Flexible platform for performing protocols on a chip utilizing surface acoustic wave (SAW) driven mixing

Yaqi Zhang¹, Citsabehsan Devendran¹, Christopher Lupton², Alex de Marco²,³, Adrian Neild¹

¹Department of Mechanical and Aerospace Engineering, Monash University, Clayton, Victoria (Australia)
²Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria (Australia)
³ARC Centre of Excellence for Advanced Molecular Imaging (Monash University, Australia)

Abstract

We present and demonstrate a dextrous microfluidic device which features a reaction chamber with volume flexibility. This feature is critical for developing protocols directly on the chip when the exact reaction is not yet defined, enabling bio/chemical reactions on chip to be performed without volumetric restrictions. This is achieved by the integration of single layer valves (for reagent dispensing) and surface acoustic wave excitation (for rapid reagent mixing). We show that a single layer valve can control the delivery of fluid into, an initially air-filled, mixing chamber. This chamber arrangement offers flexibility in the relative volume of reagents used, and so offers the capability to not only conduct but also develop protocols on a chip. To enable this potential, we have integrated a SAW-based mixer into the system, and characterised its mixing time based on the frequency and power of excitation. Numerical simulations of the streaming pattern inside the chamber were conducted to probe the underlying physics of the experimental system. To demonstrate the on-chip protocol capability, the system was utilised to perform protein crystallization. Furthermore, the effect of rapid mixing, results in a significant increase in crystal size uniformity.
1. Introduction

Integrated microfluidic systems represent an excellent tool for biological and chemical applications especially in complex reactions such as single cell analysis,\(^1\,^2\) PCR,\(^3\) protein crystallizations and nanoparticle synthesis\(^4\) where precise and sophisticated handling of extremely small amounts of sample are required. Regardless of the number of steps involved in a protocol, in most cases, in each step, the reagents must first be added to the sample and then mixed. In the context of protocol-on-a-chip systems, there are two key components that are required to scale down laboratory process: (i) micro valves, which enable control of fluid and aid complex fluid processing; and (ii) mixers which are needed at the micro scale as the laminar regime dominates and mixing by diffusion alone is a time inefficient process.

Multilayer valves\(^5\) use a control layer to apply pressure to a soft PDMS membrane which in turn deforms vertically to seal the fluid layer beneath. By use of multiple such valves, multistep integrated batch-process systems for biological and medical applications have been realised.\(^6\,^7\,^8\) Here, reagents can be loaded in each batch-processor and complex multistep reactions can be performed. To enable this, valves are opened successively to add capacity to the reaction chamber. As each valve is opened, a fixed volume (defined by the system geometry) of the next reagent is added to the sample. Subsequently, the reagents are mixed. Mixing is usually achieved by rotary mixers, in which fluid is circulated inside channel loops by sequential control of the valves.\(^9\) Whilst these systems are very elegant in their operation, they face two volume related issues. Firstly, the protocol must first be developed at the bench top and only then translated into a microfluidic environment. The systems are highly tuned to a single application, which, in turn, means that direct development of protocols on the chip is prevented. As the volume of each reagent added to the reaction chamber is predefined at the chip design stage, such systems are not versatile enough to accommodate any modification in the protocol. Secondly, the total reaction volume of each batch-processor is limited by the mixing time. The rotary mixers used typically yield mixing times measured in minutes for volumes of 5 nL,\(^10\) and even when optimised this volume is only increased to 140 nL for the same time period.\(^8\) Such a limitation is relevant in examples such as single cell protocols. Here, multiple amplification steps are needed to remove bias issues around rare signals,\(^11\,^12\) as such larger reaction volumes must be handled. In this work we seek to remove these volume related limitations, by developing a flexible system in which protocols can be developed on chip and with much faster mixing times.

