Development of an Evaluation Device for Phagocytic Activity of New Phagocytes Using Simple and pH-sensitive Particles that Do Not Require Pre-treatment

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Abstract. *Background/Aim: Phagocytic activity is affected by a number of different stress and age-dependent factors. An easy measurement of phagocytic activity is thought to allow an indicator of an individual's health. In this study, we investigated conditions of measurement to easily evaluate the activity of phagocytosis of phagocytic cells (macrophages and neutrophils) using an easy-to-use prototype, which was improved from the device by Hamamatsu Photonics K.K., to detect neutrophil activity using subtle fluorescence. Materials and Methods: pH-sensitive fluorescent particles (pHrodo-Green E. coli Bio particles, GE particles) were added to mouse-derived macrophage cell lines (J774.1) and then incubated for 2 h at 37˚C. For negative control, the phagocytosis inhibitor cytochalasin D (CyD), was added prior to culture. Next, fluorescence intensity was measured by the Prototype to evaluate the phagocytic activity of macrophages and neutrophils. Phagocytosis was also confirmed by flow cytometry. Results: The Prototype detected a steady fluorescence increase in 5 sec in J774.1 after phagocytosis, using GE particles as a negative control in the presence of CyD. Furthermore, detection was possible at 10^4 cells/test, a concentration where the flow cytometer had difficulty for detection. Conclusion: The Prototype enables measurement of the phagocytic activity within a short period of time, even with a small sample amount, thus establishing the basic conditions of measurements of phagocytosis.*

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Phagocytic cells are primarily leukocytes, such as macrophages and neutrophils. The major role of phagocytes is to maintain the body's health by recognizing foreign matter and removing it *via* phagocytosis. Recognition of foreign matter is not only limited to invading pathogens but also includes dead cells, denatured proteins, oxidized lipids, advanced glycation end products (AGEs) and unwanted substances that occur in the body, such as cancer cells (*in vivo* non-self foreign bodies) (1, 2). Such removal is known to play an important role in the maintenance of body health. However, it is reported that factors, such as aging and stress, reduce phagocytic activity, leading to accumulation of foreign substances that result in lifestyle diseases, such as cancer (3-8).

In addition to being caused by insufficient exercise, excessive calorie and fat intake, as well as deterioration of the lipid balance, lifestyle diseases also cause chronic social stress (9). Besides increases in low-density lipoprotein (LDL) and blood sugar levels due to excessive intake of calories and fat, increased biological oxidative stress caused by social stress can increase *in vivo* foreign substances, such as oxidized LDLs (10). On examination of two groups, a group comprising youth (age range=22-45 years) and an elderly group (age range=68-84years) for phagocytic activity of peripheral blood monocytes, 6.7% of youth and 50% of elderly showed a less than 80% rate of phagocytosis, clearly demonstrating the relation of age with phagocytic activity (2). Healing of skin wounds is empirically known to be reduced by aging. The phagocytic activity of wound skin lesion migrating macrophages in mice aged 22-27 months has been reported to be 25% lower than that of eight-week-old mice (3). Moreover, inhibiting alveolar macrophage cell differentiation leads to difficulty in maintaining a necessary amount of lung surfactants, resulting in pulmonary proteinosis. Taken together, phagocytes, through phagocytosis, are essential in maintaining a healthy lung function (11).

As shown in the above report, the ability of phagocytes to exclude foreign bodies is extremely important because health maintenance may be disrupted when phagocytic activity is reduced due to aging and stress. Thus, phagocytic activity of macrophages and neutrophils is an excellent method for monitoring health.

The conventional method for evaluating phagocytic activity of phagocytes is to first perform hemolysis treatment on a few milliliters of fresh blood, introduce fluorescent particles, such as latex beads, count the cells under a microscope or analyze using flow cytometry. Hence, multiple measurements of a few milliliters of blood on the same individual in a single day cause pain on patients' arms.

The aim of this study was the establishment of documentation of an easy-to-operate method using a new device to evaluate the phagocytic activity of phagocytes using whole-blood samples. The device contains an optical system already developed by Hamamatsu Photonics K.K. that easily measures neutrophil myeloperoxidase (MPO) activity in whole blood, thus allowing for high-sensitivity detection of trace fluorescence without the need for hemolysis (12, 13). pH-sensitive particles were used in this study, since a pH reduction in phagosomes is accompanied by an increase in fluorescence intensity. The use of an improved Prototype to easily measure the fluorescence intensity of a macrophage cell line phagocytic activity was examined, allowing detection on a very small number of cells without the requirement of specialized techniques.

