observed for GFP and mCh antibodies at equal concentrations possibly depend on antibody properties, e.g. affinity and clonality.

The dependence of signal strength on antibody titer is essential for clinical applications, which aim to determine antibody titers within blood sera via label-free detection. Notably, antibody titers within sera and the affinity of antibodies not only depend on the antigen, but also on the time after meeting the antigens (class switch, affinity maturation), or even vary between individuals, making an absolute quantification of antibody titers challenging (Kerbel and Eidinger, 1971; Nagel et al., 2008).

In summary, we detected the presence of different antibodies in blood sera using the antigen microarray produced from DNA samples by a single *in vitro* expression mix. Strong iRIf signals originated from spots where antigen was immobilized and the antibodies from sera matched, and no signal was visible at spots without matching antibodies, demonstrating that an unequivocal identification of the sera's antigenicity was possible. Furthermore, the iRIf signal appears to correlate with the antibody titer of the respective blood serum, which potentially could allow a quantitative analysis of multiple antibody concentrations from a single serum sample.

4. Conclusion

In this study, we used a self-prepared optimized transcription/translation mix for the *in vitro* generation of His_{10}-tagged GFP and mCh. To specifically capture the proteins without time-consuming purification steps, we designed a Ni-NTA-based glass surface that only binds the His_{10}-tagged proteins from the crude expression and translation mix. Finally, we used the protein microarray for the label-free detection of different specific antibodies directly from blood sera via iRIf (Burger et al., 2016).

Pilot studies with LOC systems and label-free readout already showed the successful detection of disease-related antibodies from sera. One example is the detection of *Salmonella* related antibodies in animal blood sera after infection (Ewald et al., 2013). Here, lipopolysaccharides of *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis were immobilized on the sensor surface to detect anti-*Salmonella* antibodies. Another example for label-free detection of infections in serum samples is the work of Nagel et al. (2008). They could show that tuberculosis-specific antibodies can be detected in human blood serum with different label-free detection methods. Furthermore, Bleher et al. (2014) showed the successful parallelized detection of many antigen-antibody interactions.

In all of the above studies, antigens were purified before immobilization on the surface, increasing the need for trained personnel and time. Using *in vitro* expression of different DNA templates and direct immobilization on functionalized surfaces allows the fast generation of multiplexed protein microarrays with minimum effort. The critical step of slide functionalization can be outsourced to specialized off-site facilities. We have shown that the specific Ni-NTA surface exclusively captures His_{10}-tagged proteins and no detectable background signal was measured for the diluted serum samples (negative control). Combining the strengths of different, well-established techniques (*in vitro* expression, substrate functionalization and iRIf), we demonstrate the possibility to reliably detect different antibodies directly from blood sera. Our approach can therefore be a valuable asset for future serological application, both from a clinical and a research point of view.

Acknowledgements

We would like to thank the whole research group of Dr. Roth, especially Jürgen Burger for his technical support conducting iRIf measurements, Philipp Meyer for his help producing PDMS flow cells and Normann Kilb, Tobias Herz and Johannes Wöhrle for their technical support and helpful advice. Additionally, we are grateful to Biametrics GmbH for providing transducers for reflectometric measurements.

Funding sources

The project was supported by the Excellence Initiative of the German Federal and State Governments (EXC 294), the GSC-4/Spemann Graduate School (to P. Schwenk) and the BMBF (VIP+ 01200, Biomolecule Copier, G. Roth) enabling us to use the iRIf devices.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2018.05.022.

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