

Single step, direct fluorescence immunoassays based on metal enhanced fluorescence (MEF-FIA) applicable as micro plate-, array-, multiplexing- or point of care-format

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ABSTRACT

Although Enzyme Linked Immuno Sorbent Assay (ELISA) technology is approaching its 45th year of existence since first described in 1971, it is still the main diagnostic tool in clinical research and routine diagnostics.

However, despite its broad usage it suffers from some drawbacks, limiting its use especially in more advanced assay formats like multiplexing platforms, point of care devices or protein arrays. Those limitations result from the need for an enzyme label, a soluble enzyme substrate, washing steps (multiplexing, point care, arrays) and in some cases also insufficient sensitivity, because the majority of circulating proteins and thus potential biomarkers may be found in lower sub-picomolar concentrations.

We hereby present a new assay platform based on metal enhanced fluorescence (MEF), that remedies these problems since it eliminates the need for washing steps, for using enzyme labels and allows detection of analytes down to sub-picomolar concentrations. In addition this technology is fully compatible to standard fluorescence reader equipment as it is found in many laboratories nowadays.

Since our present work is focused on single biomarker evaluation, we chose a 96 well plate format for convenience, but any other format like antibody arrays, strip-like point of care devices etc. is feasible too.

Introduction

Although Enzyme Linked Immuno Sorbent Assay (ELISA) technology is approaching its 45th year of existence since first described by Engvall and Perlman [1] in 1971, it is still the main diagnostic tool in clinical research and routine diagnostics.

However, despite its broad usage it suffers from some drawbacks, limiting its use especially in more advanced assay formats like multiplexing platforms, point of care devices or protein arrays.

Those limitations result from the need for an enzyme label, a soluble enzyme substrate and washing steps (multiplexing, point care, arrays) and in some cases also insufficient sensitivity, because the majority of circulating proteins and thus potential biomarkers may be found in low- or sub-picomolar concentrations [2].

We hereby present a new assay platform based on metal enhanced fluorescence (MEF), that remedies these problems since it eliminates the need for washing steps, for using enzyme labels (Fig. 1) and allows detection of analytes down to sub-picomolar concentrations.

In addition this technology is fully compatible to standard fluorescence reader equipment as it is found in many laboratories nowadays.

Since our present work is focused on single biomarker evaluation, we chose a 96 well plate format for convenience, but any other format like antibody arrays, strip-like point of care devices etc. is feasible due to the generality of the concept.

Material and methods

Reagents

All chemicals and biologicals were used as received from the respective suppliers.

Metal enhanced fluorescence measurements and substrates

The principles of metal enhanced fluorescence are well understood and described in literature [3–5]. Briefly, the fluorescence of molecules approaching a surface with metallic nano-structures can be greatly enhanced, if those nano-structures have the right shape, size and distance to each other [6]. Silver and gold are the preferred metals used and there are a growing number of academic papers investigating

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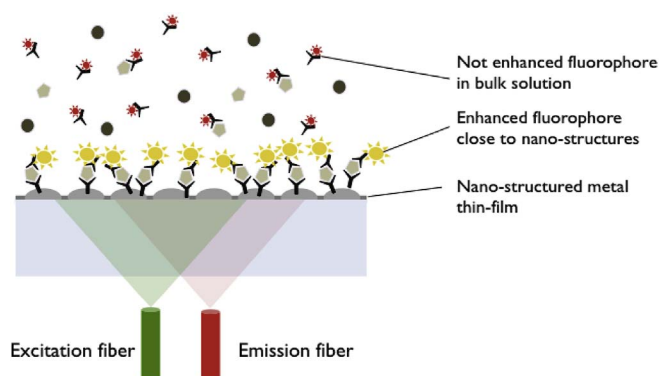


Fig. 1. Principle of Metal Enhanced Fluorescence: Only the fluorescence of molecules (yellow stars) close to the nanostructures is enhanced. Those further away (> 50 nm distance) do not increase in emission. This discrimination between bound/unbound molecules (e.g. fluorescent labeled antibodies) allows detection in the presence of the bulk solution, thus eliminating the need for washing steps. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

different aspects of MEF.

