Acoustomicrofluidic separation of tardigrades from raw cultures for sample preparation

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Tardigrades are microscopic animals widely known for their ability to survive in extreme conditions. They are the focus of current research in the fields of taxonomy, biogeography, genomics, proteomics, development, space biology, evolution and ecology. Tardigrades, such as Hypsibius exemplaris, are being advocated as a next-generation model organism for genomic and developmental studies. The raw culture of H. exemplaris usually contains tardigrades themselves, their eggs, faeces and algal food. Experimentation with tardigrades often requires the demanding and laborious separation of tardigrades from raw samples to prepare pure and contamination-free tardigrade samples. In this paper, we propose a two-step acoustomicrofluidic separation method to isolate tardigrades from raw samples. In the first step, a passive microfluidic filter composed of an array of traps is used to remove large algal clusters in the raw sample. In the second step, a surface acoustic wave-based active microfluidic separation device is used to deflect tardigrades continuously from their original streamlines inside the microchannel and thus isolate them selectively from algae and eggs. The experimental results demonstrated the efficient separation of tardigrades, with a recovery rate of 96% and an impurity of 4% algae on average in a continuous, contactless, automated, rapid and biocompatible manner.

ADDITIONAL KEYWORDS: acoustic radiation force – Hypsibius exemplaris – surface acoustic wave.

INTRODUCTION

Tardigrades, which are microscopic animals found on all continents, are widely known for their cryptobiotic abilities to survive in extreme physical conditions (Guidetti et al., 2011; Møbjerg et al., 2011), such as temperature (Hengherr et al., 2009a, b), pressure (Seki & Toyoshima, 1998; Ono et al., 2008, 2016), space vacuum (Jönsson et al., 2008), ultraviolet (Altiero et al., 2011; Horikawa et al., 2013; Giovannini et al., 2018) and γ-ray exposure (Beltrán-Pardo et al., 2015). Much effort has been devoted to characterizing the taxonomy (e.g. Kosztyla et al., 2016; Jørgensen et al., 2018), biogeography (e.g. Pilato & Binda, 2001), evolution and ecology (e.g. Nelson, 2002), genomics (e.g. Yoshida et al., 2017), proteomics (e.g. Schokraie et al., 2012), development (e.g. Smith et al., 2016; Gross et al., 2017) and space biology (e.g. Rebecchi et al., 2009; Weronika & Łukasz, 2017) of tardigrades in an effort to gain a better understanding of this group of microscopic organisms. Recently, the recovery and reproduction of a tardigrade that had been frozen for > 30 years attracted a great deal of attention (Tsujimoto et al., 2016). Tardigrades have been found to produce specific proteins (e.g. CAHS, SAHS, MAHS, RvLEAM) under external stresses to enable survival (Yamaguchi et al., 2012; Tanaka et al., 2015). A unique DNA-associated protein (Daup) from a tardigrade has been expressed in human cultured cells to improve human radiation tolerance and reduce X-ray-induced damage of the cellular DNA by 40% (Hashimoto et al., 2016).

With the aforementioned characteristics, tardigrades have been suggested as potential model...
organisms for space research (Jönsson, 2007) and developmental and genomic studies, to be investigated along with two other commonly used model organisms: Caenorhabditis elegans (Nematoda) and Drosophila melanogaster (Arthropoda) (Tenlen et al., 2013; Goldstein & King, 2016; Martin et al., 2017). The tardigrade Hypsibius exemplaris (Gasiorek, 2018) has been specifically investigated as a new model animal for evolutionary developmental research because it shares characteristics with the two model animals mentioned above: a short generation time and ease to culture continuously for decades (Gabriel et al., 2007). The genome of H. exemplaris has been sequenced to investigate the evolution of molecular and developmental mechanisms. It was suggested erroneously that a horizontal gene transfer from other animals has had an undesirable impact on the structure of the genome of H. exemplaris (Boothby et al., 2015). However, some follow-up studies by subsequent researchers refuted this earlier claim and contended that the false finding was attributed to bacterial contamination derived from uncontrolled sample preparation (Arakawa, 2016; Bemm et al., 2016; Koutsovoulos et al., 2016). To prevent such false-positive results in experimental data, it is imperative to develop effective separation methods for selective isolation of tardigrades from raw cultures.

