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# A multi-purpose ultrasonic streaming mixer for integrated magnetic bead ELISAs

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**Abstract**

We present an ultrasonic streaming mixer for disposable and on-chip magnetic bead ELISAs. The ultrasonic transducer is placed at system-level to keep cost per chip as low as possible, and is coupled to the chip by means of a solid ultrasonic horn. The system provides mixing of liquids, as well as dispersion of the superparamagnetic beads in the ELISA. Additionally it can be used clean the chamber surface from nonspecifically bound proteins during the washing steps in the ELISA protocol. Using our system the time for the ELISA protocol has been greatly reduced down to 30 min.

Keywords: lab-on-chip, ELISA, immunoassay, mixing, dispersion, cleaning, horn

(Some figures may appear in colour only in the online journal)

## 1. Introduction

Enzyme linked immunosorbent assays (ELISAs) are one of the most important tools for immunological analysis today [1]. They are able to detect the presence of antigens in biological samples of very low concentrations and with high specificy. ELISAs can be used for detection of all diseases that provoke an immune system response, such as HIV [2], cancers [3], infections [4], markers for heart-failure [5] and even pregnancy [6], potential allergens [7] such as nuts or fruit and for drug screening [8]. This makes ELISAs an inevitable resource in clinical laboratories and biological research.

Traditionally, ELISAs are carried out on multi-well plates by skilled technicians and consume hours of time both hands-on and passively. In consequence, they are expensive, which limits their use in clinical point-of-care settings.

Moving from traditional well-plates to lab-on-chips provides many new opportunities such as the reduction of the amount of liquids needed, and the resulting reduction of assay time. On the other, it hand requires mastering new design challenges by scientists and engineers such as the proper on-chip mixing of the used reagents, the efficient dispersion of the used superparamagnetic beads, and a low non-specific background signal during measurements.

Since microfluidic devices mostly operate in low Reynolds and low Peclet number regimes, the mixing in channels and chambers is mostly of diffusive nature, with little convective mixing. Special care has to be taken for proper mixing.

 

Various mixers for microfluidic applications [9] have been developed over the years, for example passive [10], lamination [11] or herringbone [12] mixers, as well as active micro stirrbars [13]. The superparamagnetic beads used in the ELISA have also been used for mixing by moving them through the chamber with electromagnets. This design unfortunately has two major drawbacks, one being the rapid decay of the magnetic field in nonmagnetic materials, such as chamber walls and liquid, and the other being the large amount of waste heat [14] created when sufficient field gradients are produced to move beads and liquids. Additionally the beads cluster together under the influence of the magnetic field.

Ultrasonic agitation provides a more efficient method for mixing, since it can propagate well through the materials used. Ultrasonic mixing has been done before with high frequency (450 MHz) driven, integrated transducers [15], which is efficient for moderate flow-rates up to 30 $\mu$l min$^{-1}$ in a straight channel, but requires a large and expensive HF-generator as well as integration of piezoelectric materials. Streaming on trapped bubbles in hydrophobic channels [16] has the advantage of efficient mixing, but needs special materials and careful filling of the device, to keep the bubbles intact. The same expensive manufacturing problem applies to mixing in etched silicon channels [17] and surface-accoustic-wave (SAW) mixing [18], as well as other devices with on-chip ultrasonic transducers [19–21]. Most of those devices exhibit three fundamental flaws for our application. Firstly, because of the dimensions [22] of the thin-film piezoelectric material, the resonant frequencies are very high. Secondly, they dissipate heat directly into the chip and liquid, which may be prohibitive for biomedical applications. Additionally, the integration of piezoelectric materials increases the production cost per device, which is problematic for disposable bio-chips. In the worst case such an integration means having to operate full clean-room facilities compared to being able to create the disposable chips by injection molding.

When superparamagnetic beads are exposed to a magnetic field to collect them during washing and liquid exchange steps, they become magnetic themselves and thus attract each other. Although they lose their magnetization [23] after the magnetic field is turned off, the clusters tend to stay intact, reducing their surface area, and rendering them useless. This can be attributed to the hydrophobicity of their surface and of the proteins attached to it. To operate an efficient magnetic bead based ELISA, it is very important to quickly and uniformly re-disperse the magnetic beads in the reaction chamber to take advantage of the unique properties of the beads.

Mechanical motion such as vibration in vortexers or mixing during pipetting has been used to redisperse beads in off-chip wellplate ELISAs. Since microfluidic assays do not permit such a manual intervention, other possibilities for redistribution have to be found. Movement of the magnet is not an option for redispersion the beads, as they will still be magnetically attracted to each other under the influence of the magnetic field. The cluster of beads will move in the liquid without breaking up.

Ultrasonication, as a means for dispersing colloids, is one of most common and effective methods used in laboratory and industry-scale experiments [24] and provides the answer for the aforementioned problems.

The dispersion of particles with ultrasound works on the principle of acoustophoresis [25], the motion and attraction of particles into the anti-nodes of standing waves of ultrasonic fields. Accoustophoresis has been extensively studied as a way to collect [26], sort [27] and attract [28] particles in microfluidic chambers.