Mixing methods can be broadly categorised into two kinds, passive and active. The first uses physical structures\(^13\,^14\) or repetitive expansions\(^15\) within a channel to induce mixing as the fluid flows through. Clearly, in a chamber containing a finite volume, these kinds of flow-based methods are not feasible. Therefore, to improve mixing speeds in finite volumes, beyond what can be obtained through diffusion or by rotary mixers, active mixing becomes the only viable option. Here, energy is delivered into the system using mechanisms such as magnetic forces acting on suspended beads,\(^16\) electrical potentials on conductive flow medium,\(^17\) low frequency vibration of the system,\(^18\) or ultrasonic actuation.\(^19\,^20\)

In microfluidic systems, acoustic actuation has become a popular method of interacting with fluids, interfaces and suspended matter. As an ultrasonic wave passes through a fluid volume it causes acoustic radiation forces which act on the interface between two immiscible fluids,
and can be used for droplet generation and handling and on suspended matter which has found widespread applications in cell patterning and particle sorting. In addition, body forces are created which act directly on the fluid and result in swirling motions, these can be used to capture cells or nanoparticles in flowing fluids, manipulate particles in sessile droplets, to detach adherent cells and can be applied for rapid mixing. For the latter, ultrasonic mixing is achieved in microfluidic channels with an externally imposed flow, rather than the finite volume case studied here.

Acoustic actuation can involve the use of bulk piezoelectric actuation which can be used for mixing in conjunction with structural discontinuities, such as pointed edges, fluid/gas interfaces and holes in membranes. An alternative approach is the use of patterned inter digital electrodes on a piezoelectric substrate to create surface acoustic waves (SAW), which couple efficiently into fluids. The typically high frequency of operation creates tight spatial focussing of the acoustic energy which gives rise to rapid swirling flows. Here, we make use of SAW to drive mixing within a reaction chamber, and combine this with microvalves for dispensing the required volumes into the chamber.

In this work we present a system which offers flexibility in the reagent quantities used and incorporates active mixing. We took advantage of the ease of fabrication of single layer valves. Here, a thin membrane at the sidewall of the fluid channel is deformed by pressurisation until the channel is blocked, this approach has previously been used for droplet size and composition modulation and droplet sorting. These valves are used to control the delivery of fluid into, an initially air-filled, 150 nL mixing chamber. The progressive filling of which places no requirement for prior knowledge of the optimum microscale protocol.

Acoustic streaming was utilized to drive fluid recirculation inside the mixing chamber for the achievement of rapid and homogeneous mixing. We studied the effects of frequency (48 MHz, 70 MHz and 130 MHz) on mixing time and supported our findings with numerical simulations. The effect of applied power on mixing time was also examined at 70 MHz, the optimum operating frequency. Finally, to demonstrate the performance of the system, on-chip protein crystallisation was conducted. This work offers a flexible on-chip protocol platform with the potential for developing protocols ad hoc.

2. System principles

SAWs are generated when an oscillating electrical signal is applied to patterned, metallic electrodes on a piezoelectric substrate (shown in Fig. 1), termed an interdigital transducer (IDT). At the optimum frequency of excitation, the waves emerging from each finger pair constructively interfere, maximising the amplitude of the SAW. This occurs when \( f = \frac{c_s}{\lambda_{SAW}} \), where \( c_s = 3990 \text{ m s}^{-1} \) denotes the speed of wave propagation along the substrate (lithium niobate, LiNbO\(_3\), 128° Y-cut X-propagating) and \( \lambda_{SAW} \) is the wavelength of the SAW. In the presence of a fluid volume, the acoustic energy will couple into it at the Rayleigh angle, \( \theta_R = \sin^{-1} \left( \frac{c_f}{c_s} \right) \), where \( c_f \) is the speed of sound in the fluid (1497 m s\(^{-1}\)). This energy transfer into the fluid, results in a SAW amplitude decay by a factor of \( e \) (Euler’s number) over the attenuation length, \( \alpha^{-1} \), where \( \alpha^{-1} = \frac{\rho_s c_s \lambda_{SAW}}{\rho_f c_f} \), in which \( \rho_s = 4700 \text{ kg m}^{-3} \) and \( \rho_f = 997 \text{ kg m}^{-3} \) denotes the density of the substrate and fluid, respectively. As such the attenuation length is approximated to 12.5 \( \lambda_{SAW} \).
attenuation of the SAW, also causes a spatial decrease in the energy coupled into the fluid, the resulting pressure field gradient gives rise to a body force which acts on the fluid. This, in turn, causes a steady net flow, termed acoustic streaming.\(^4\) It is this effect which we utilise for reagent mixing in the reaction chamber (shown in Figure 1). The IDTs are aligned such that the beam of SAWs will propagate along the straight wall of the chamber, the net motion of fluid this causes is balanced by a reflux flow which occurs outside the area of the beam, the intention is that a single vortex is formed.

