INTRODUCTION

Neutrophils are one of the key species involved in the front-line immune defence against invading pathogens, and their bactericidal activity is crucial in protecting the host from harmful bacteria (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012). However, when a host is subjected to severe surgical stress, such as invasive surgery, trauma or a thermal injury, its neutrophil functions are easily impaired, thereby resulting in an immunocompromised host (Kinoshita et al., 2011; Miyazaki, Kinoshita, Ono, Seki, & Saitoh, 2015). Although neutrophils function as instructors of the immune system in the context of not only bacterial infections but also various inflammatory diseases such as autoimmune disorders, neutrophils can be considered as professional killers of invading pathogens, and in particular, microorganisms. Interestingly, psychological stress has also been reported to affect neutrophil functions (Bartlett, Demetrikopoulos, Schleifer, & Keller, 1997; Duggal, Upton, Phillips, Hampson, & Lord, 2013; Reiche, Morimoto, & Nunes, 2005). However, this effect is presumably slight compared to that observed following severe surgical stress. Thus, to evaluate the immunocompromised condition of a host, antibacterial activity can be employed as a good indicator.

In this context, the antibacterial activity of neutrophils can be measured by their incubation with bacteria and comparison of the number of bacteria before and after co-incubation. However, the conventional bacterial killing assay of neutrophils is particularly time-consuming, as it requires 4–6 hr for the co-incubation stage followed by 6–8 hr to count the colony forming units (CFUs) of the bacteria on the culture plates. In contrast, the direct sensing of molecules related to cell activity can remarkably shorten measurement times, and microdevice technology enables analysis using only a small volume of the desired sample solution. Among reported methods, impedimetry is a direct method for measuring the concentration of bacterial or animal cells (Altintas, Akgun, Kokturk, & Uludag, 2018; Etayash, Jiang, Thundat, & Kaur, 2014; Ward et al., 2018), while changes in the pH (Pourciel-Gouzy, Assié-Souleille, Mazenq, Launay, & Temple-Boyer, 2008), superoxide concentration

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Abstract
Impaired neutrophil functions, and in particular their antibacterial activity, are known to reflect the immunocompromised condition of the host. To investigate the potential for prompt on-site measurement of this condition, we analysed the antibacterial activity of murine neutrophils by measuring the change in oxygen consumption in a 10 μl sample solution in which bacteria and neutrophils were co-incubated. A critical point was the use of freeze-dried Escherichia coli (E. coli), which remarkably facilitated handling because of its ability to be stored at room temperature for extended periods and the ease in which its respiratory activity could be re-established. This therefore allowed the antibacterial activity of murine neutrophils to be measured following their co-incubation with E. coli. Indeed, we successfully detected a reduction in the antibacterial activity of murine neutrophils in mice subjected to water-immersion restraint stress.

KEYWORDS
Escherichia coli, neutrophils, oxygen electrode, respiratory activity, stress
(Kimura, Fukuda, Tajima, & Suzuki, 2012), oxygen content (Ito et al., 2016; Yamagishi et al., 2017) and metabolite concentrations (Sanger et al., 2017) of the environment can also be measured. Impedimetry is widely used to measure the concentration of bacterial or animal cells. On the other hand, the changes in the number of live cells can be ascertained by measuring the changes in the respiratory activity of cells. For evaluating the bactericidal activity of neutrophils, the number of bacterial cells killed must be known, which is not necessarily related to the total number of cells. Therefore, for our purpose, a more sensitive method to measure the oxygen concentration or the respiratory activity of cells is required.