So far industrial application of MEF in life sciences was hindered by issues like stability of the metal structures in aqueous solution, reproducibility of the metallic nano-structures or insufficient fluorescence enhancement.

The MEF-structures used for the experiments in this paper were developed in cooperation with our partner STRATEC Consumables GmbH (former Sony DADC BioSciences GmbH) using proprietary manufacturing techniques derived from Blu-Ray production.

Briefly the manufacturing process can be described as a standard injection molding process but instead of creating macroscopic structures, micro-to nano-sized patterns are generated (Fig. 2).

The realized nanostructures consist of pit-like structure arranged in periodic hexagonal array. The pit have diameters below 500 nm, an aspect ratio of about 2, and a spacing of several hundred nanometers. The master was manufactured by laser lithography, copied into a Nickel - stamp by electroforming, and replicated in a disc format by a variothermal injection compression process. The process is similar to the production optical disc (CD, DVD or BD). As injection material a cyclic olefin polymer was used because of its a high transparency and its low level of background fluorescence in the visible region.

Subsequently, the polymer substrates were coated with a thin homogeneous Ag layer by a site directed DC sputtering process to form metal coated spots, that will become the bottom of microplates. After milling out the MEF-structures in microscope slide format, they are

laser-welded to a 96 well standard microplate frame thus forming the bottom of the microplate (Fig. 3).

The determining factors in terms of the order of enhancement (up to 300 fold) have been the distance (period) of the pit-like structures, the thickness of the silver layer and the type of fluorescent dye used [7].

The MEF-MTP format allowed fluorescence measurements to be easily performed by using a standard microplate reader (TECAN F200 pro) equipped with suitable fluorescence filters and a Xenon flash lamp by simply adding the fluorophore containing solutions under investigation to the wells of the plate (MEF-MTP). Measurements were done either in bottom (excitation and emission detection from below the plate) or top configuration (excitation and emission detection from above the plate) which are built in features of the reader used. To fully exploit bulk fluorescence suppression by MEF, bottom measurements were applied during assays without washing. If assays were washed, the measurement mode (bottom/top) had no impact on results. Standard measurement parameters were 25 flashes per well of the Xenon lamp with an integration time of 20 μ s. Gain (setting 1–255) of the built in photomultiplier tube was set to achieve at least 10000 fluorescence units (F.U.) between the lowest and highest result. For FITC, Cy3, Cy5 and AlexaFluor680 detection, filters with (Ex/Em) 485/535 nm, 535/590 nm, 635/680 nm and 670/720 nm having a bandwidth of 20 nm were used. The obtained enhancement of a specific assay was assessed by performing the identical assay on standard microplates from Greiner BioOne (Immuno-Strips, F-Form, 1×8 , High Binding, VWR 737-0195) or on the MEF-MTP plates without silver coating.

Adsorptive binding of fluorescence labeled antibodies to MEF-MTPs to assess basic system properties

Antibodies against rabbit IgG raised in goat and labeled with FITC, Cy3, Cy5 and AlexaFluor680 were purchased from Thermo Fisher Scientific, diluted in buffered solutions and incubated on MEF-MTPs, Greiner ImmunoStrips HB and MEF-MTPs without silver coatings. Binding of the antibodies was monitored in bottom configuration for a period of 2 h. Then plates were washed 3 times with 250 μ l sodium phosphate buffer (50mM/150 mM NaCl) pH 7.0 containing 0.1% (v/v) Triton X100 (Sigma-Aldrich, T9284) and measured again in bottom and top configuration.