Unspecific bindings of proteins and antigens to the chamber walls during the ELISA protocol are a major problem for immunoassays, especially if the read-out of the luminescence is done in the same microfluidic chamber as the incubation of the sample. The incubation attaches non-specifically bound proteins to the measurement chamber and since the luminescent substrate will react with specifically bound antibodies as well as non-specifically bound protein a background signal is introduced. This signal is often many times higher than the actual specific detection signal. As a countermeasure, the surface of the reaction chamber is normally blocked with non-reacting protein, such as bovine serum albumin (BSA) [29]. Alternative chamber materials, or coating with hydrophilic polymers [30] can also be used. All of these methods are costly, as additional chemicals and steps during manufacturing are necessary.

The aim of this paper is to describe non-integrated ultrasonic streaming mixing for on-chip magnetic-bead ELISAs [31], forming a diagnosis system for distinct markers of sepsis. The system addresses three major problems in on-chip magnetic bead ELISAs with one simple measure.

Through the application of ultrasound from the outside of the chip by means of a low-cost, low-power ultrasonic system, we manage to enhance the mixing of the liquids on chip, facilitate the dispersion [32] of superparamagnetic beads used in the ELISA, and provide a reduction of non-specific signal [33] in the ELISA.

To validate the performance, all three steps have been analyzed separately, always taking care that they can be combined to support the ELISA protocol. The ELISA measurements, containing all three enhancements, prove the usefulness of the system.

## 2. Materials and methods

### 2.1. Ultrasonic device and system

*2.1.1. Piezo.* The piezoelectric transducer used is a commercial low-cost Prowave M165D25 [38] hard piezo-ceramic type originally made for medium power (30 W) liquid atomization. It has a thickness of $t = 1$ mm, a diameter of $d = 25$ mm and its main resonance frequency at $f_{\mathrm{res}} = 1.65$ MHz.

*2.1.2. Driver.* For off-resonance and swept-frequency drives a variable frequency driver is needed. Drivers for power ultrasonic applications usually use a self resonating oscillator such as a Hartley-Oscillator [39], with the piezoelectric device as frequency defining and actuator element. Since we want to create ultrasound with different modes, and not only at the main resonance frequency of the piezo this approach is futile.