The other aspect of the system is the metering of the fluid into the reaction chamber using single layer valves. These consist of a thin membrane located between the feed channel and a control channel (see Figure 1). When the control channel is pressurised the membrane deforms and fully constricts the main channel. The dimension of the membrane was found to be optimal at 20 μm in thickness, 120 μm in height and 800 μm in length, with these values providing the best combination of membrane flexibility and robustness under pressure. The fluid feed channel was also designed to improve the performance of the valve through the use of a bottleneck at the valve, in which the channel width is reduced from 70 μm to 15 μm.

The rounded geometry of the mixing chamber we present has a total reaction volume of 150 nL, and is intended to enhance fluid circulation by enabling the vortex generated by SAW to extend and encompass the whole chamber, with sharp corners being minimised. The chamber is fed through a delivery channel, where the single layer valve is located and an outlet channel through which air escapes as the chamber is progressively filled. The length of this outlet is intended to minimise evaporation. The feed channel branches of the main channel (not shown in Fig. 1) through which fluid is pumped constantly. This main channel also has a single layer valve. In order to dispense fluid into the chamber for the first time, the valve in the feed channel is depressurised and so opens, and that in the main channel is pressurised and so closes, this causes the main channel flow to divert along the feed channel. Subsequently, once the valve channel is fully wetted, further dispensing of the fluid into the chamber can occur without the use of the valve in the main channel, by depressurising the feed channel valve, the main channel was designed such that a sufficient pressure head exists to divert flow into the chamber. The branched nature of the main and feed channel allows for the future
addition of multiple chambers on a chip. Other reagent types were introduced by changing the fluid pumped along the main channel. In this manner, the mixing chamber can be progressively filled with different reagents allowing protocols to be followed.

3. Methods

3.1 Experimental set up

The device is comprised of a microfluidic channel formed within soft polydimethylsiloxane (PDMS) and the LiNbO₃ piezoelectric substrate. The layer of PDMS containing the valve and channels was cast on a master mould which was fabricated by standard SU-8 photolithography. To facilitate membrane deformation the PDMS was made softer than usual by using a non-standard mix ratio (20:1, ratio of the polymer and curing agent). Throughout the chip, channels were designed to have a height of 120 μm and a width of 70 μm, with the exception of the location of the valves at which a constriction was included to reduce the width to 15 μm.

IDTs were patterned on the piezoelectric substrate using E-beam evaporation to deposit 5 nm of chromium as an adhesion layer and 250 nm aluminium, a 400 nm SiO₂ was then deposited across the whole wafer. Focused IDTs were designed with a focal point at 1000 μm from the last finger pair, and with SAW wavelengths of 30 μm, 55 μm and 80 μm, in each case, the aperture was fixed at 240 μm. A 10 μm layer of PDMS was spin coated on the substrate. This was to aid the bonding of the thin membrane structures in the top layer of PDMS, and to better resist the pressures applied in the valve to these structures (there were no features in this layer).

To initialise the system, the valve control channels were prefilled with water and subsequently connected to a pressure-controlled pump (Fluigent, MFCS-EX). In the main channel network, fluid was pumped using a syringe pump (KD Scientific Legato 210, Holliston, MA, USA). When pressurised, the valves prevented the filling of the reaction chambers, with the continuously pumped fluid going to waste, upon deactivation the initially air-filled chambers were progressively filled with fluid. Two different fluids, pure water and fluorescent dye (fluorescein, Fluka Analytical), were used in the main channel alternately. This allowed two distinct samples to be introduced into the reaction chamber sequentially, and the mixing performance of the SAW actuation to be examined. The SAWs were actuated using an RF signal generator (Rohde & Schwarz, HAMEG HM8134-3) and amplified by a power amplifier (Amplifier Research, 25A250A). In all experiments, the device was actively cooled using a Peltier cooler integrated into a 3D printed mount. Videos were recorded using a digital CCD camera (PixeLink, PL-B782U) mounted on an upright fluorescent microscope (Olympus BX43).