To conduct the killing assay, live bacteria need to be prepared. To this end, a common procedure is to store the bacteria suspension in a deep freezer and then thaw it prior to use. Although this may be acceptable for laboratory experiments, this procedure is unsuitable in the context of on-site analyses, due to the strict storage requirements. Ideally, the bacteria should be stored at room temperature and employed in a similar manner to chemical reagents. To address this issue, we herein examine the use of freeze-dried E. coli, which could be restored to a viable state by simply dissolving in an aqueous solution. We therefore investigate the antibacterial activity of murine neutrophils following their co-incubation with freeze-dried E. coli using our micro-fluidic device and attempt to measure the reduced antibacterial activity of neutrophils in mice following water-immersion stress, which is a representative murine stress model (Azlin, Kamisah, Chua, Ibrahim, & Qodriyah, 2015; Takahashi et al., 2012).

2 | EXPERIMENTAL SECTION

2.1 | Materials, reagents, E. coli and animal subjects

The reagents and materials employed for device fabrication and characterization were obtained as follows: glass wafers (no. 7740, 3 in, 500 μm thick, Corning Japan), polyimide prepolymer solution (Semicofine SP-341, Toray Industries), thick-film photore sist (SU-8 25, MicroChem), poly(methyl methacrylate) (PMMA) substrate (500 μm thick, Mitsubishi Rayon, Tokyo, Japan), poly(dimethyldiylxiane) (PDMS, KE-1300T, Shin-Etsu Chemical), isotonic buffered salt solution, Gibco® Hanks’ Balanced Salt Solution (HBSS, Thermo Fisher Scientific), Luria-Bertani (LB) broth (Lennox, Sigma-Aldrich), aminoethylaminopropyltrimethoxysilane (AEAPS, Sigma-Aldrich), polyoxymethylene (20) sorbitan monola rate (Tween 20, Wako Pure Chemical Industries) and E. coli (strain B, ATCC 11303, Sigma-Aldrich). The murine neutrophil samples were obtained from C57 BL/6 mice (male, 8 weeks old, 20–25 g) purchased from Japan SLC, which were subjected to water-immersion restraint stress.

2.2 | Preparation of the freeze-dried E. coli

Prior to use, the E. coli was stored in 20% glycerine (2 × 10^10 CFU/ml) at –80°C. After thawing, a sample of the E. coli suspension (1 ml) was washed twice with phosphate-buffered saline (PBS) (5 ml) by centrifugation for 5 min at 1,500 r.p.m. and 4°C. The precipitated E. coli was then re-suspended in PBS containing 5% trehalose (700 μl), and the resulting suspension was frozen rapidly using liquid nitrogen and then dried over 6 hr using a freeze-dryer (FreeZone 4.5 Labconco). The freeze-dried E. coli was stored with a desiccant at either 4°C, 37°C or room temperature prior to use.

2.3 | The mice water-immersion restraint stress model

All animal experiments were conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Japan (permit No. 14055). The mice were exposed to water-immersion restraint stress by immersion in water for 2 hr at 37°C using a vertical plastic restrainer (30 mm diameter, 80 mm length, Figure S6). The mice were immersed up to their shoulders and were considered immobilized when they ceased to struggle, and limb movements reached a minimum. The control mice were removed from their home cages and placed in new breeding cages for 2 hr.

2.4 | Isolation of the murine neutrophils

As described previously (Kinoshita et al., 2011; Yano et al., 2012), a whole blood sample was withdrawn into a heparinized syringe from the abdominal inferior vena cava of each mouse under lethal anaesthesia with pentobarbital. The anaesthesia itself reportedly does not influence host immune cells (Isobe, Okajima, Harada, Liu, & Okabe, 2004; Ohta et al., 2015). The whole blood sample was mixed with dextran in a 4:1 volume ratio, then laid flat for 30 min. Neutrophils were collected from the supernatant and were re-suspended in HBSS according to the following procedure. After centrifugation for 5 min at 430 g and 4°C, the precipitate was re-suspended in lysis buffer. Following lysis of the red blood cells, the neutrophil suspension was filtered using a cell strainer (40 μm mesh), washed with HBSS and re-suspended in HBSS to give a neutrophil density of 2 × 10^6 cells/ml.