Increased research interest in tardigrades has demanded advancements in supportive technologies to facilitate experimentation and the development of standard protocols. Several studies on the laboratory-scale culture methods of tardigrades have been reported (Altiero & Rebecchi, 2001; Suzuki, 2003; Horikawa et al., 2008; Altiero et al., 2015; Tsujimoto et al., 2015). The growth cultures of tardigrades contain tardigrades of different age groups (adults, neonates and juveniles of variable sizes), eggs, exuviae and algal food. It is important to prepare homogeneous samples of tardigrades having a uniform age or size distribution for further experimentation (Gabriel et al., 2007). The separation of tardigrades from food, eggs, exuviae or unwanted debris in a raw sample is laborious and time-consuming work, in which individual tardigrades must be selected manually using a wire Irwin loop (Sands et al., 2008), a micropipette (Degma et al., 2011) or a needle (Gross et al., 2017) under a microscope. These labour-intensive methods require a skilled person to locate and dexterously capture a single tardigrade in a sample under a microscope, provide a limited separation throughput and are susceptible to contamination. In contrast, tardigrades can also be isolated in batches from their algal food using the Baermann filtration technique, in which raw cultures are repeatedly passed through a filter paper (Koutsovoulos et al., 2016). Despite the simple operation and low cost of the filtration method, it has been reported to have inherent limitations, such as the long processing time and its dependence on sample volume (Van Bezooijen, 2006). The manual and filtration methods described above do not offer continuous, automated, non-contact, on-demand control over the separated constituents.

In recent years, microfluidic approaches to separate microscale objects have been proved to be effective and have shown great potential for many medical, biological, chemical and industrial applications (Sajeesh & Sen, 2014; Shields et al., 2015). Microscale flow within a microchannel allows precise control over the flow and suspended objects and allows continuous sample processing. A variety of microfluidic separation techniques (Bhagat et al., 2010), including passive methods that use hydrodynamics forces arising from the microchannel geometry and active methods that rely on external force fields, have been reported to offer rapid, continuous, automated, biocompatible sample processing. Inertial microfluidics (Hur et al., 2010), hydrodynamic filtration (Choi et al., 2008), magnetophoresis (Alnaimat et al., 2018), optofluidics (Jung et al., 2014), dielectrophoresis (Mathew et al., 2016) and acoustofluidics (Ding et al., 2013; Destgeer & Sung, 2015) are common techniques for isolating micro-objects based on size, magnetic, optical, electrical or mechanical properties. In particular, acoustofluidic platforms are widely used for numerous applications, including cell sorting (Ma et al., 2017), microparticle separation (Ahmed et al., 2017a, 2018), microparticle patterning (Destgeer et al., 2016, 2019; Collins et al., 2018), microscale flow mixing (Nam et al., 2018; Ahmed et al., 2019), chemical and thermal gradient generation (Destgeer et al., 2014b; Ha et al., 2015), protein isolation (Ahmad et al., 2017), droplet handling (Park et al., 2017a, 2018b), in-droplet microparticle manipulation (Park et al., 2017b, 2018a), nebulization (Winkler et al., 2017) and microorganism manipulation (Ding et al., 2012). Microfluidic platforms can be used for selective isolation of tardigrades from complex samples to address the limitations of the conventional tardigrade separation techniques based on pipettes, sieves, filter papers or Irwin loops.