Materials and Methods

Cell culture. The murine macrophage-like cell line J774.1 was obtained from Riken Cell Bank (Tsukuba-shi, Tsukuba, Japan). Cells were maintained at 37˚C in a 5% CO_2–95% air atmosphere in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich), 100 U/ml penicillin (Sigma Aldrich) and 100 μg/ml streptomycin (Sigma Aldrich).

Device detecting trace fluorescence. The device, developed by Hamamastu. K.K. (Hamamastu-shi, Shizuoka, Japan), is of extremely high sensitivity to detect trace fluorescence (the Prototype). An excitation light is emitted on the side of the test tube by light-emitting diode (LED) from the lower portion of the device and a fluorescence is detected by a photomultiplier (PMT) at the upper portion from the test tube (Figure 1). A long pass filter, designed to minimize the effect of scattering due to co-existing cells, was also installed (13).

Confirmation of pH-dependent fluorescence intensity of pH-sensitive fluorescent particles in the Prototype. pH-sensitive green fluorescence labeling phagocytosis particles (GE particles) (pHrodo, Green-*Escherichia coli*; Molecular probes, Carlsbad, CA, USA) were added to a final concentration of 5 μg/ml in Na_2HPO_4 and citric acid buffers at pH 2-9. Each buffer sample was transferred into measurement tubes where the Prototype was measured 10 times (5 sec).

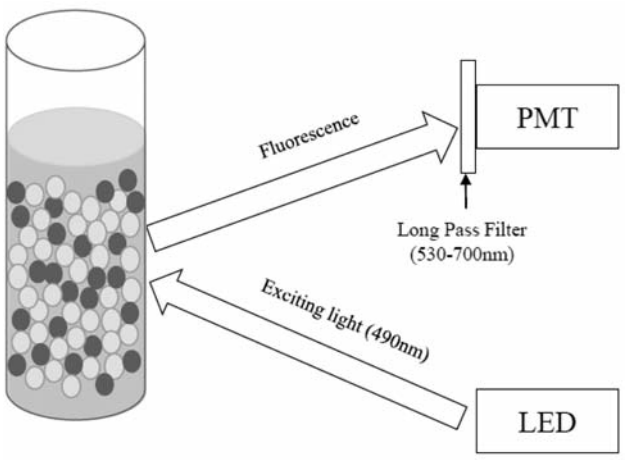

Figure 1. *Image of the fluorescence detection optical system path of the prototype. An excitation light (490 nm) is emitted on the side of the test tube by light-emitting diode (LED) from the lower portion of the device and a fluorescence (530-700 nm) is detected by a photomultiplier (PMT) at the upper portion from the test tube.*

Observation of pH-sensitive fluorescence particles in J774.1 cells under a microscope. J774.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) at 37˚C in a CO_2 incubator. For microscopic observation, 250 μg/250 μl of GE particles was added to 5×10^5/250 μl of cells, with cytochalasin D (CyD) (Wako, Osaka, Japan) at an 8-μM final concentration, which was added simultaneously at the end as negative control. The reactions (total volume 500 μl) were carried out in a CO_2 incubator at 37˚C for 1 h. The cells were then recovered and subsequently observed under a fluorescence microscope.

Evaluation of phagocytic fluorescence intensity using the Prototype. In one test, GE particles were added at 10 μg/100 μl into 1×10^4/100 μl of J774.1 cells. For the negative control, CyD was added at the end of the incubation period at a concentration of 20 μM in 200 μl final volume in RPMI medium and then incubated in a CO_2 incubator at 37˚C for 2 h to allow the reaction to occur. Subsequently, 1,300 μl of PBS was added after phagocytosis, transferred into 1.5-ml glass test tube (micro tube; Maruemu Corporation Inc., Osaka, Japan), and measured 10 times using the Prototype.