Chromogenic detection of adsorbed rabbit IgG to determine coating efficiency

In order to compare the antibody coating efficiency of the developed MEF-surfaces to standard microplates from Greiner (see 2.3.) and Costar (1×8 Strip Well High Binding Plates, Sigma-Aldrich, CLS2592),

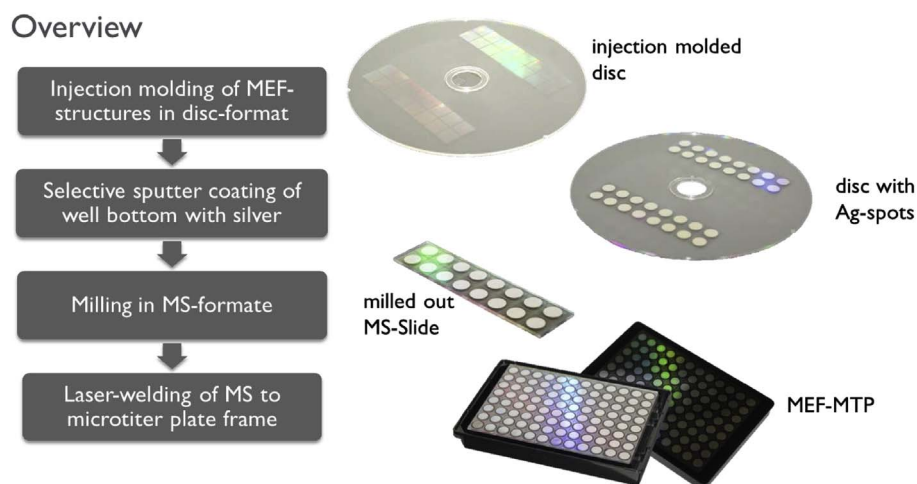


Fig. 2. Manufacturing process of MEF –MTPs.

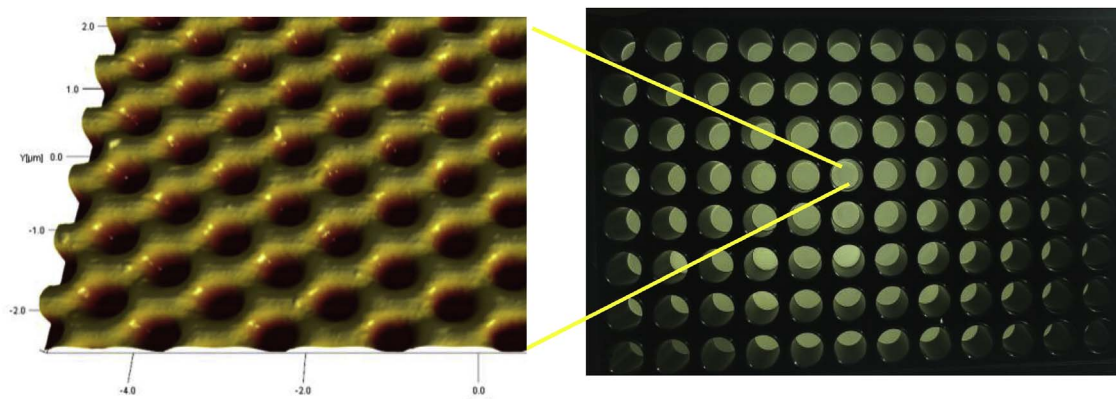


Fig. 3. Metal enhanced fluorescence micro titer plate (MEF-MTP, to the right). The pop out shows the silver coated nanostructure (to the left) at the bottom of the well that generates the MEF-effect.

wells were incubated with 50 μ l of a solution of immune purified rabbit IgG (0.125–2.0 μ g/ml, I5006 SIGMA) in sodium phosphate buffer (50mM/150 mM NaCl) pH 7.4 (PBS) for 2 h at room temperature. Plates were washed 3 times with 250 μ l PBS containing 0.1% (v/v) Triton and blocked with 100 μ l/well 2% (w/v) cold water fish skin gelatin (Sigma-Aldrich, G7041) for 1 h at room temperature. Following another washing step, the amount of adsorbed rabbit IgG was quantified (50 μ l/well) using an anti-rabbit IgG antibody labeled with alkaline phosphatase (A3687 SIGMA) diluted 1:5000 in PBS. After a final washing step the wells were filled with 100 μ l p-Nitrophenyl phosphate solution (SRE0026 Sigma) and the optical density read after 20 min of incubation.