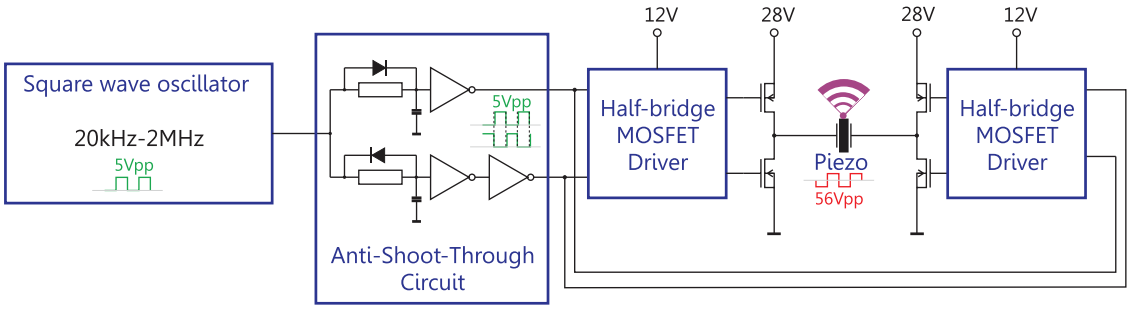


**Figure 1.** Block diagram of the driver circuit.

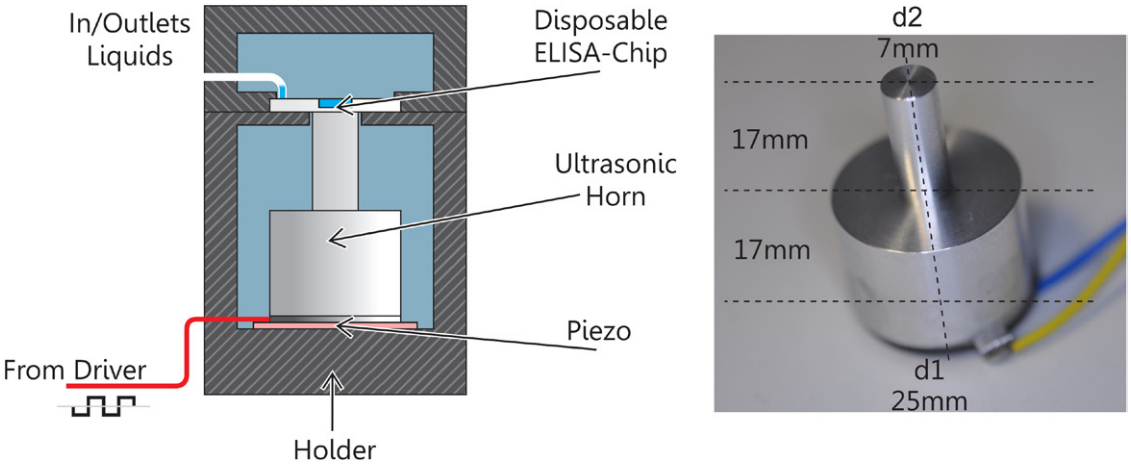


**Figure 2.** Design of the horn and schematic of the horn and piezo in setup.

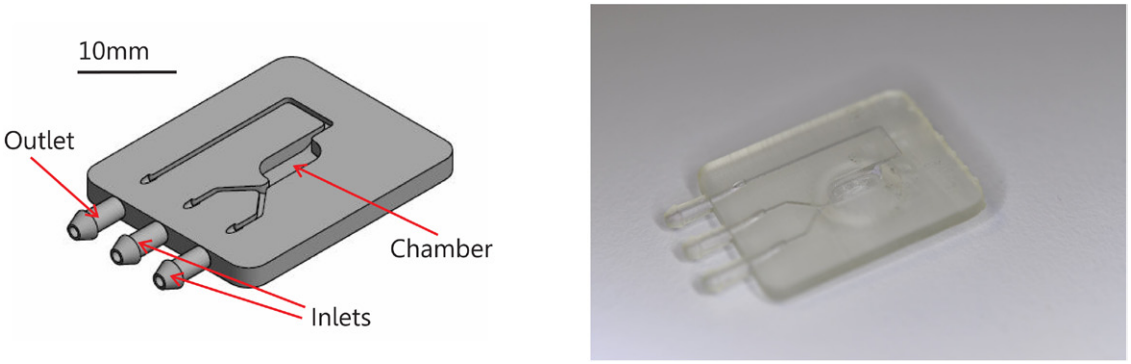


**Figure 3.** CAD-drawing and photograph of the microfluidic mixing chip.

Analog drivers for the desired frequency and power range are large and expensive since the driving transistors have to dissipate the power while they are turning on slowly to create the sinusoidal analog modulation. To keep the driver simple and to reduce cost and size, we use a switching driver. The design of the driver is shown in figure 1.

A frequency generator, providing a 5 V, 50% duty-cycle, variable 20 kHz–2 MHz square wave provides the starting point for the ultrasonic generation. The following anti-shoot-through circuit prevents both the upper and lower transistors of the output H-bridge from turning on at the same time, by delaying the rising edges by the time the transistors need to safely turn off. It also provides an inverted signal for the following driver circuit. ISL2111 half bridge drivers by Intersil create the gate voltage necessary for the final output stage, composed of four FDMS86200 n-channel MOSFETs. The H-Bridge configurations inverted switching provides twice the supply voltage over the transducer.

*2.1.3. Horn.* An ultrasonic horn [40], or sonotrode couples the ultrasonic waves from the large piezo into the small microfluidic chamber.

While usually $\lambda/2$ sized [41], the horn used for this setup was designed to adapt the different sizes of piezoelectric transducer and microfluidic chip and direct the sound pressure towards the chamber. It is lathed from aluminum and has a total length of 34 mm. This means that each part of the horn, as seen in figure 2, is 17 mm long. The 25 mm diameter of the piezo and the size of the microfluidic chamber with 7 mm diameter, determines the diameters of the two parts of the horn.