Phagocytic activity, measured by the Prototype, was determined using the formula:

Phagocytic Fluorescence Intensity (PFI)=[Fluorescence intensity of cells without CyD, CyD (–)] − [Fluorescence intensity of cells with CyD, CyD (+)]

Evaluation of phagocytic activity using a flow cytometer. In one test, GE particles were added at 10 μg/100 μl into 1×10^4/100 μl of J774.1 cells. For the negative control, CyD was added at the end of the incubation period at a concentration of 20 μM in 200 μl final volume in RPMI medium and then incubated in a CO_2 incubator at 37˚C for 2 h to allow the reaction to occur. The rate of phagocytosis was measured using a Beckman Coulter Gallios flow cytometer using the Kaluza software (Beckman Coulter, Indianapolis, IN, USA).

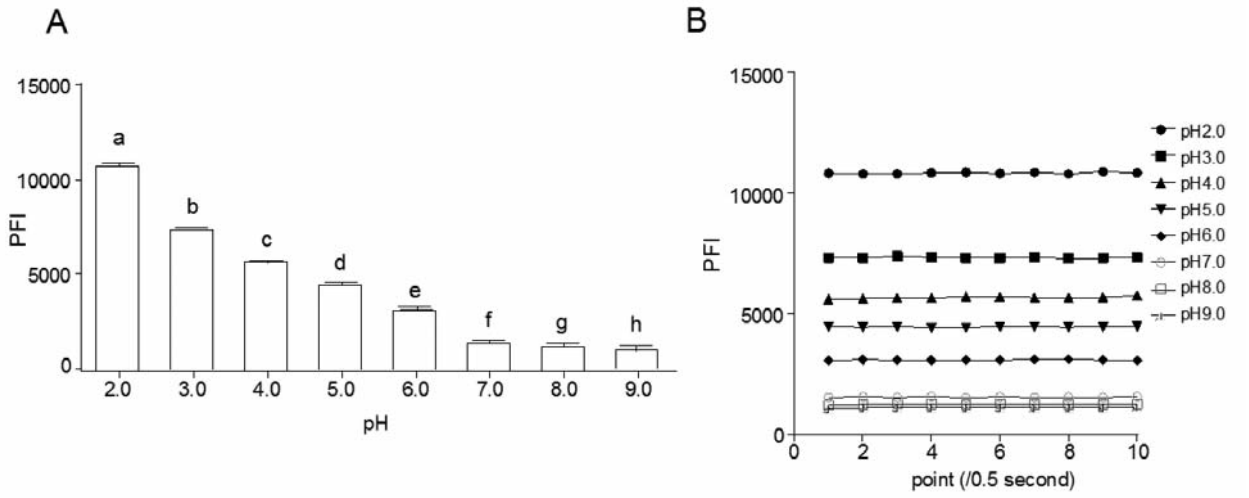

Figure 2. *Fluorescence intensity of pH-sensitive fluorescence particles under each pH. Each pH solution includes 5 μg/ml of GE particles. Each fluorescence intensity is measured 10 times and the mean variation of pH-dependent fluorescence intensity is determined. A: pH-dependent column and bar shows average of 10 measurements and standard deviation. B: Raw data of the Prototype of GE particles in each pH condition. a~h indicate a significant difference based on one-way ANOVA followed by Tukey's multiple comparison test (n=10), p<0.001.*

Statistical analysis. All results are expressed as the means±standard deviation (SD). If the difference based on one-way analysis of variance (ANOVA) was significant, Tukey's multiple comparison test was used for paired comparisons using Graph Pad Prism 6.02 for Windows (Graph Pad Software, Inc., La Jolla, CA, USA). The results were considered to be significantly different at $p<0.05$.

Results

Confirmation of a pH-dependent fluorescence intensity increase of pH-sensitive fluorescence particles by the Prototype. To confirm the changes in fluorescence intensity of pH-sensitive fluorescence particles with changes in pH, GE particles were added to all pH buffers. Each solution was transferred into a glass tube and measured 10 times using the Prototype. Figure 2A shows the data of 10 measurements that lasted 5 sec. The data were stable and had an error within a 3.4% margin. This figure also shows the correlation between pH and fluorescence intensity, with a reduction in pH being accompanied by a reduction in fluorescence intensity (Figure 2B).