Metal enhanced fluorescence immuno assays (MEF-FIAs)

Sandwich immunoassays were created by adsorptive coating of 50 μ l (0.25–2.0 μ g/ml) of the respective capture antibodies in buffered solutions pH 5–9 over night at 4 °C. Plates were washed 3 times with 250 μ l PBS pH 7.4 containing 0.1% (v/v) Triton X100 (Sigma-Aldrich, T9284, “PBSTx”) and unspecific binding blocked with 100 μ l/well 2% (w/v) cold water fish skin gelatin (Sigma-Aldrich, G7041) or 1% (w/v) skim milk powder (Sigma-Aldrich, 70166) in PBS with 2.5% saccharose for 1 h at room temperature. The blocking solution was aspirated and the plates were allowed to dry over night at room temperature in a drying cabinet (relative humidity < 10%).

The next day 25–50 μ l of detection antibody diluted in PBSTx containing 5% polyvinylpyrrolidon (“PBSTx”, MW = 10.000 g/mol PVP10, Sigma) at concentrations between 125n/ml and 500 ng/ml depending on assay type either labeled with FITC, Cy3, Cy5 and AlexaFluor680 or used in combination with suitable host specific fluorescence labeled antibodies were pipetted into the wells of a MEF-MTP. After addition of 10–20 μ l of sample or calibrator in human serum, plates were incubated for 4 h at 37 °C or overnight at room temperature. Fluorescence was read without any further processing as described above (2.2.). For comparison of the signals obtained with and without washing, plates were washed a 3 times with 250 μ l PBSTx and read again.

Results

Characterization of the developed MEF- micro titer plates

Fig. 4 depicts titration curves of FITC, Cy3, Cy5 and AlexaFluor 680 labeled goat anti-rabbit IgG on MEF-MTPs and high binding MTPs from Greiner as described under 2.3.

As can be seen, by using the MEF-MTPs it was possible to detect bound antibody with a sensitivity at least three orders of magnitude higher than on conventional micro plates. Increasing the gain of the

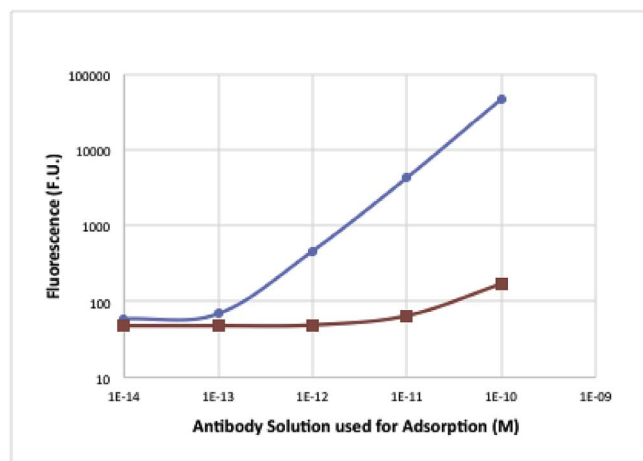


Fig. 4. Signal enhancement of adsorbed, Cy5 labeled goat anti-rabbit antibody adsorbed to a MEF-MTP. The signal obtained on a MEF-MTP was compared to the signal obtained on commercially available high binding MTPs. Adsorption was done in PBS pH 7.4 for 2 h at RT and the retained fluorescence measured after washing the plate with PBS pH 7.4 containing 0.1% Triton X-100. Similar titration curves were obtained for FITC, Cy3 and AlexaFluor680 labeled antibodies.

fluorescence reader, as expected, did not have any influence on the sensitivity of antibody detection, which clearly indicates that by the MEF effect a significant increase of signal to noise ratio was obtained. In order to quantify the enhancement in relation to the fluorophore used, signal noise ratios at 10^{-10} M antibody concentration (the only concentration detectable on conventional MTPs) were calculated (Fig. 5).