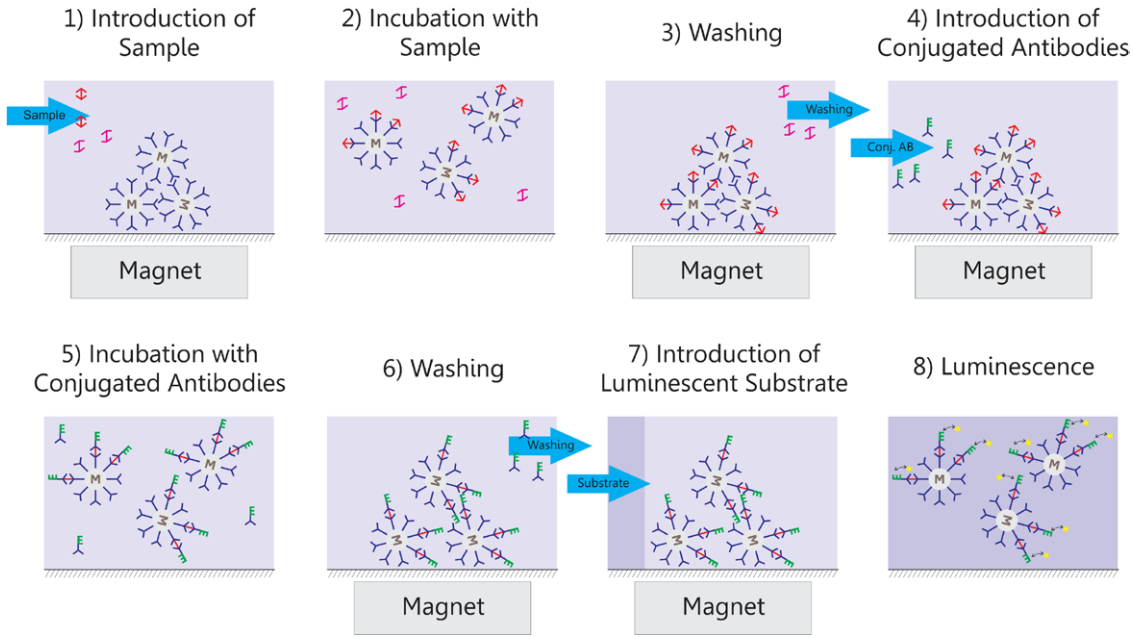


**Figure 4.** Steps in the magnetic bead based ELISA protocol. (1) Introduction of sample. (2) Incubation with sample. (3) Washing. (4) Introduction of conjugated antibodies. (5) Incubation with conjugated antibodies. (6) Washing. (7) Introduction of luminescent substrate. (8) Luminescence.

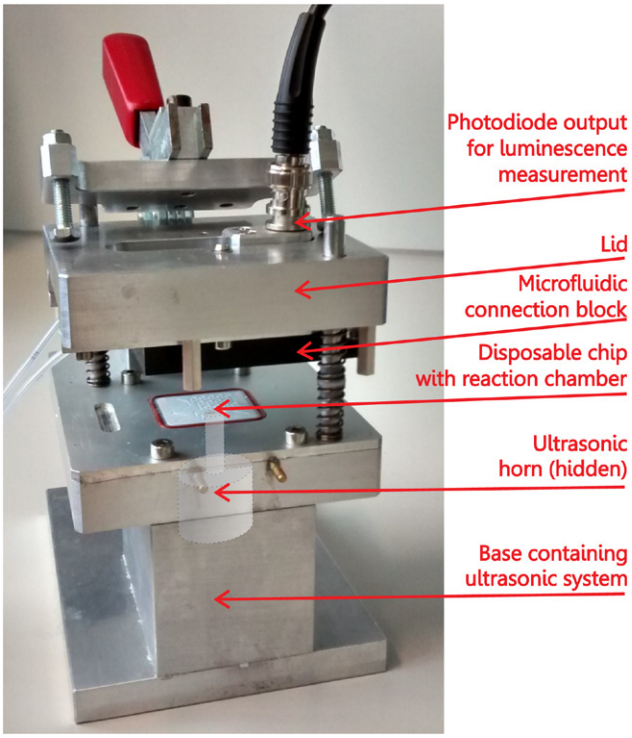


**Figure 5.** Photograph of the ELISA system loaded with the chip.

Additionally, the horn acts as a passive heat sink, reducing the waste heat influence on the sensitive biological samples.

### 2.2. Microfludic chip

The microfluidic chip, as seen in the system in figure 5, is designed to provide channels and chambers for the on-chip ELISA. It is made from a poly-methyl-methacrylate acrylonitrile-butadiene-styrene (PMMA-ABS) blend and with design consideration to make the final device injection moldable. Prototypes are created through milling or stereo-lithography.

For the characterization of the ultrasonic system a special chip design, containing a chamber connected to two inlets and one outlet is fabricated. A CAD-drawing and photograph of the device is shown in figure 3.

### 2.3. Micromixing of liquids

The system operates at Reynolds numbers between $\mathrm{Re} = 0$ for stopped flow and $\mathrm{Re} \leqslant 0.1$ for a flow-rate of 100 $\mu$l min$^{-1}$, and Peclet numbers between again $\mathrm{Pe} = 0$ and $\mathrm{Pe} \leqslant 2000$ in the reaction chamber. This means that it operates in the laminar flow regime ($\mathrm{Re} < 2300$), and that convective mixing ($\mathrm{Pe} > 1000 \cdot \mathrm{Re}$) only plays a role for the largest expected flow-rates.

Since the device should be able to operate when no new liquid is introduced, i.e. with stopped flow, an active micro mixer is the only suitable solution.

Acoustic streaming [34] with the aforementioned off-chip transducer is used to mix the liquids in the reaction chamber. It can be used with and without beads inside the microfluidic chamber and is independent of the size or shape of the beads, since its effect creates motion in the liquid itself. By applying so called Schlichting–Rayleigh-streaming [35], circular flows are created in the center of the chamber. These streams are the main contribution to the liquid mixing. Cavitation induced streaming [36] should be prevented because that causes clogging of the channel though the cavitation bubbles.