Fluorescence detection of pH-sensitive particle phagocytosis in J774.1 using fluorescence microscope. Samples were observed with 400× magnification using a microscope. Although phagocytosis-produced fluorescence could not be detected in cells with CyD (Figure 3A), emitted fluorescence from phagocytosed particles was confirmed in cells without CyD (Figure 3B).

pH-sensitive fluorescence particle phagocytosis measurements using the Prototype and flow cytometer. Phagocytic activity of GE particles (10 μg/test) added to J774.1 cells (1×10^5/test) was measured using the Prototype. Compared to cells to which CyD (20 μM) was added, the cells without CyD had an increase of 11,446 PFI in fluorescence intensity (Figure 4A). To determine whether the fluorescence intensity was caused by phagocytosis, the rate of phagocytosis was confirmed using a flow cytometer. Cells without CyD exhibited an increased rate of phagocytosis in the order of 84% (Figure 4B).

Correlation between the Prototype, flow cytometer and cell numbers. After adding GE particles into 1×10^4, 2×10^4, 5×10^4 and 1×10^5 J774.1 cells/test, samples were incubated for 2 h in a 37˚C CO_2 incubator. The recovered samples were added to tubes and measured using the Prototype. As shown in Figure 5A, at a certain amount of GE particles, changes in fluorescence intensity due to increased cell numbers were found, with the values increasing proportionately with cell numbers. Significant values in fluorescence intensity were observed even at 10^4 cells. Once the measurement with the Prototype was completed, flow cytometry was performed. It was found that a correlation existed between phagocytic activity measured by the Prototype and the rate of phagocytosis measured by a conventional flow cytometer (Figure 5B). There was a strong correlation ($R^2=0.9299$) between the rate of phagocytosis and increases in cell number and fluorescence intensity.

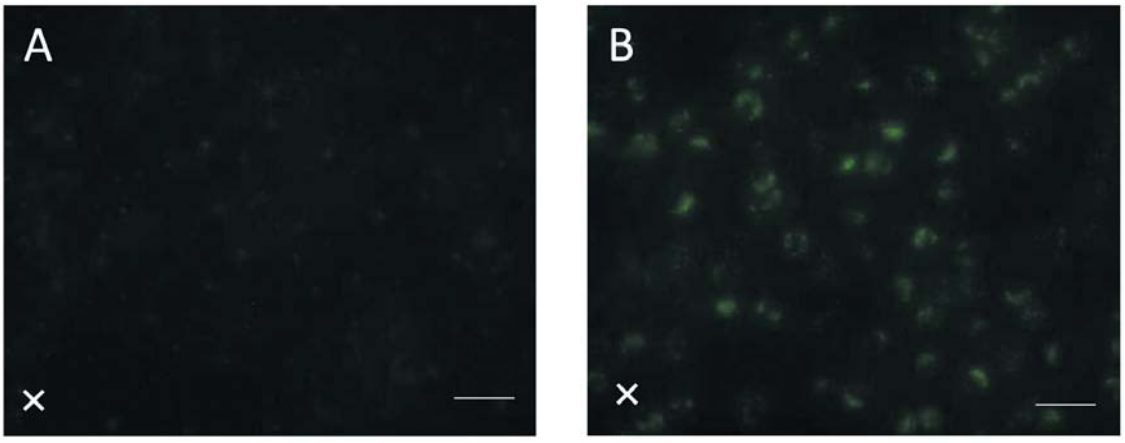

Figure 3. *Fluorescence microscope confirmation of GE particles phagocytosis by macrophages. GE particles were added to mouse-derived macrophage J774.1 cells and then placed in a 37˚C incubator for 1 h to allow the reaction to occur. Cytochalasin D (CyD; 4 μM) was added at the end of the incubation period. The samples were subsequently observed using a fluorescence microscope at 400× magnification. A: Negative control CyD (+); B: positive control CyD (–). The bars indicate 50 μm.*