3.2 Data analysis

Quantification of the system’s mixing performance was obtained using a frame by frame analysis of the fluorescent intensity captured from the mixing chamber. Typically, the experimental sequence would involve, firstly, de-actuation of the feed channel valve to allow one of the fluids (e.g. water) to flow from the main channel into the reaction chamber. Subsequently, the valve would be actuated (i.e. feed channel valve closed) and the chamber sealed and the fluid in the main channel changed to the second fluid (e.g. fluorescein solution). The feed channel valve would be de-activated again to allow the delivery of a
desired quantity of the second fluid into the chamber. Finally, mixing would either be diffusion driven or activated using SAWs. The area containing fluorescent dye was defined through thresholding of the intensity in each frame. The variation in the intensity of these pixels was used to assess mixing. Initially the mixing index (MI) was used for this task. It compares the standard deviation of the intensity of fluid at each time point with that in the initial, unmixed state:

\[
MI = 1 - \frac{\sqrt{\frac{1}{N} \sum_{i=1}^{N} (I_i - \bar{I})^2}}{\sqrt{\frac{1}{N} \sum_{i=1}^{N} (I_i' - \bar{I}')^2}}
\]

where \(N\) corresponds to the number of pixels analysed, \(I_i\) represents the grayscale intensity of the current frame and \(I_i'\) the intensity of the initial frame (unmixed state). The MI value can be expected to start at zero and increase towards 1 (perfect mix) over time. Fig.2 (a) shows the MI plotted against time for a typical experiment. The insets show a chamber partially filled with water initially, and subsequently an equal volume of fluorescein solution was added. Over the duration of the actuation, the intensity is seen to become uniform over the wetted area of the chamber. In the mixing index plot, it can be seen that SAW actuation results in a rapid increase of the MI (region with white background). Subsequently, at a value of approximately 0.85, the MI levels off (blue background), indicating a fair degree of mixing. However, when the SAW is first turned off (transition from blue to grey background), a jump is observed in the MI, with the MI reaching a value above 0.9, usually associated with good mixing. This rapid increase is a visual artefact, in the sense that the fluid is not instantaneously better mixed. Rather, it is due to a region close to IDT, the colour of which is darkened during operation. The SAW couples from the substrate into the fluid before impinging on the roof of the channel, we believe this discoloration is due to the deformation of the roof of the channel, fabricated using a mix of PDMS designed for its enhanced flexibility. As a consequence, the mixing time has been evaluated using a technique which examines a region of the chamber which suffers no discolouration during operation.
During the mixing process the interface between high intensity and low intensity pixels moves outwards in the case shown in Fig. 2a. As such a region of pixels was selected which passes from the corner of the chamber nearest to the IDTs, to the contact line of the fluid at the opposite edge of the chamber (bottom right to top middle in Fig 2b). The intensity of the pixels was averaged across a width of five pixels for each point along the length of this area. This intensity information is plotted against time in Fig 2c. Firstly, this plot shows that at low y-values, i.e. near the IDT, the measured intensity is low (time 0.5 to 6 s, y-values less than 5) until the SAW is turned off (second vertical dotted line), this is due to the distortion of the image, as the SAW is actuated, it causes a step change in the MI analysis. In addition it shows how the interface from brighter (red/yellow; Fig 2c) to darker (blue; Fig 2c) pixels moves to higher y-values over time, until this interface, more clearly shown in the thresholded image in Fig 2d, reaches the boundary of the chamber (first vertical dotted line). This occurs at the mixing time comparable to that determined by the MI, but offers a more robust method across our experiments, avoiding the issue of discoloration near the IDTs and more clearly defining a time at which mixing can be considered complete.

**Numerical simulation**

SAW aids the mixing process via the exploitation of acoustic streaming, which generates a steady state flow in the fluid domain. We invoked numerical simulations to aid comparison and optimisation of operational frequencies. The resultant fluid velocity profile at the three SAW wavelengths used experimentally were analysed. Specifically, we wish to study the
interplay between two key aspects as they vary with frequency. First, as the SAWs propagate they couple from the substrate, therefore the SAW intensity attenuates exponentially. The rate of decay is wavelength dependent, with studies showing that an inverse relationship with SAW wavelength.\textsuperscript{42, 46} In order to have a sufficient reaction chamber volume, the mixing chamber must be relatively large, as the height is limited by the need to fabricate a valve membrane, the difficulty of which increases considerably with height. Hence, the distance at which the SAW attenuates to an insignificant strength is likely to be significantly smaller than the chamber itself. The role this plays in the strength of the streaming needs to be established.