In this paper, we have combined passive and active microfluidic separation techniques to isolate H. exemplaris tardigrades from raw samples. In the first step, a laboratory culture of tardigrades was passed through a passive microfluidic filter to capture large algal clusters. The passive filter was composed of an array of micropillars that acted as traps and were built inside a polydimethylsiloxane (PDMS) microchannel to allow passage of micro-objects (i.e. tardigrades, eggs, algae) smaller than the trap pore size while trapping larger objects (i.e. algal clusters).
The sample collected from the outlet of the passive filter contained tardigrades, exuviae, eggs and algal food, and it was then passed through an active separation device for the second step. The active microfluidic platform was composed of an interdigital transducer (IDT) mounted on top of a piezoelectric substrate and a PDMS microfluidic chip. Surface acoustic waves (SAWs) produced from the IDT were transformed into compressional waves (CWs) inside the microchannel and exerted an acoustic radiation force (ARF) on the tardigrades in the direction of wave propagation. The deflected tardigrades were thereby isolated from any remaining algae, exuviae and eggs, with the achievement of a recovery rate of 96% and algal impurity of 4%. The experimental demonstration has proved that the proposed method is a promising technique for the preparation of tardigrade samples in a continuous, automated, rapid, biocompatible, non-contact, on-demand manner.

MATERIAL AND METHODS

Tardigrade culture
Cultures containing the tardigrade *H. exemplaris* and *Chlorococcum* sp. algae were purchased and grown according to the protocol provided by the supplier (Sciento). The *H. exemplaris* tardigrades were grown in Chalkley’s medium plus soil extract at 16 °C, with a light–dark cycle of 14 h–10 h (Arakawa *et al.*, 2016). The *Chlorococcum* algae, used as tardigrade food, were prepared in Bold’s basal medium plus soil extract at 25 °C with a light–dark cycle of 16 h–8 h. Every 4–6 weeks, subcultures of the tardigrades were prepared, because they had consumed their algal food. On average, a new generation of tardigrades was produced every 2 weeks. These tardigrades laid eggs inside their exuviae. The exuviae of a healthy tardigrade might contain up to 36 eggs that hatched within 4–5 days. The juveniles matured into adult tardigrades within 2 weeks, at which point they were ready to lay eggs (Gabriel *et al.*, 2007).

Tardigrade separation process
Figure 1 shows the workflow of the two-step tardigrade separation process composed of the passive filtration of algal clusters and the active separation of tardigrades from eggs and algae.

Unicellular *Chlorococcum* algae tend to flocculate to form large clusters in the tardigrade growth cultures. These aggregated clusters even exceed tardigrades in size; thus, they often cause microchannel clogging and produce pulsating fluctuations that significantly disturb the laminar flow inside the microfluidic channel. For these reasons, the algal clusters present in the growth cultures should be disintegrated by vigorous shaking or removed for the effective separation of tardigrades before the acoustofluidic tardigrade separation.

In the first step, the raw sample was filtered passively in the passive microfluidic device, in which the large clusters of algae were removed by an array of algal cluster traps, each of which consisted of four micropillars. As a result, the amount of algae present in the raw tardigrade sample could be decreased significantly. The micropillars of the traps also broke the flocculated algal clusters into smaller units to facilitate the active microfluidic separation of the tardigrades.

In the second step, the filtered sample was pumped continuously into an active separation device to separate the tardigrades selectively from the remaining algae and eggs. The SAW-induced ARF acting on the tardigrades deflected them from their original laminar streamlines inside a microchannel and thus sorted them into a desired outlet, whereas the algae and eggs were collected at a different outlet without being affected by the acoustic field. The detailed underlying physics of selective separation of tardigrades will be discussed in detail in below.
Design and Fabrication of the Device

Passive and active microfluidic devices were designed and fabricated separately, in parallel, before conducting the experiments in series.

A passive filtration device was designed to have a total of 218 algal cluster traps composed of four micropillars, each 200 μm in diameter. The pillars were arranged in a polar array having a radius of 500 μm, angle of 40° and distance of 142 μm between adjoining pillars, which determined the pore size of the traps, as shown in Figure 2.