To test the mixing capabilities, the microfludic chip was attached to the horn. Phosphate buffer (PBS, pH 7.4, clear) and bromocresol-purple in citric acid solution (pH 4.0, yellow), are brought together in the reaction chamber. The pH of the citric acid solution is set so that a complete 1:1 mixture will yield

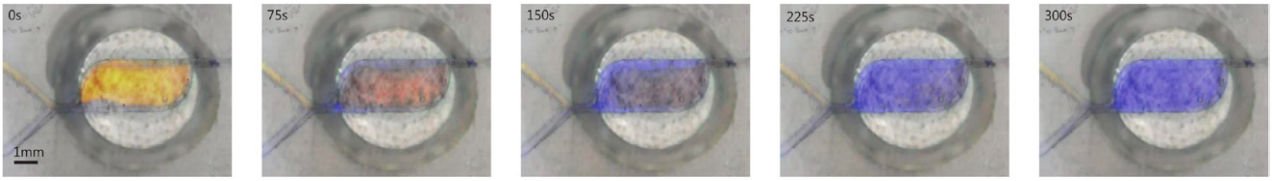


**Figure 6.** Mixing under stopped flow condition without ultrasound (diffusion).

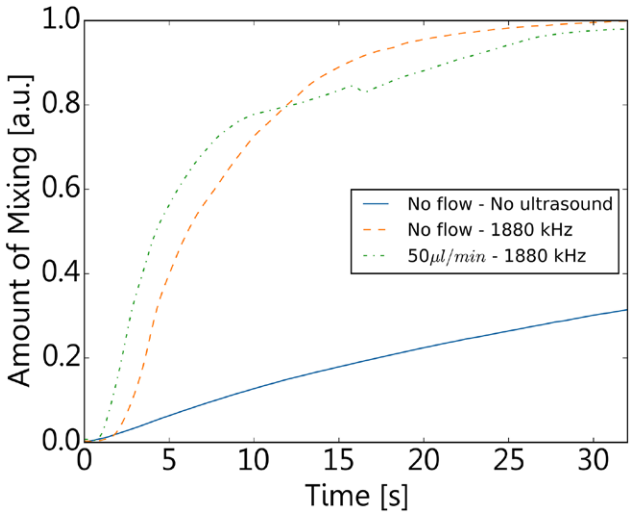


**Figure 7.** Analysis of mixing experiment.

a full color change. The resulting concentration is $c = 0.1$ mmol $l^{-1}$ citric acid. Unmixed solution is yellow, turning red during partial mixing and blue for full mixed solutions. The chamber is filled with both liquids at constant flow-rate by a KD-Scientific Legato 180 syringe pump. The experiments are recorded through the top of the device with a digital SLR-camera (Nikon D90). When an unmixed steady state is reached the mixing experiment can be started.

The driving frequency of the ultrasonication system is set to 1880 kHz. This frequency was chosen as it lies in a resonant frequency-range of the piezoelectric transducer, and its wavelength is a multiple of both the size of the ultrasonic horn and the height of the chamber, resulting in efficient energy transfer.

### 2.4. Dispersion of beads

Acoustophoresis is normally used as a way to aggregate and concentrate particles in liquids. Since dispersion and not concentration is required, the formation of long-lasting standing waves in the chamber has to be prevented. To achieve this, the sonication frequency has to be rapidly altered, to switch between different modes with different shapes of standing waves. This is accomplished by a frequency sweep.

Particle dispersion was analyzed by first introducing Invitrogen Dynabeads M-280 (tosylactivated) superparamagnetic beads into the chamber. After attracting and clustering them with a permanent magnet and removing the magnet, the measurement is started. Again, the measurement is observed with a digital SLR-camera (Nikon D90) and analyzed with Matlab.

### 2.5. Washing of unspecific bindings

Ultrasonic cleaning is a technique using acoustic energy for the removal of difficult to remove contaminations, especially, but not exclusively, in biology.

To prove efficiency of this operation, a microfluidic device was incubated with fluorescently stained (Qubit by Life Technologies) BSA of 5 mg $ml^{-1}$ concentration in phosphate buffer (PBS) for 30 min. It was connected to the system and ultrasound with the same parameters as above was activated. During the washing the liquid in the chamber was exchanged with clean PBS at a flow-rate of 50 $\mu$l $min^{-1}$. Every 30 s the measurement was observed and measured using a fluorescence microscope (Nikon AZ100). Continuous measurement was not used to avoid photo-bleaching.

In addition to the direct measurement of the washing, a negative ELISA i.e. 0 pg $ml^{-1}$ Interleukin 8 (IL-8), was performed with the system in three different ways: (a) with blocking by 24 h incubation of the chip with BSA (the common method in off-chip ELISAs), (b) without blocking of any kind and without ultrasound, and (c) finally without blocking, but with ultrasound applied during washing.

### 2.6. ELISA

In superparamagnetic bead based ELISAs, such as the on-chip ELISA for the detection of sepsis markers [42] for which the ultrasonic support system is presented here, the antibodies used for capture and detection are not attached to the detection chamber walls, but instead to the surface of micrometer-sized superparamagnetic beads. The steps needed for the bead based ELISA are described in figure 4. Mixing of different liquids is necessary whenever new fluids are introduced, i.e. at the steps 1, 3, 6 and 7 to introduce the new liquid as uniformly as possible and to keep the assay time low. After each washing step (3 and 6), a dispersion step is necessary to distribute the beads in the chamber. Ultrasonic cleaning can also be used during the last washing step (6) to remove non-specifically bound proteins from the reaction chamber walls.