Second, streaming is driven by body forces (i.e. Reynolds stresses) acting on the fluid, generated by the propagation of the ultrasonic waves coupled from the substrate into the fluid, it can be derived from equation below: \textsuperscript{47}
\[ F = \rho_f \left< \nabla U + U \nabla U \right> \] (2)
where \( U \) denotes first order velocity results from SAW oscillation and \( \rho_f \) is the fluid density. The strength of these body forces is frequency dependant when comparing actuation at a constant input energy.

With a view to probing these two effects, in a computationally efficient manner, a simplified, two-dimensional system to mimic the condition when the chamber was fully filled. To achieve this, a two-stage study was carried out in COMSOL Multiphysics v5.2. The acoustic velocity used in equation (2) to calculate body force was obtained by solving the Helmholtz equation (acoustic module in COMSOL Multiphysics). In the experimental setup, the SAW couples into the fluid through PDMS layer from the LN substrate and propagates at the Rayleigh angle through the fluid layer. This cannot be captured in a two dimensional model in which the plane of interest is the parallel to the substrate – this is the plane in which the mixing takes place. So, in the acoustic pressure module, a wave front is applied to the boundary of the domain with a curve matching the shape of the smallest IDT finger, this leads to an oscillation which propagates along the plane of the model. To capture the inherent SAW attenuation which occurs due to energy leaking into the fluid, the wave is attenuated with a damping coefficient in accordance to the attenuation length \( \alpha^{-1} \), along the chamber length. The magnitude of initial pressure was chosen such that same energy was applied across models. The other boundaries of the acoustic domain were impedance matched, as PDMS is well matched acoustically to water. In the second stage, a laminar flow steady state study was then conducted to calculate the streaming velocity fields for each corresponding frequency. In the fluid model, the boundary conditions of the sidewalls were set to be no slip and the streaming field was obtained by solving the Navier-Stokes equations driven by the calculated body forces (from equation 2). This approach will not capture all features of the full 3D experimental system, but will allow for the probing of competing effects. Specifically, the effect of streaming strength as a function of frequency, namely at raised frequencies we obtain higher \( U \) (for constant power input), but also lower penetration of the wave into the chamber due to an increased attenuation.

**Protein crystallization**

Lysozyme from chicken egg was purchased from Hampton Research (Product number: HR7-110) and was dissolved in 50 mM sodium acetate (Sigma Aldrich) pH 4.5 to a final concentration of 50 mg/ml. The protein was dissolved and the solution passed through a 0.22 \( \mu \)m centrifugal filter (Merck-Millipore) to remove any aggregates. The protein solution was
then mixed with the crystallisation solution containing 50 mM sodium acetate pH 4.5, 15% (w/v) PEG 5000, and 3.5M NaCl at a 1:1 volume ratio and crystals allowed to form in the reaction chamber via the batch method.

4. Results and discussion

Experiments were conducted to determine the efficacy of the valve, to establish the mixing times which can be achieved, and to determine the optimum excitation conditions for mixing, the latter being supported by numerical simulation.

In the main channel the fluid is pumped along the main channel at a constant flow rate. Initially, the reaction chamber is air-filled, and this remains so whilst the valve is actuated. Upon depressurisation of the valve, the membrane retracts, and fluid can flow from the main channel into the chamber via the feed channel. This controlled leaking from the main channel into the chamber is driven by the pressure head at the entrance to the chamber, and hindered by the energy required to move the contact line at the air-liquid interface. By ensuring a sufficient flow rate and resistance in the main channel the latter becomes insignificant. In Fig. 3 the volume of fluorescein solution in the chamber is plotted against time as the valve is repeatedly opened and shut. At a valve gauge pressure of 1400 mbar, it can be seen that there is no undesired fluid flow into the chamber, the volume remains constant when the valve is sealed. It can also be observed that the flow into the chamber starts slowly, due to the hysteresis of the PDMS forming the valve, it then increases to a rate of 41 ± 3.9 nL/s. When opening the valve, the pressure drops to atmospheric pressure, a more rapid opening could be achieved, if desired, by lowering this pressure or by lowering the flow resistance in the dead-end control channel which forms the valve.
Fig. 3 Volume of fluorescein inside chamber over time. The reaction chamber was filled with fluorescein in an increasing manner, controlled by the valve operation. The delivery channel was shut when the valve was pressurized at 1400 mbar, holding fluorescein within the reaction chamber for a time period. The valve opens when depressurized, allowing fluid into the reaction chamber.