A soft lithography technique was used to fabricate PDMS microfluidic chips, on which a microchannel was patterned (Friend & Yeo, 2010; Destgeer et al., 2013). Polydimethylsiloxane was selected as the microchannel material, because it is biocompatible, optically transparent, gas permeable and chemically stable (Weibel et al., 2006). A negative photoresist (SU-8 2150; MicroChem), coated on a silicon wafer using a spin coater (Midas System) and sequentially baked on hot plates (65 and 95 °C, respectively), was selectively exposed to ultraviolet through a chrome/glass mask (NEPCO) mounted on the stage of a mask aligner (Midas System). The height of the algal cluster traps in the passive microfluidic filter was defined as the thickness of the photoresist layer. After baking, the wafer was placed in an SU-8 developer solution to remove the uncured photoresist and thus obtain the desired microchannel pattern. The PDMS base and curing agent (Sylgard 184A and 184B; Dow Corning) were thoroughly mixed, with a weight ratio of 10:1, and the PDMS mixture was poured onto the patterned SU-8 mould on the silicon wafer placed inside a square Petri dish. The Petri dish was placed in a vacuum chamber and degassed for bubble removal. After the PDMS solution was cured in an oven at 65 °C for 2 h, PDMS microfluidic chips were peeled off the Si substrate and cut into appropriate sizes. The inlet and outlet ports were punched (Harris Uni-Core) through the microchannels for sample injection and collection. The surfaces of the PDMS microchannels and a glass slide were treated with oxygen plasma for 2 min (Covance, Femto Science) and gently placed in contact to achieve irreversible bonding.

The active tardigrade separation device consisted of an IDT deposited on a piezoelectric substrate and a PDMS microfluidic chip, on which a desired microchannel was patterned. The IDTs were fabricated by spin-coating a photoresist layer onto a 128°-rotated Y-cut, X-propagating LiNbO3 substrate (MTI Korea) and patterning the resist in the reverse shape of comb-like transducers. A bimetallic conductive layer composed of a 30-nm-thick layer of chrome that promoted adhesion of a 100-nm-thick (1000-Å-thick) gold layer was deposited by the e-beam evaporation process onto the piezoelectric substrate, followed by a lift-off process to remove the excess metals and photoresist. Two IDTs with uniform electrode width and spacing were formed on the substrate.

Two sets of the electrodes were fabricated such that the electrode width was equal to electrode spacing between the electrodes. The resonant frequencies of the IDTs used in the experiments were 45 and 72 MHz. A 200-nm-thick SiO2 layer was deposited to cover the electrodes to protect them from mechanical damage (Destgeer et al., 2015).

Polydimethylsiloxane microfluidic chips were fabricated using the soft lithography process described above. A thin PDMS membrane was used to seal the PDMS microfluidic chip to investigate the effects of the SAW-induced ARF on tardigrades, as shown in Figure 3 (Park et al., 2018b). The 38-μm-thick SiO2 layer was deposited to cover the electrodes to protect them from mechanical damage (Destgeer et al., 2015).

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![Figure 2](https://example.com/figure2.png)  
**Figure 2.** A passive filtration process for the removal of algal clusters from the raw tardigrade sample before the active separation of tardigrades. A, a polydimethylsiloxane (PDMS) microfluidic filtration device placed on a microscope. B, the microchannel layout. C, a magnified view of algal cluster traps composed of four micropillars. D, a microscopic snapshot, in which algal clusters were filtered by the trap, whereas a tardigrade passed through it along with smaller algae and eggs.
and width of the PDMS microfluidic channel were 190 and 500 µm, respectively. The PDMS microchannel was placed directly on top of the IDT (72 MHz) to bond it reversibly to the LiNbO₃ substrate covered with a layer of SiO₂. For tardigrade separation, a PDMS microfluidic chip 163 µm high and 500 µm wide was used. This microchannel was not sealed using a PDMS membrane, but permanently bonded to a SiO₂-covered LiNbO₃ substrate such that the IDT (45 MHz) was positioned outside the microchannel (see Fig. 4).

**RESULTS**

**Passive filtration**

Raw cultures of tardigrades contain numerous algal clusters along with unclustered algae, tardigrades and their eggs. For effective and efficient acoustofluidic separation of tardigrades, the algal clusters should be removed by passive microfluidic filtration, in order to reduce the amount of algae present in the sample significantly.