At longer excitation/emission wavelengths (e.g. AlexaFluor680 $\text{Ex}_{\text{max}} = 679$ nm $\text{Em}_{\text{max}} = 702$ nm) the relative enhancement was significantly higher. This was a result of the higher background fluorescence shown by the cycloolefine polymer at shorter wavelength. However it did not have a significant impact on the detection limit of e.g. adsorbed FITC labeled antibody since the 150 fold enhancement was still sufficient to reach picomolar or sub-picomolar detection limits.

Comparison of antibody coating density at a given concentration by detection of the bound antibody (see 2.3.) with a host-specific, alkaline phosphatase labeled secondary antibody (data not shown) revealed that the MEF-MTPs were able to bind approximately a 1/6th to 1/8th of the maximum amount of antibody capable of binding to a conventional MTP. In other words, the monitored enhancements are definitely not the result of higher antibody binding to the MEF-MTP. In the contrary, it has to be stressed, that although there is less antibody present, the sensitivity for antibody detection on the MEF-MTP is much higher than that of standard MTPs, leaving even more room for improvement in terms of achievable detection limits, if antibody binding on MEF-MTPs

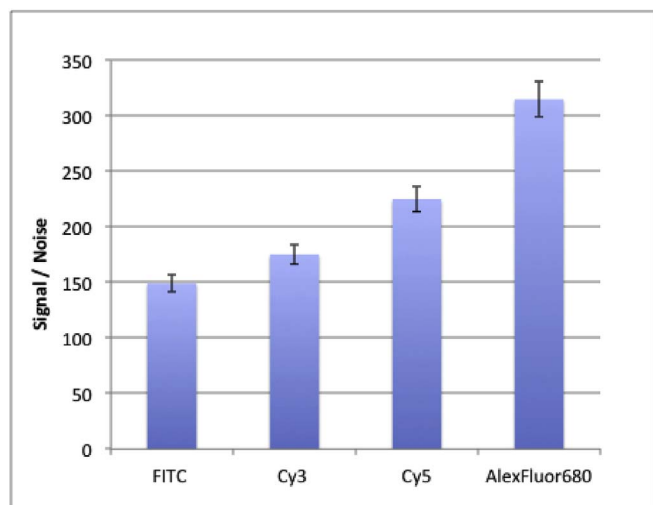


Fig. 5. Dependency of enhancement on fluorophore used. Signal/noise ratios on MEF-MTPs and conventional high protein binding MTPs defined as signal obtained at an antibody concentration of 10^{-10} M divided by blank signal (PBS) are compared. A 150 to more than 300 fold enhancement of the fluorescence signal was achieved.

can be increased.

It is well known [8] that the amount of enhancement in MEF shows a distinct dependency on distance of the fluorophore to the metallic nanostructures being the main reason why this technology allows performing assays without any washing steps.

In order to assess the relation between obtained fluorescence at given fluorophore concentration and distance the MEF- surface, additional layers of SiN_3 (5/20/50 nm) were applied on top of the nanostructures by vapor deposition. Afterwards adsorption of fluorophore labeled antibody was carried out in the usual way and the signal measured (see Fig. 6).

It was found, that within the range of 20 nm from the surface the signal is only diminished by 10–15% whereas a sharp decline in fluorescence was monitored between 20 and 50 nm distance. However, this does not limit application of the developed MEF-platform since any immune- or molecular biology-assay would not significantly range further from the surface given the fact that antibodies, molecular probes or protein analytes are not much bigger than 5–7 nm each. In other words a range of 20–25 nm is more than sufficient to set up any biomarker assay.

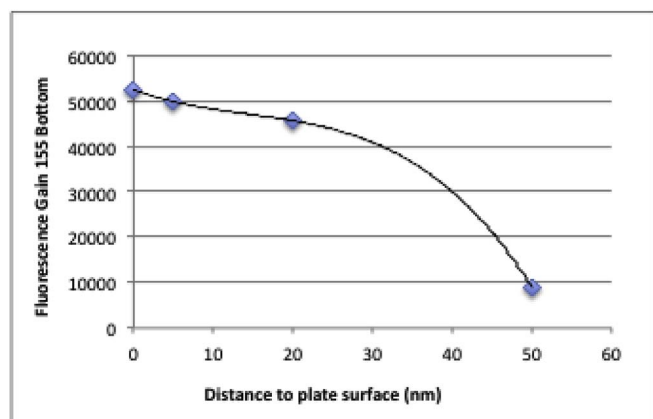


Fig. 6. Range of MEF on MEF-Surfaces, Fluorescence Signal of 10^{-10} M goat anti-rabbit antibody labeled with AlexaFluor680 adsorbed to MEF-surfaces additionally coated with SiN_3 (5/20/50 nm).