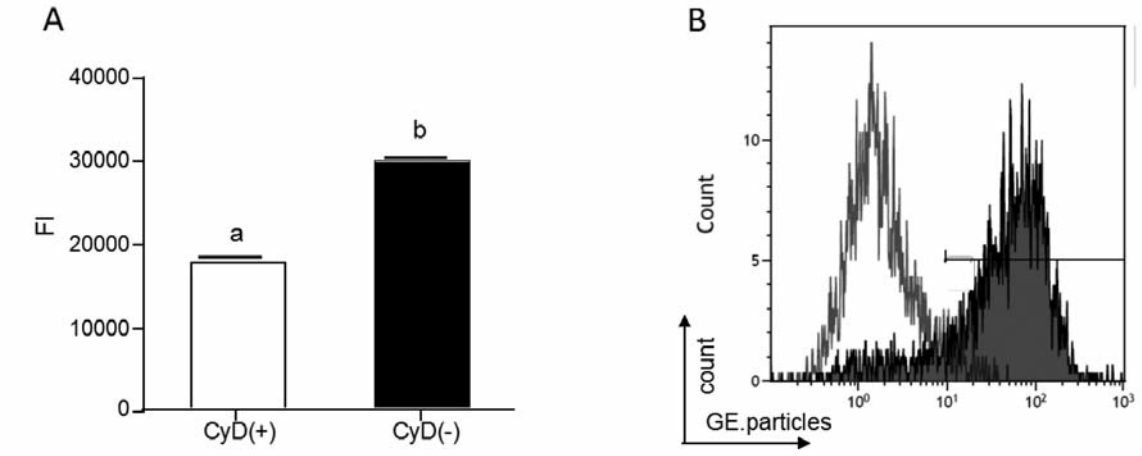

Figure 4. *Measurement results of J774.1 using the Prototype and flow cytometer. A: The fluorescence intensity in 10^5/test J774.1 cells after adding GE particles (10 μg/test) was measured using the Prototype. B: The rate of phagocytosis was measured using a Beckman Coulter Gallios flow cytometer using the Kaluza software. *Significant difference from negative control group CyD (+), ***p<0.001. (n=10). Line indicates negative control CyD (+); Gray area indicates positive control CyD (–).*

Discussion

The present study evaluated the phagocytic activity of 10^4 phagocytes using an improved version of the Prototype by Hamamastu Photonics K.K. (13) for trace fluorescence detection; this device was initially developed to measure neutrophil activity.

As the time required for measurement is only 5 sec for 10 counts of multiple measurements, this approach is much quicker than quantifying using a flow cytometer or counting under a microscope. Since the Prototype can carry out 500 measurements of fluorescence intensity and yield stable data, an average of 10 measurements was deemed sufficient for stable data measurements (Figure 2B). Furthermore, GE particles'-phagocytosed fluorescence intensity was measured using a conventional fluorescence spectrophotometer; however, the measurement data was unstable due to scattering caused by the presence of cells and particles (data not shown).

In this measurement system, pH-sensitive fluorescence labeled particles play an important role in allowing easy evaluation of phagocytosis. Phagocytosed particles taken into phagosomes, concomitant with the formation of