As shown in Figure 2A–C, the passive microfluidic filter was designed to have an array of 218 traps composed of four micropillars to capture the large algal clusters, whereas the tardigrades, their eggs and algal food would pass readily through the gaps between the pillars. The raw tardigrade sample, including algal clusters, tardigrades, eggs and algae, was injected into the passive microfluidic device at a flow rate of 15–20 mL min⁻¹ (7.5–10 s per round). The distance between the adjacent pillars was 142 µm, meaning that the tardigrades (50–100 µm in length), eggs (40–50 µm in diameter) and algae (10–20 µm in diameter) were allowed to pass through the algal cluster traps, as shown in Figure 2D. Once a trap was filled with an algal cluster, the increased flow resistance caused the following fluid streams carrying large algal clusters and tardigrades to be diverted away from the occupied trap and towards the empty traps downstream of the device.

For validation of the removal of algal clusters, we measured the optical density (OD) of the tardigrade cultures.
sample before and after the passive microfluidic filtration with a spectrophotometer (GeneQuant 1300) at 600 nm; the measured OD value of the tardigrade sample decreased significantly by 76.53 ± 2.88% from 2.198 ± 0.177 to 0.512 ± 0.037 after the algal cluster filtration, on average, for ten independent repeated experiments.

**ACOUSTIC RADIATION FORCE ON TARDIGRades**

For effective acoustofluidic tardigrade isolation, the tardigrades should be affected selectively by SAW-based ARF while the eggs and algae remain unaffected. We investigated the effects of the SAW-induced ARF on the filtered tardigrade sample using a parallel-type acoustofluidic device (Park et al., 2017, 2018b), where the wave propagation direction was opposite to the flow direction. Figure 3A shows the top and side views of the device, in which the filtered sample was injected through the inlet of the microchannel. The bottom of the microchannel was sealed by a thin PDMS membrane to ensure that the tardigrade sample was not in direct contact with the IDT, which might induce undesired side-effects from the electric field formed around the electrodes. When alternating current signals were applied to the IDT at its resonant frequency, SAWs were produced from the electrodes and immediately transformed into CWs.

Figure 3B shows a series of experimental images where the passively filtered tardigrade sample fluid was continuously passing through the microchannel at a flow rate of 100 μL h⁻¹ when 72 MHz SAWs at 17.2 peak to peak voltage (Vpp) were produced from the IDT placed underneath the microchannel. The experiments were conducted in the parallel-type device to confirm that the SAW-based ARF was selectively effective on the tardigrades, for isolation from the eggs and algae. As each tardigrade approached the IDT, the magnitude of the ARF acting on the tardigrade was increased until it was located at the equilibrium position of two counteracting forces: the flow-induced drag force (F_D) and ARF (F_AR), as shown in the figure (t = 7.5 s). The vertical component of the ARF continuously pushed the tardigrade towards the ceiling of the microchannel, while the horizontal component of the ARF matched the drag force (Ahmed et al., 2017a, b, 2018). In contrast to the tardigrade, trapped at the equilibrium position right next to the IDT, the eggs

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**Figure 4.** A, schematic diagram illustrating the continuous separation of tardigrades from algal food and eggs based on surface acoustic wave (SAW)-induced acoustic radiation force (ARF). The sample fluid containing tardigrades, their eggs and algal food was pumped through a central inlet and focused by two sheath flows. The SAW-based ARF deflected the tardigrades away from their original path to separate into a desired outlet port. The eggs and algae were unaffected by the force and thus moved along their original streamlines. B, when the SAWs were not applied, a focused culture sample fluid passed through the microchannel and was collected at outlet 1, without deflection of the tardigrades. Images B1 and B2 of the collected samples at outlets 1 and 2 show the presence and absence of tardigrades in addition to optical density (OD) values for algae quantification at respective outlets. C, the ARF produced by 45 MHz SAWs originating from the interdigital transducer (IDT) pushed the tardigrades from their original path to sort them into outlet 2, with a recovery rate of > 95%. The algae and eggs were mostly unaffected by the force and thus collected at outlet 1. Images C1 and C2 of the outlet samples show the absence and presence of tardigrades and OD values for the quantification of algal impurity at outlets 1 and 2, respectively.
and algae present in the sample passed freely through the microchannel, verifying our hypothesis that the SAW-induced ARF can separate the tardigrades from the eggs and algae (see also Supporting Information, Movie S1). We observed that the tardigrades collected after being exposed to the acoustic field were moving actively (see also Supporting Information, Movie S2) and succeeded in reproduction.