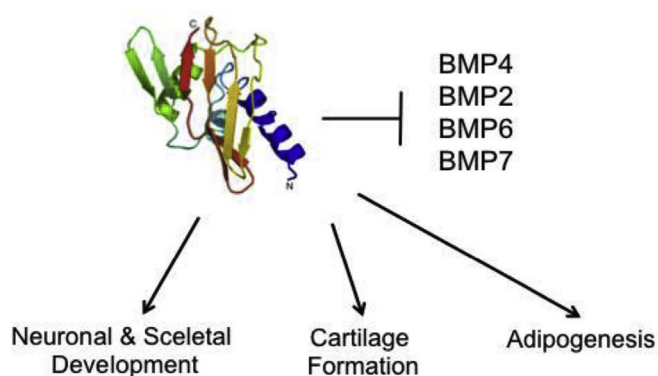


Fig. 7. Biological functions of Noggin.

MEF-FIA for human NOGGIN protein

An immunoassay for human NOGGIN protein was created as described under 2.5. to demonstrate the high sensitivity and the advantages in methodology of our MEF-platform compared to conventional ELISA. NOGGIN acts as Bone Morphogenetic Protein (BMPs 2,4 and 7) inhibitor and as such has an impact on bone/cartilage regeneration, limb development and fracture repair. (Fig. 7).

Although this molecule has been linked to several diseases like ankylosing spondylitis [9], pulmonary arterial hypertension [10] or osteolytic bone metastasis [11], its value as a biomarker remains to be established due to a lack of sufficiently sensitive immunoassays. The later, made NOGGIN an ideal candidate for demonstrating proof of principle for our MEF-platform. The final assay protocol consisted of pipetting 25 μl of fluorescence (FITC, Cy3, Cy5 or AlexaFluor680) labeled detection antibody to all wells required, followed by adding 20 μl of human serum/plasma sample or calibrator. The MEF-plate was swirled gently to mix the reagents and incubated for 4 h at 3 $^{\circ}\text{C}$ or overnight at room temperature. Then the plate was read in bottom configuration (BOT) without any further processing at the Ex/Em wavelength fitting to the used detection antibody. Afterwards the contents of the wells were discarded, the plate washed and read again in BOT and TOP configuration to compare results with and without washing.

Fig. 8 shows a typical calibration curve in human serum and the comparison to performing the same assay (identical reagents) on a conventional high protein binding MTP.

As can be seen, no signal at all was obtained on conventional MTPs. Only the MEF effect allows detection of the analyte in picomolar

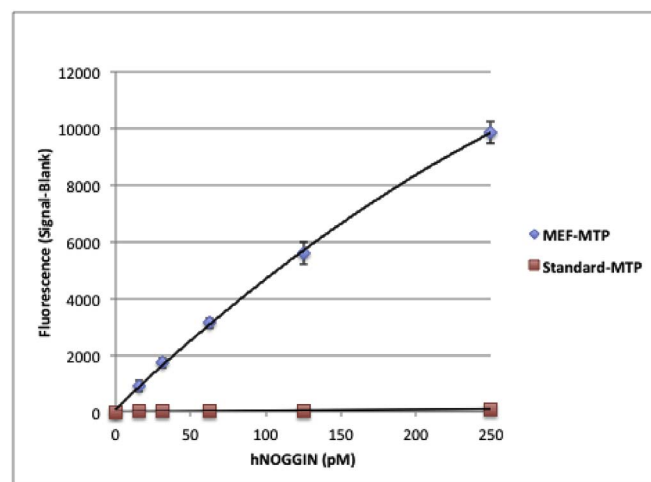


Fig. 8. Calibration curve for human NOGGIN in serum. Detection-antibody was labeled with AlexaFluor680. Identical curves were obtained for antibodies labeled with FITC, Cy3 or Cy5.