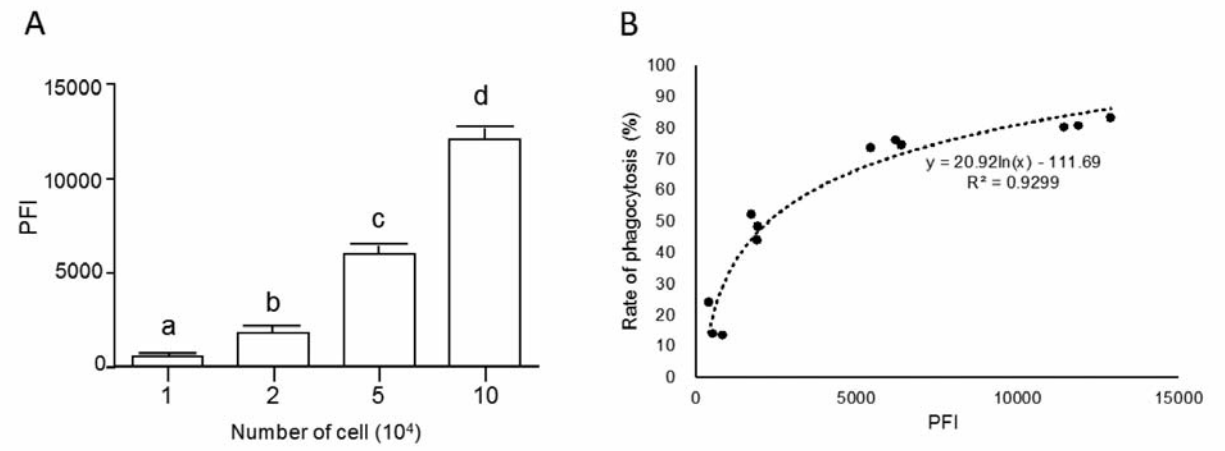

Figure 5. *Correlation data of phagocytic activity and rate of phagocytosis using flow cytometer at each cell number. Using the same phagocytosis reaction conditions as described for Figure 4, GE particles were added at 10 μg/test to every 1×10^4, 2×10^4, 5×10^4 and 1×10^5 cells. A: After the reaction, measurements were performed using the Prototype and phagocytic activity was determined using fluorescence intensity differences (n=3). a~d indicate a significant difference based on one-way ANOVA followed by Tukey's multiple comparison test (n=10), p<0.001. B: Correlation between fluorescence intensity differences acquired by the Prototype and the rate of phagocytosis obtained by the flow cytometer (n=12).*

Table I. *Pros and cons concerning flow cytometer and the Prototype.*

	Flow cytometer (Gallios, Beckman coulter)	The prototype
Measure	High level analytical capability	Simple
Measuring time	Less than minutes	5 sec
Size	724(W)×495(D)×298(H)mm	20(W)×25(D)×20(H)mm
Weight	104 kg	2.7 kg
Blood requirement	Several mililiters	Several microliters
Hemolysis	Necessary	Not necessary
Measuring time	About 4 h	2.5 h
Reagent	Fluorescecence particles Hemolysis Antibodies Reagent-related flow cytometer	pH fluorescecnce particles

phagolysosomes and a reduction in pH (to about 4.5~5.0), produce fluorescence. In fact, fluorescence emission in the phagosomes can be observed using a fluorescence microscope (Figure 3). Furthermore, pH decreases, leading to fluorescence activity increases, were confirmed (Figure 2A). With this feature, an evaluation of the phagocytic activity of phagocytes using fluorescence intensity was possible without removing the fluorescence particles. Furthermore, changing conventional media into PBS facilitated the suppression of background fluorescence (data not shown). The optimum concentration of CyD as a phagocytosis inhibitor is 20 μM (data not shown). Also, phagocytosis inhibition was confirmed using a flow cytometer (Figure 4B).

The evaluation of phagocytic activity in cells is considered to reflect the *in vivo* foreign body exclusion ability of the cell. Since this evaluation system can identify several markers that may be indicators of the onset of many lifestyle diseases, such as *in vivo* accumulation of oxidized LDL (10), it may be implemented as a risk factor indicator for the development of such diseases. However, there are few publications reporting the changing trend in phagocytic activity in the light of lifestyle diseases. One reason for this is that conventional methods used to evaluate phagocytic activity are not simple, requiring for expensive measuring equipments. Measurements of the rate of phagocytosis using flow cytometry commonly involve drawing of several milliliters of peripheral blood, isolation procedures of white

blood cells to phagocytose fluorescent latex particles and, subsequently, treating the obtained sample with antibodies (*e.g.*, CD11b) that detects phagocytes. Such evaluation methods require expensive reagents and technical expertise for detection. In addition, the requirement of venipuncture involves circadian rhythm and inflicts a lot of pain on the test subjects, thus adding parameters that may interfere with the evaluations. Conversely, the Prototype that allows evaluation of self-collected volumes (a few microliters) of blood is currently in development (manuscript in preparation). In addition, the disadvantage of the flow cytometer method is its unportable size, whereas the Prototype is portable and weighs approximately 3 kg (Table I). Furthermore, measurements by flow cytometry require several minutes, whereas the Prototype requires merely five seconds to acquire results. Therefore, this equipment can easily measure trace amounts of blood and does not require for special technology or technical expertise. It can repeatedly perform measurements within a day, including diurnal evaluation, and can monitor an organism's health. Using these features, one example of its potential applications is its implementation as an evaluation system for homeostasis maintenance (health maintenance function) during exercise or ingestion of agricultural products.

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Conflicts of Interest

The Authors have no financial conflicts of interest.

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