2.5 | Device fabrication

A schematic representation of the device employed herein is shown in Figure 1. The cathode (platinum) and anode (Ag/AgCl) for the oxygen electrode were formed on a glass substrate, where the metals were deposited by sputtering, then patterned using the photore sist lift-off method. The electrode active areas were then delineated with a polyimide layer. For the anode, we used a Ag/AgCl electrode structure, where AgCl was grown from 16 pinholes (50 μm diameter) in the polyimide layer (Suzuki & Taura, 2001). This structure provided a stable anode potential over long periods of time. A compartment to accommodate the electrolyte solution and connecting flow channels to introduce the electrolyte solution were formed using the SU-8 thick-film photore sist. The height of the structure was 50 μm. To facilitate introduction of the electrolyte solution, the
The electrode-bearing glass substrate was treated with a 0.001% solution of Tween 20 for 5 min. After rinsing with pure water, the surface was dried under a flow of nitrogen gas.

The measurement chamber and connecting flow channels were formed from PMMA using a laser engraving machine (8015 Rayjet-50, Trotec Laser Japan). These structures were pierced in the PMMA substrate, which was then bonded to the other PMMA substrate to close one side of the structure. The completed measuring chamber (960 nl volume) and flow channels measured 1.56 mm in width and 500 μm in depth. The opposite side of the structure was then sealed using a silicone rubber membrane (50 μm thickness), which functioned as an oxygen-permeable membrane for the oxygen electrode. For this purpose, the surface of the bonded PMMA substrate was initially activated by exposure to oxygen plasma (power: 100 W, oxygen flow: 30 mg/ml, oxygen pressure: 30 Pa) for 60 s. The PMMA substrate was then immersed in a heated 5% solution of AEAPS for 30 min at 80°C. Following incubation, the PMMA substrate and the silicone rubber membrane were activated by exposure to oxygen plasma once again (power: 20 W, oxygen flow: 20 mg/ml, oxygen pressure: 30 Pa) for 10 s. After this time, the membrane was immediately placed on the PMMA substrate to bind the two materials together (Vlachopoulou et al., 2009).

To complete the device fabrication, the silicone rubber membrane of the PMMA substrate was placed on the SU-8 structure of the glass substrate. The stacked structure was then inserted between two PMMA plates, which were fixed with springs, bolts and nuts (Figure S7). A 0.1 M Tris-HCl buffer solution (pH 8.5) containing 0.1 M KCl was used as the electrolyte solution for the oxygen electrode, which was introduced into the appropriate compartments using a microsyringe.

2.6 | Examination of cell viability of the freeze-dried E. coli and measurements of the dissolved oxygen levels of the E. coli suspensions

To examine the cell viabilities of the freeze-dried E. coli samples stored at a range of temperatures (i.e., 4°C, room temperature, and 37°C), the bacterial stock was suspended in HBSS and incubated on an agar culture plate overnight. The number of E. coli colonies was then counted.

For the on-chip measurement of dissolved oxygen levels of the bacterial suspensions, solutions of the diluted bacterial suspensions obtained from the freeze-dried or freezer-stored (i.e., at −80°C) E. coli samples were prepared in HBSS and then incubated for 30 min at 37°C. Subsequently, the solution of interest was introduced into the device flow channel to measure the oxygen consumption. For operation of the oxygen electrode, the contact pads for the cathode and anode were connected to a potentiostat (Autolab PGSTAT12, Eco Chemie), and the current was measured following the application of −1.0 V to the cathode (vs. the Ag/AgCl anode). All measurements were carried out at room temperature.