**Acoustofluidic Separation of Tardigrades**

**Figure 4A** shows a schematic diagram of the cross-type acoustofluidic device, where the wave propagation direction was perpendicular to the flow direction, which was used to prepare pure samples of tardigrades from raw cultures. The filtered tardigrade sample fluid was injected into the central inlet, along with the sheath flows 1 (left) and 2 (right) of deionized water from the two other inlets, to focus the sample flow hydrodynamically at the desired location. The flow rates were 2000 (sample flow), 1000 (sheath 1) and 3000 μL h⁻¹ (sheath 2). The sheath flow 2 from the right-hand inlet was used to pinch the sample flow close to the microchannel sidewall, while the shear flow 1 was introduced to prevent the suspended constituents in the tardigrade sample from being located in the anechoic region, where the effective acoustic field was not formed (Destgeer et al., 2015). The IDT was placed beside the microchannel within an acoustic window to minimize acoustic wave damping, meaning that the acoustic waves could be coupled with the fluid inside the microchannel in an energy-efficient manner (Shi et al., 2009; Destgeer et al., 2013). The SAWs radiating from the IDT propagated along the surface of the piezoelectric substrate and penetrated into the microchannel passing through the narrow (100-μm-thick) PDMS wall.

**Figure 4B** shows the stacked experimental image, in which the hydrodynamically focused sample fluid was collected at outlet 1 (left) without the acoustic field applied to the microchannel. The microscopic images of the samples collected from outlets 1 and 2 clearly show the presence (**Fig. 4B1**) and absence (**Fig. 4B2**) of the tardigrade sample, including the tardigrades, eggs and algae. The average OD values of the samples collected at both outlets were measured to be 0.1948 (outlet 1) and 0.0162 (outlet 2), confirming our experimental observation of all the components of the tardigrade sample flowing into outlet 1.

In contrast, **Figure 4C** shows the stacked microscopic image when 45 MHz SAWs at 17.2 V_w were applied to the microchannel. As shown clearly in the figure, the SAW-induced ARF, acting on the tardigrades exclusively, deflected them from the original streamlines, meaning that the tardigrades were transferred from the filtered sample to sheath flow 2. Consequently, the tardigrades could be separated into outlet 2, whereas the algae and eggs followed their original streamlines and were collected at outlet 1 (see also Supporting Information, Movie S3). In five independent repeated experiments with > 100 tardigrades, the average recovery rate was measured to be 95.76 ± 2.42%, with an average throughput of 400 tardigrades h⁻¹. The average OD values of the samples at outlet 1 and 2 were 0.1736 (**Fig. 4C1**) and 0.0203 (**Fig. 4C2**), respectively. Considering that the average OD value of the filtered tardigrade sample was 0.512, the algae impurity was 3.96 ± 1.67% in the sample collected at outlet 2 after the acoustofluidic tardigrade separation.

**Discussion**

When the SAWs interacted with the fluid inside the microchannel, these waves were transformed into CWs and propagated through the fluid at a Rayleigh angle θ_n = sin⁻¹(c_s/c_0) ≈ 22°, where c_s and c_0 are the speeds of sound inside the fluid and the substrate, respectively (Collins et al., 2014; Fakhfouri et al., 2018). When micro-objects are suspended in the microchannel and exposed to the CWs in the path of the wave propagation, these waves are scattered in an inhomogeneous manner at the interface between the liquid and the object. As a result, the suspended micro-objects experience the ARF in the direction of wave propagation. The magnitude of the ARF acting on the micro-objects depends strongly on the dimensionless Helmholtz number, k = πd_m/λ_f, where d_m is the representative length of the objects and λ_f is the acoustic wavelength. If d_m > λ_f, asymmetric wave scattering off the micro-object results in a significant ARF in the direction of the wave propagation, whereas the ARF acting on small objects compared with the acoustic wavelength (d_m < λ_f) is negligible because of homogeneous wave scattering (Skowronnek et al., 2013, 2015; Destgeer et al., 2014a; Devendran et al., 2016).