This provides the ELISAs with increased sensitivity, since the beads provide a vastly larger surface area for attachment of the antibodies. Hence, they create an increased contact probability between analyte and immunosorbent surface compared to standard ELISAs. Since the beads can move freely in the liquid analyte, they are able to collect a large amount of antigens, even if the antigen concentration in the sample is very low.

During the washing and liquid exchange steps, the beads are collected and immobilized by a strong permanent magnet

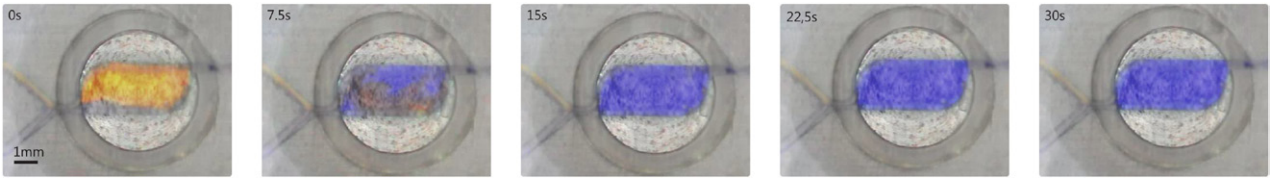


**Figure 8.** Mixing under stopped flow condition with ultrasound.

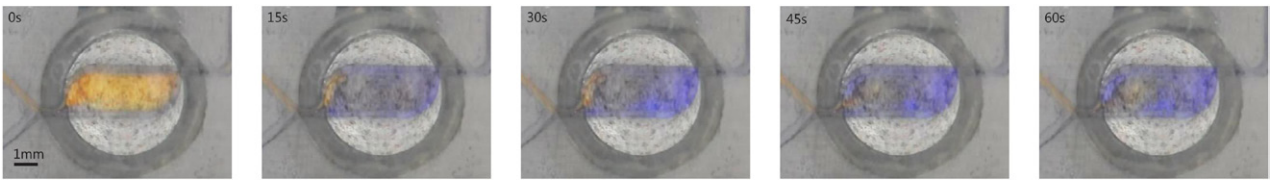


**Figure 9.** Mixing under 50 $\mu$l min$^{-1}$ flow with ultrasound.

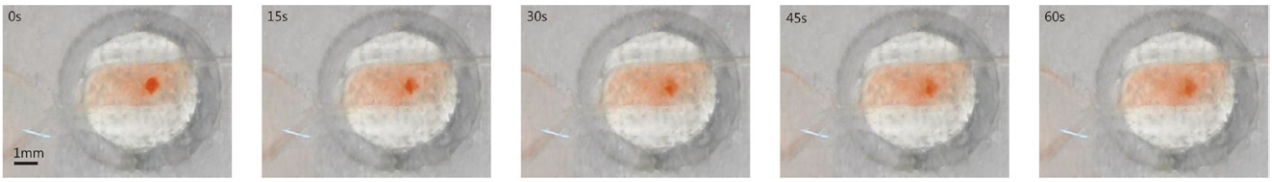


**Figure 10.** No dispersion of beads without ultrasound.

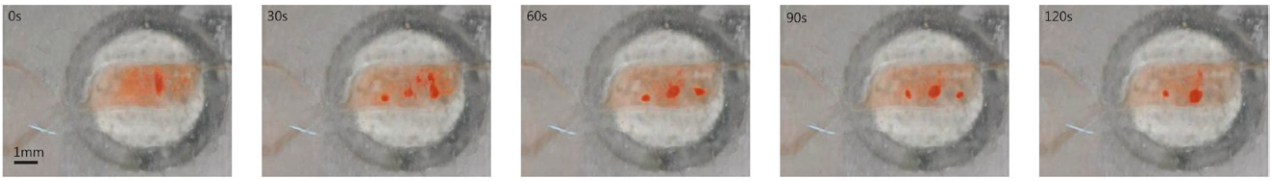


**Figure 11.** Attraction of beads to ultrasonic anti-nodes.

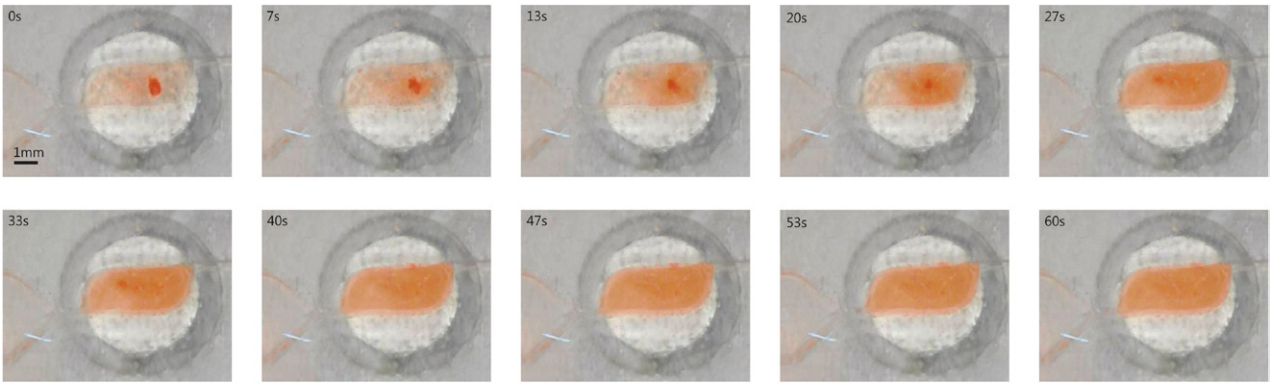


**Figure 12.** Dispersion of beads through ultrasonic frequency sweep.

placed outside of the chamber. This enables an exchange of the surrounding liquid while the beads and the attached antibodies are not removed.

Additionally to the separate ultrasonic enhacement measurements, our system and chip—as depicted in figure 5—has also been proven in an ELISA experiment [42]. An Interleukin 8 (IL-8) standard curve has been recorded with the protocol mentioned above and the ultrasonication system presented here. Interleukin 8 is an inflammatory marker used for the detection of sepsis in intensive care units. The measurement was done with 10% diluted blood plasma, which was spiked with the necessary concentration of IL-8 antigens.

## 3. Results and discussion

### *3.1. Micromixing of liquids*

Measurements with flow, without flow and as reference without ultrasound have been undertaken. The reference diffusion mixing process—without ultrasound—can be seen in figure 6. It has to be noted that even after 300 s, the liquids

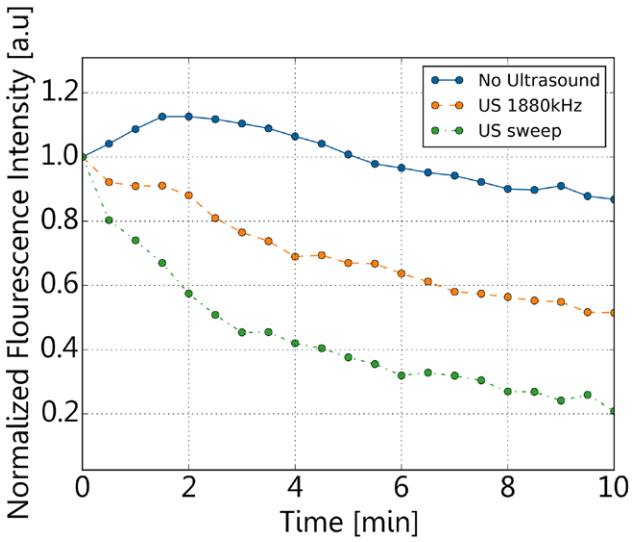