After establishing that the valve operates as intended, we examined the mixing performance of the SAW at three operational frequencies. To do this, the fluid in the main channel is altered, whilst the feed channel valve is closed, such that we can deliver an equal volume of fluorescein solution and water into the reaction chamber. The mixing time is plotted in Fig. 4 against the total volume of fluid present in the chamber, for an excitation power of 26.4 dBm. The order of fluid introduction (i.e. initially water filled or fluorescein filled) was reversed and as expected this had no effect on the measured mixing time, indicating a robust analysis procedure. Based on the images in Fig 4b, the fluorescein can be seen to vary in intensity despite the constant level of illumination, this is due to photobleaching. Upon initial actuation of the SAW, these images show that a plume of the fluid added second to the chamber (i.e. closest the chamber entrance) is formed in the direction of SAW propagation, this is especially evident for the case of the lowest frequency. As time progresses the curved boundary between the two intensity levels moves radially outwards as mixing ensues. As expected, larger fluid volumes take longer to mix, it is also noteworthy that, regardless of volume, the fastest mixing is achieved by the middle frequency, that of 70 MHz.

To examine why 70 MHz offers more rapid mixing than either 130 MHz or 48 MHz, we conducted a numerical simulation. Here, what is key is the distance the wave penetrates the relatively large chamber (measuring 1740 µm or 58 wavelengths for the 130 MHz case). As the SAW beam propagates under the fluid filled cavity, energy will couple into the fluid in the form of acoustic waves, it is these coupled waves which drive the acoustic streaming required for mixing. However, this means that the amplitude of the SAW drops with propagation distance (over a distance of $12.5 \lambda_{\text{SAW}}$, the displacement amplitude drops to $1/e$ of the original value). As shown in Fig 5 a, c and e, larger the acoustic pressure was generated in high frequency case while it decays faster spatially. These plots are shown at the same scale as the reaction chambers shown in Fig 5 b, d and f. In each simulations the input power is equal.
Fig. 5 Numerical results of first order velocity in reaction chamber (a,c,e) for λ=30, 55, and 80 μm at 130, 70, and 48 MHz, respectively. Resultant streaming velocity magnitude field overlaid with streaming velocity vectors across each respective frequency (b, d and f). Comparison of velocity at fluid domain boundary under each frequency, density of streamline reflecting magnitude of circulation of fluid (g). $V_{n}$: streaming velocity normalised to maximum value in 70 MHz frequency. $D_{b}$: distance from domain boundary in direction 45° away from SAW propagation.

There are two effects at play, the penetration distance of the SAW which effects the distance over which body forces are generated, this is better for a lower frequency, and, secondly, the of the streaming strength which has a relationship with the frequency when equal excitation powers are compared, this favours high frequencies. The result of this interplay is shown in Fig 5 b, d and f, the stream lines showing the fluid circulating inside the chamber, whilst the highest frequency has the highest maximum flow speed, it can be seen that the streamlines
are concentrated near the IDTs, showing the poor penetration of the SAW waves into the fluid volume across whole chamber. Note that, as the transmission process of SAW through PDMS film which causes more energy loss in higher frequency in this process was not considered in this simplified model, the extent of streaming will be overestimated for the higher frequency. What limits mixing time is the fluid circulation in the corner furthest away from IDT. In order to probe this effect, the velocity close to boundary was compared for each frequency along direction 45° from SAW propagation, as shown in Fig 5 g, it can be seen from this data that the middle frequency has the highest velocities in this region and so can expect the fastest mixing speeds.

![Fig. 6](image)

Fig. 6 (a) Raw intensity along x direction on three consecutive time points (left), time lapse micrographs (right) showing fine mist of fluid sprayed after t=0 s when SAW is turned on. (b) Composite image of before (green) and after (purple) actuation of SAW, comparing change in interface curvature. Scale bar 200 µm.