2.7 | On-chip measurement of the antibacterial activities of murine neutrophils

A stock sample of freeze-dried E. coli was resuspended in HBSS, and the concentration was adjusted to 1 × 10⁷ CFU/mL. Neutrophil samples were obtained from normal mice and from mice subjected to water-immersion restraint stress for 2 hr. A sample of the E. coli suspension (500 μl, final concentration = 5 × 10⁶ CFU/ml) and the neutrophil suspension (500 μl, final concentration = 1 × 10⁶ cells/ml) was mixed in a 5:1 cell number ratio, and co-incubation was carried out over 6 hr at 37°C. When using the microfluidic device, the E. coli/neutrophil ratio was 5:1, which enhanced the output; this ratio was reversed as compared to that when using conventional assay (1:5) described in the next section. The antibacterial activities of the neutrophils were measured at predetermined times by introducing an aliquot of the mixture (10 μl) into the device. The dissolved oxygen levels of the co-incubation media were measured up to 60 min.

2.8 | Conventional bacterial killing assay of neutrophils

As previously described (Kinoshita et al., 2011; Miyazaki et al., 2015; Yano et al., 2012), murine neutrophils (5 × 10⁶ cells/ml) were co-incubated with E. coli (1 × 10⁵ CFU/ml) in a cell number ratio of 5:1 for 6 hr. The number of live bacteria (i.e., CFU) on the agar plate was then counted.
RESULT AND DISCUSSION

3.1 Properties of freeze-dried E. coli

Morphological and biological properties of freeze-dried E. coli were examined and compared with those of E. coli stored at −80°C following the conventional method without freeze-drying. The freeze-dried E. coli were rod-shaped and grew well on various media, similar to E. coli stored using the conventional method (Figure S1). On brain heart infusion (BHI) agar, they formed grey-white, round colonies. On Drigalski agar, they formed yellow colonies, indicating lactose-fermenting ability. On MacConkey agar, they formed pink-purple colonies, indicating resistance to bile salts. In indole pyruvic acid Kliglers’ iron (IKL) agar tubes, they formed brown-yellow colonies and produced gas, indicating lactase activity. The freeze-dried E. coli also gave positive results in the oxidase test (Figure S2). We also compared the growth of E. coli prepared by the two methods. E. coli (1 × 10^6 CFU) were incubated in 1 ml of 10% fetal bovine serum (FBS)-Roswell Park Memorial Institute (RPMI)-1640 medium in a 5% CO₂ environment at 37°C, and the number of the bacteria was counted at predetermined time points using the CFU assay. The growth of both bacteria was very similar (Figure S3). These results suggest that the properties of E. coli were not lost upon freeze-drying.

Since the total respiratory activity of E. coli is determined by the number of live E. coli cells, the number must be controlled until measurement begins, to ensure that the initial total respiratory activity remains the same. An orthodox method to maintain bacteria in the live state and switch off respiratory activity is to store the bacteria in a deep freezer at −80°C. However, for on-site measurements at places like an athletic stadium or a swimming pool, we cannot use this method. The ideal scenario would be that the bacteria are like chemical reagents and can be stored at room temperature without using a deep freezer or refrigerator for ease of handling by the end.

**FIGURE 2**  Response curves for the oxygen electrode. (A) A saturated Na₂SO₃ solution (a) and air (b) were introduced into the measuring chamber composed of PMMA. (B) Variation of the current of the untreated (orange) and Tween 20-treated (blue) oxygen electrodes

**FIGURE 3**  Variation in the oxygen electrode response with the active E. coli concentration. (a) Variation in the recorded current with time for E. coli suspensions of different concentrations prepared from the freeze-dried samples. Numerals in the figure indicate the density of E. coli (×10^6 CFU/ml). “HBSS” indicates the case without E. coli and with only HBSS. The inset shows the same data with a wider current range. (b) Dependence of the current decrease on the density of E. coli resuspended from the freeze-dried state (black) and the frozen state (white). Values shown are averages ± standard deviation (n = 5) calculated from values at 180 s. The values of the ordinate are shown as the difference between the current decreases recorded with and without E. coli (only HBSS)
users. However, storage of bacteria in a medium initiates growth and it is impossible to maintain the initial respiratory activity. Freeze-drying the bacteria solves all these problems. They can be stored at room temperature without changing their respiratory activity. Moreover, their handling is very simple: they only need to be dissolved in an aqueous solution to be activated.