**Figure 13.** Washing of luminescently labeled proteins.

are not completely mixed, as analysis of the colored area with Matlab reveals. Figure 7 shows the relevant part of this analysis in comparison with the ultrasonic mixing. The time constant of the mixing can be estimated as $\tau = 176$ s as the time it takes to reach 63.2% of complete mixing.

Mixing with ultrasound speeds up the process by a factor of more than 15 to a time-constant of $\tau = 10$ s as the photographs in figure 8 and the analysis in figure 7 reveal.

Ultrasonic mixture of the two liquids under constant flow of 50 $\mu$l min$^{-1}$, as in figure 9, shows an unmixed area at the entrance of the chamber, where new liquid is introduced, and complete mixing at the fluid exit. From the image analysis in figure 7 the time-constant can be estimated as $\tau = 4.5$ s.

This fast mixing is of great advantage during the liquid exchange phases of the ELISA, since washing and cleaning times can be reduced while still maintaining efficient operation.

### 3.2. Dispersion of beads

The reference measurement without ultrasonication is shown in figure 10. It is clearly visible that the beads stick together and form a large cluster. This cluster does not break up or disperse over time.

Ultrasound, again with 1.88 MHz, is activated and the result is observed over time as shown in figure 11. The acoustophoretic aggregation of the beads in the anti-nodes is clearly visible. No dispersion is happening.

If the ultrasound frequency is swept from 1 MHz up to 2 MHz within 30 s, returning down to 1 MHz in the same time, complete dispersion is achieved. Figure 12 shows that the cluster is breaking up and dispersion takes place which illustrates that the frequency sweep is absolutely necessary for efficient dispersion of the magnetic particles.

Only an aggregation-free immunoassay can provide for repeatable and highly sensitive magnetic bead immunoassay, since the high surface area advantages of the particles can only be used if they are distributed through the chamber. Ultrasound sweeping sums out to be an excellent method to accomplish the required dispersion.