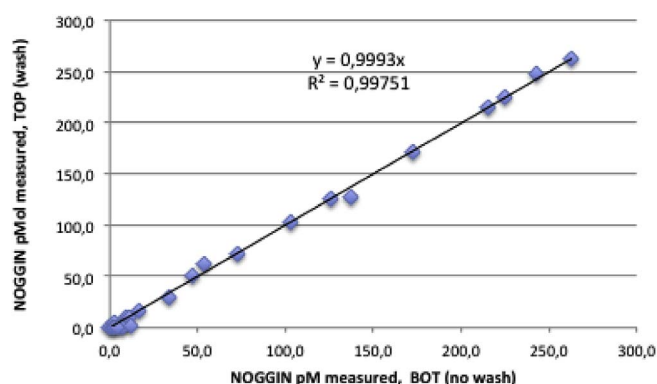


Fig. 9. NOGGIN dosage obtained with and without washing.

concentrations. Detection limit ($0 \text{ pM} + 3\text{xSD}$) was established as $1,5 \text{ pM}$. Intra-assay precision was evaluated by testing 4 samples of known concentrations 3 times within 1 assay run and inter-assay precision by testing of 2 samples of known concentrations duplicates within 3 different assay runs and ranged from 2–5% and 3–7% respectively. The recovery of NOGGIN was evaluated by adding 2 known concentrations of human recombinant NOGGIN to 4 different human serum samples and ranged from 81 to 112%. Finally 4 human serum samples were spiked with recombinant NOGGIN und diluted 1 + 3 and 1 + 9 with the same buffer used for the detection antibody preparation and the mean linearity determined as 93%.

Identity of results in both assay variants (wash/no wash) was tested by correlation of measurements with serum-based calibrators and 18 human serum samples (Fig. 9).

In contrast to ELISA systems, which lose their signal because the colored product of the enzymatic reaction degenerates, MEF-FIAs show a constant signal over time once equilibrium of antigen binding is achieved (Fig. 10).

It is also not possible to “over-incubate” or “over-develop” a MEF-FIA like it could happen in ELISAs during incubation with the enzyme substrate leading to a loss of sensitivity because background becomes too high. Once equilibrium is reached, the fluorescent labeled antibody at the surface does not show any change in signal over time as it is the case in all fluorescence immunoassay of course.

As mentioned earlier, NOGGIN acts as inhibitor of the biological function of BMPs by binding to those molecules and preventing interaction with their receptors. Therefore it is possible to check specificity of the NOGGIN assay by titration of the serum-based calibrators with human recombinant BMP-2, -4 and -7. If the antibodies used for the assay bind to NOGGIN **outside** the binding pocket, then incubation

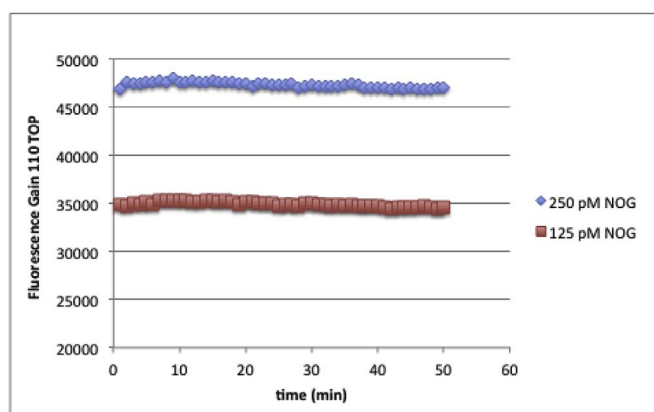


Fig. 10. Signal stability of the MEF-FIA for NOGGIN. Detection antibody was labeled with AlexaFluor680. Each time point corresponds to measurements with 25 flashes of the reader's Xenon Lamp.