Considering that the average length of the tardigrades that we targeted to manipulate in our experiments was ~100 μm, we used IDTs with their resonant frequencies > 36 MHz, such that the tardigrades experienced an ARF strong enough to overcome the flow-induced drag force. The 72 MHz SAWs were used in the experiments above to prevent standing SAWs formed in the vertical direction within the parallel-type acoustofluidic device. As shown in **Figure 3B**, 72 MHz SAWs, whose wavelength was smaller than the tardigrades but larger than the eggs and algae, imparted the ARF selectively to the tardigrades. As a consequence, only the tardigrades remained stationary, whereas the other components of the tardigrade sample after microfluidic filtration passed freely through the acoustic field. As previously
reported (Wiklund, 2012; Li et al., 2015), the ARF-based acoustofluidic manipulation was found to be biocompatible and imparted no harmful stresses to the tardigrades, because they moved actively (see also Supporting Information, Movie S2) and reproduced successfully.

The average recovery rate of the acoustofluidic tardigrade separation was > 95%. A few tardigrades did not experience the ARF sufficiently to be deflected towards outlet 2, for the following reasons. First, a few tardigrades might have passed through the acoustic anechoic region, where the acoustic field was not effective, owing to unintended flow disturbance induced by pressure fluctuations and clogging within the microchannel. Second, the characteristic length of some tardigrades was smaller than the acoustic wavelength ($\lambda = 80 \mu m$ for 45 MHz SAWs), meaning insignificant acoustic radiation occurred around the tardigrades. Third, when several tardigrades were passing through the acoustic field close to each other, insufficient magnitude of the acoustic field was applied to the tardigrades located farther from the IDT, leading to insignificant wave scattering.

In the acoustofluidic tardigrade separation, the average time required to collect 100 tardigrades was measured to be 15 min (~400 tardigrades h$^{-1}$). However, the throughput can be varied depending on the number of tardigrades per unit sample volume injected into the active separation device.

In addition, the algal impurity of the tardigrade sample after acoustofluidic separation was measured to be slightly < 4% based on the OD measurement. A small amount of the algae and eggs in the sample flow were unintendedly separated along with the tardigrades for the following reasons. First, the acoustic streaming flow-induced drag force was imparted to the eggs and algae. Second, unintended flow disturbance caused the tardigrade sample flow to be guided imperfectly to outlet 1 by the sheath flows. Third, some algae attached to tardigrades were separated together with the tardigrades that were affected by the SAW-based ARF.

The acoustofluidic tardigrade separation device can easily be switched on and off, simply by controlling the electrical signal applied to the IDT. The on-demand control of the device can be used to prepare tardigrade samples with the desired number of tardigrades.

CONCLUSION

We developed a two-step tardigrade isolation method to separate tardigrades from raw culture samples in a continuous, automated, contactless, on-demand manner. In the passive microfluidic filtration step, large algal clusters were removed by an array of 218 traps composed of four micropillars, with an OD value decreased significantly, by 77% on average, to facilitate the acoustofluidic tardigrade separation.

The effects of the acoustic waves on the tardigrade sample were examined in a parallel acoustofluidic device. In our experiments, tardigrades were found to experience a SAW-based ARF that was significant enough to overcome the flow-induced drag force acting on the tardigrades, whereas the eggs and algae remained unaffected by the acoustic field. Biocompatibility of the acoustic waves applied to the tardigrades was confirmed by active movement and reproduction of the tardigrades after exposure to the acoustic field.

In the active tardigrade separation device, the passively filtered tardigrade sample was injected into the cross-type acoustofluidic device. We demonstrated that the tardigrades could be deflected selectively by the SAW-induced ARF and thus collected at a separated outlet at a recovery rate of 95.76%, with an algal impurity of 3.96%.

The proposed acoustomicrofluidic approach to tardigrade separation is expected to aid tardigrade research and might be extended to the selective isolation of tardigrades in different age groups.

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