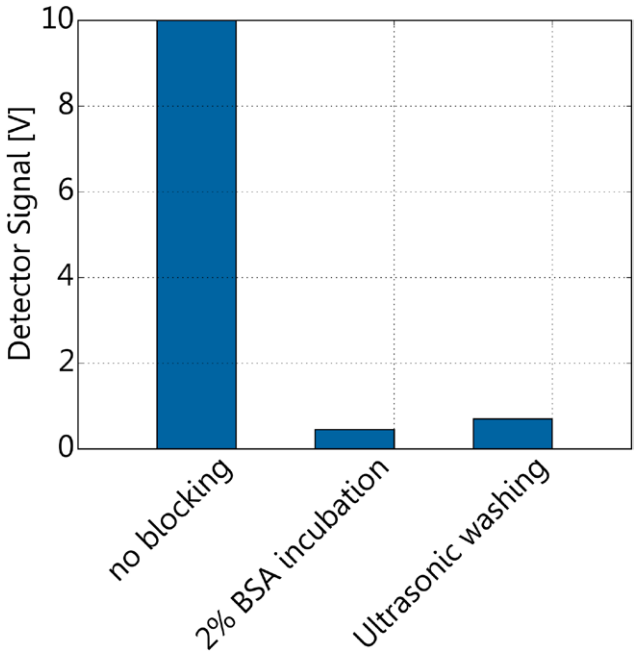


**Figure 14.** Signal for 0 pg ml$^{-1}$ sample IL-8 ELISA with different blocking schemes.

### 3.3. Washing of unspecific bindings

The normalized intensity of the fluorescence in relation to the background is shown in figure 13. There is almost no cleaning visible without ultrasound. The single frequency ultrasound at 1880 kHz provides some cleaning of the surface, but is not as efficient as the sweeping actuation.

The results in terms of luminescence intensity are shown in figure 14. The measured intensity without blocking saturates the detecting photo-diode of the system, and gives a background signal far larger than any valid sample concentration. Its signal is equivalent to more than 2000 pg ml$^{-1}$ IL-8. The ultrasonic washing results in a background signal of 0.7 V, which is comparable to the BSA blocked chamber with 0.45 V.

Since the non-sepcifically bound proteins bind mostly through hydrophobic forces [37], they are removed, even by comparatively low power ultrasound. No cavitation, as used in classical ultrasonic cleaning machines, is necessary. This keeps, in addition to the already mentioned advantages, the stronger specific bindings between beads and antigens intact and the normal ELISA procedure is not disturbed.

### 3.4. ELISA

The results for the Interleukin 8 (IL-8) standard curve are shown in figure 15. They show the usability down to clinically relevant levels and our system is able to run a full ELISA in less than 30 min [42], while commercial ELISA-kits require much more time (e.g Luminex [43] with 8 h assay time). The advancements in the immunoassay technology, especially the reduced processing time, is a result of the advantages in mixing and dispersion archived with the ultrasonication system.

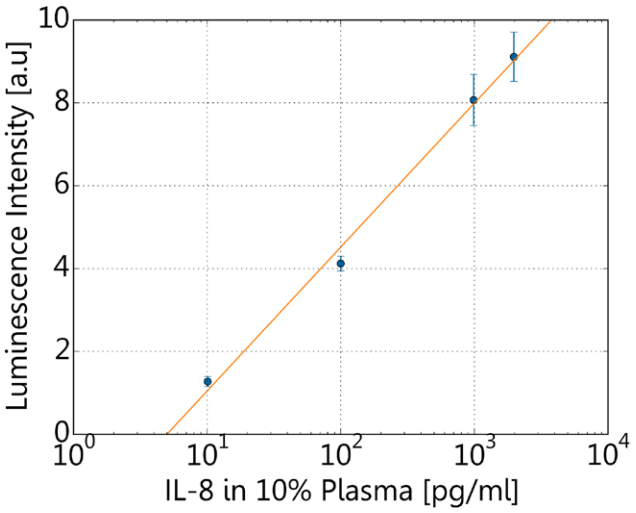


**Figure 15.** Signal for spiked IL-8 ELISA in 10% blood plasma.

## 4. Conclusions

The ultrasonic system for integrated ELISAs accelerates the fluidic mixing by a factor of 15 compared to a diffusion based approach. It is able to disperse superparamagnetic beads in less than 30 s throughout the chamber. Ultrasonic cleaning in less than 10 min is also possible to greatly reduce the background in the ELISAs without blocking of the chamber. The system provides a low-cost and efficient method, that is usable with injection molded disposable ELISA chips. The variable frequency drive gives the possibility to use it for different purposes depending on the actuation. In particular, the sweeping actuation is useful in cases where standing waves have to be avoided.

The ultrasonic streaming mixer greatly improves the functions of on-chip ELISAs and allows a significant time gain for the protocol. The functionality has been proven separately for each step, and integrated into the ELISA protocol. It has to be noted that the greatest advantage of this system is found in the speed and convenience gained. With small sample volume and 30 min assay time, this microfluidically enhanced immunoassay is ideally suited for point-of-care diagnostics.

## Acknowledgments

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