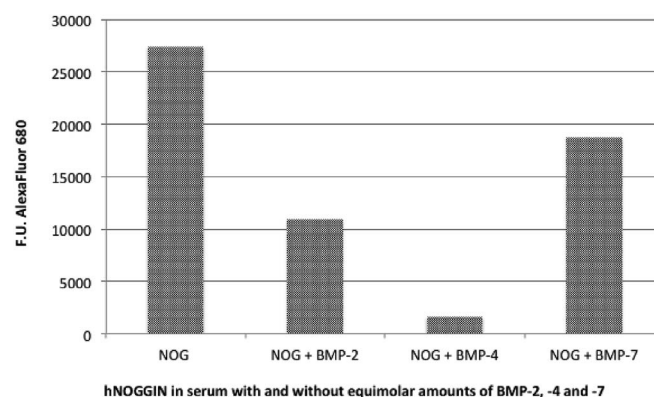


Fig. 11. MEF-FIA for human NOGGIN - Proof of Specificity by Checking Reactivity with BMPs.

with BMPs should not affect the assays signal. The assay then would detect NOGGIN in complex with BMP as well as free, not bound NOGGIN. If the recognized epitopes lie **within** the binding pocket, then addition of BMPs would reduce signal, since the antibodies could no longer bind to NOGGIN. As can be seen from Fig. 11, addition of BMPs significantly reduces the signal of the calibrator, which demonstrates that the assay only detects **free, bioactive** NOGGIN.

When compared to commercially available ELISA systems for NOGGIN on the market, the MEF-FIA was found to have 30–300 fold higher sensitivity (Table 1).

Testing of serum samples with recombinant NOGGIN added in concentrations of 250 and 125 pM, i.e. at the upper calibrator levels of our MEF-FIA, with the ELISA kits from supplier 2 and 5 (data not shown) resulted in no signal at all, supporting that their sensitivity indeed is much too low for measuring pico-molar concentrations of NOGGIN.

As last step of assay validation, a panel consisting of serum samples from 48 blood donors was analyzed to establish reference values for NOGGIN in the general population (Table 2).

All samples were detectable and showed NOGGIN serum concentrations in the low pico-molar range. No influence of gender, age or sample type (i.e. serum or plasma) was found, although it must be stressed, that the number of samples was not high enough to draw final conclusions on the distribution of NOGGIN serum levels in the general population. Further studies with larger sample cohorts of healthy people and of patients who suffer from pathologies that might be linked to NOGGIN have to be performed to establish the value of this protein as biomarker.

Conclusion

We successfully demonstrated, that the developed MEF-platform is suitable for the creation of high sensitivity immunoassays that can be used in clinical research. In addition to the dramatic improvement of signal/noise ratio, it also allows performing single step assays due to its unique feature of bulk fluorescence suppression. The rational for

Table 1

Comparison of detection limits of immune-assays for human NOGGIN according to information given in the respective instructions for use.

Supplier	Assay Type	LOD (pM)	rel Sens.	x fold Sens.
FIANOSTICS	MEF-FIA	1,5	100,0%	n.a
Supplier 1	ELISA	47	3,2%	31
Supplier 2	ELISA	47	3,2%	31
Supplier 3	ELISA	47	3,2%	31
Supplier 4	ELISA	236	0,6%	157
Supplier 5	ELISA	473	0,3%	315

Table 2

Serum NOGGIN levels in apparently healthy blood donors.

	Serum NOGGIN (pM)	
	female (n = 25)	male (n = 23)
90th Percentile	7,6	8,3
70th Percentile	5,7	5,3
Median	5,0	4,5
30th Percentile	4,4	3,2
10th Percentile	2,6	2,2

presenting this new technology in a microplate format lies in the compatibility with standard microplate readers and its suitability for investigation of small to medium sized patient cohorts in clinical research. Since this technology is in no way limited to a special format, we believe that it will become a very valuable contribution to various areas of life- and medical-sciences (e.g. point of care diagnostics, micro arrays or high throughput screening) where ease of handling, time to result or sensitivity is an issue.

Competing interests

Authors declare no competing financial interests.

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