A secondary effect of the longer penetration distance at the lowest frequency is that when the chamber is only partially filled, significant acoustic amplitudes can impinge on the air fluid interface. The effect of this is shown in Fig 6 a, where the intensity is plotted at three time points along a line across the chamber. At low x values, the intensity is outside the wetted area, as x is increased a step change is seen as the air-fluid boundary is crossed. Firstly, over time it can be seen that the intensity of the fluid decreases slightly as the fluorescence decays. More importantly, it can be seen that despite this decay, the intensity outside the air-fluid boundary increases. This is due to a small degree of atomisation at the interface which leads to a spray of droplets. In addition, at smaller volumes, and low frequencies, a significant motion of the air-fluid interface can be observed, as seen in Fig 6 (b).
Fig 7. The effect of power applied on mixing speed for a range of fluid volumes under given frequency 70.9 MHz. Inserts are experimental images at linked data points illustrating initial condition and after SAW mixing.

Fig 7 shows the role of power in determining the mixing time. For a range of volumes, which can be seen visually in the insets, the mixing time has been found for three powers at 70.9 MHz. Inevitably, the mixing time drops with increased power and volume. At 28 dBm mixing times of between 2 and 5 s are recorded over the range of volumes examined. However, whilst the power increases from 24 dBm (251 mW) to 26.4 dBm (436 mW), a significant drop in mixing time is observed. Further increase of power to 28 dBm (631 mW) had a relatively modest effect. Clearly there are diminishing gains to be made at further increased powers, and it is noteworthy that at this frequency the onset of atomisation can be observed at 28 dBm, whilst this involved only very small volumes of fluid being ejected from the sample, it is indicative of an upper limit. Consequently, the fastest mixing was observed across the volume range of 40 to 160 nL and occurred over a time period of between 2 and 7 s.

Fig. 8 comparison of free interface distribution (a) and protein crystal formed with SAW mixing (b)
The system represents a platform to develop and run chemical reaction protocols on a chip. Its design consists of a central channel to which multiple reaction chambers can be connected. At the junction between the central channel and the reaction chambers single layer valves control the access of fluid. The reaction chambers can be seen as an open vessel and therefore can be partly or totally filled. Anytime, after or while filling, the SAW based mixing can be activated to facilitate and speed up mixing. These features are essential for the reproducible performance of chemical reactions. As an example, here we performed a batch protein crystallisation reaction. Lysozyme was used because the desirable conditions are known and in order to obtain a highly uniform pool of crystals, whilst minimising crystal size, the mixing time is critical. We tested the crystallisation under passive and active mixing and we could clearly see in Fig 8 that the size distribution of the micro-crystals changes dramatically between the two tests. Specifically, in the case of the diffusive mixed sample, the crystal has a size variation of $8.2\pm3.8\,\mu m$ as compared to $10.3\pm1.6\,\mu m$ for the SAW mixed case.

5. Conclusion
We have demonstrated a flexible platform for performing various protocols on the chip. The mixing process was enhanced by acoustic streaming and the customized delivery of fluid into the mixing chamber was controlled by a single layer valve. Whereas, within protocol specific microfluidic devices, the fluid process flow are predefined. The added dexterity in terms of fluid delivery potential utilising this platform, allows flexibility to perform varies protocols. The optimum operating condition has been identified by evaluating the effect of frequency and power applied on the mixing time. Three frequencies (48.75 MHz, 70.9 MHz and 130 MHz) were tested and experiments indicate that the middle frequency (70.9 MHz) has the best performance, resulting from the trade-off between the effects of frequency on attenuation length and body forces, consistent with the findings obtained via numerical simulations. The effect of applied power was tested at the middle frequency, resulting in a reduction of mixing time to 5s for 153 nL and 1.5s for 44.3 nL at an excitation power of 28 dBm. On chip protein crystallization has been conducted to demonstrate the capability of performing biological reactions, resulting in more uniform protein crystal sizes as compared to the case without SAW mixing, demonstrating the need for rapid and efficient mixing.

Acknowledgement
We gratefully acknowledge the support received from the Australian Research Council, Grant No. DP160101263. This fabrication required for this work was performed in part at the Melbourne Centre for Nanofabrication (MCN) in the Victorian Node of the Australian National Fabrication Facility (ANFF). This research was undertaken with the assistance of resources from the National Computational Infrastructure (NCI), which is supported by the Australian Government.

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