### 3.2 | Viability of the freeze-dried E. coli

To examine the storage stability of the freeze-dried E. coli samples that had been stored at 4°C, 25°C, and 37°C, freeze-dried E. coli was dissolved in PBS, and the number of live bacteria was counted using the CFU assay (original E. coli concentration = $2 \times 10^{10}$ CFU/ml) (Figure S4). The variation in the number of live E. coli bacteria following storage at the three different temperatures was small over 60 days, and we expect that the observed variations were likely caused by calculation errors due to variations in the sample volume following aliquot removal. In addition, we note that the addition of trehalose to PBS produced a microenvironment that resembled a hydrated surface, and thereby prevented damage to the cell membranes upon freeze-drying (Crowe & Crowe, 2000). Interestingly, we found that the number of live E. coli bacteria detected in these samples was comparable with the number observed following storage at −80°C. This result demonstrates that freeze-dried E. coli can be successfully stored at room temperature without any decrease in its activity and so could be used as a replacement for frozen E. coli.

### 3.3 | Measurement of oxygen consumption by E. coli

To determine the respiratory activity of E. coli via measurement of the consumption of dissolved oxygen, we employed the device shown in Figure 1. Prior to use for the measurement of E. coli respiratory activity, the performance of the device was considered. Figure 2a shows the response profile of the oxygen electrode when air and a saturated Na$_2$SO$_3$ solution were introduced alternately into the measurement chamber. The Na$_2$SO$_3$ solution was employed to remove any oxygen dissolved in the aqueous solutions. The residual current at the zero oxygen level was approximately 10% with respect to the current recorded under air. In the experiments described herein, the decrease in the oxygen electrode current below the baseline was used as an indicator for the number of bacteria. To achieve a suitably stable baseline for this purpose, the issue relating to the presence of air bubbles trapped in the cathode compartment of the oxygen electrode must be addressed (Figure S5a), as in the presence of such bubbles, sudden increases in current are often observed (Figure 2b). Indeed, upon examining the cathode compartment under a microscope, air bubbles were always observed, and we found that once a bubble was trapped, it gradually increased in size, causing an unusual elevation of the oxygen electrode current. To address this issue, treatment with the Tween 20 surfactant prior to device construction was found to be effective, as the electrolyte solution could be smoothly introduced into all compartments, and bubbles were rarely observed in the cathode compartment (Figure S5b). As shown in Figure 2b, a stable current was maintained for a number of minutes, which facilitated the precise measurement of small changes in the dissolved oxygen concentrations.

The oxygen consumption by E. coli was then measured using the device and a series of suspensions prepared from the freeze-dried E. coli, with cell concentrations ranging from $5 \times 10^5$ to $1 \times 10^7$ CFU/ml. Thus, Figure 3a shows the variation in the transient current with time after the introduction of an E. coli suspension into the measurement chamber and the application of a potential to the cathode of the oxygen electrode. As indicated, the transient current decreased upon increasing the E. coli concentration, thereby suggesting an increase in the dissolved oxygen consumption due to the elevated respiratory activity through increased numbers of E. coli cells. In addition, Figure 3b shows the dependence of this current decrease on the density of E. coli. Indeed, a linear relationship was observed in a concentration range of $5.0 \times 10^5$ to $1.0 \times 10^7$ CFU/ml. For comparison, the oxygen consumption of E. coli stored at −80°C was also measured, and only very slight differences were observed, thereby confirming that the respiratory activity of E. coli could be restored by resuspending the freeze-dried bacteria in HBSS. In the context of clinical diagnosis, measurement of bacterial numbers should be as rapid as possible, and so the real-time polymerase chain reaction (RT-PCR) is commonly employed for this purpose. However, the DNA amplification stage of the RT-PCR process is time-consuming. Although advanced RT-PCR devices can accelerate DNA amplification, a minimum of 30 min are required for such processes. In addition, information regarding the bacterial viability cannot be obtained by PCR, as this method only measures the quantity of DNA. In contrast, our device can determine the number of viable bacteria within 2 min and so can be considered a superior technique in terms of bacterial analysis.

### 3.4 | Measurement of the antibacterial activity of murine neutrophils

The antibacterial activities of the neutrophils obtained from the control mice and the mice subjected to water-immersion restraint stress were then measured. Thus, Figure 4a shows the variation in the current of the media containing the co-incubated E. coli with murine neutrophils, E. coli alone and HBSS. More specifically, the current recorded for the medium containing E. coli and the normal murine neutrophils (blue line, Figure 4a) was larger than that of the E. coli medium (red line, Figure 4a), which suggests that the neutrophils damaged the bacteria, thereby reducing their oxygen consumption. Interestingly, when E. coli was co-incubated with neutrophils from the stressed mice, the oxygen consumption (as indicated by a decrease in the current) was larger than that observed for the E. coli co-incubated with normal neutrophils, thereby suggesting that the neutrophil antibacterial activity was reduced in the stressed mice population. Furthermore, Figure 4b shows the decrease in current with time for each group following the beginning of the incubation process. These values were obtained by subtracting the current of each group from the cathode compartment. The antibacterial activities of the neutrophils obtained from the control mice and the mice subjected to water-immersion restraint stress were then measured. Thus, Figure 4a shows the variation in the current of the media containing the co-incubated E. coli with murine neutrophils, E. coli alone and HBSS. More specifically, the current recorded for the medium containing E. coli and the normal murine neutrophils (blue line, Figure 4a) was larger than that of the E. coli medium (red line, Figure 4a), which suggests that the neutrophils damaged the bacteria, thereby reducing their oxygen consumption. Interestingly, when E. coli was co-incubated with neutrophils from the stressed mice, the oxygen consumption (as indicated by a decrease in the current) was larger than that observed for the E. coli co-incubated with normal neutrophils, thereby suggesting that the neutrophil antibacterial activity was reduced in the stressed mice population. Furthermore, Figure 4b shows the decrease in current with time for each group following the beginning of the incubation process. These values were obtained by subtracting the current of each group from the cathode compartment.
that of HBSS. The current decrease obtained for the stressed mouse population was larger than that observed for the normal mice.

We also measured the bactericidal activities of the two kinds of neutrophils using a conventional method. More specifically, the number of live bacteria in each sample solution was counted by the CFU assay approach following overnight co-incubation (Figure 4c). The number of live E. coli remaining in the mixture was significantly larger after co-incubation with neutrophils obtained from the stressed mice, thereby further suggesting that the neutrophil antibacterial activity was impaired in the stressed mice. This result coincides with the decrease in current observed using our microfluidic device, which confirms that this device is suitable for use in the rapid (i.e., ~20 min) evaluation of murine neutrophil antibacterial activities upon co-incubation with freeze-dried E. coli.

4 | CONCLUSION

As impaired neutrophil functions such as their antibacterial activity are known to reflect the immunocompromised condition of the host, we examined variations in the respiratory activity and number of live E. coli bacteria co-incubated with different murine neutrophils to reflect their antibacterial activities with high sensitivity. Furthermore, we found that freeze-dried E. coli stored long-term at room temperature could be employed for this purpose, as its activity was successfully restored by simple dissolution in PBS phosphate-buffered saline. The usefulness of freeze-dried E. coli was then demonstrated using mice as hosts, where the reduced antibacterial activity of neutrophils obtained from mice subjected to water-immersion restraint stress was successfully measured. We therefore concluded that freeze-dried bacteria may be useful in the rapid evaluation of host immunocompromised conditions.

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