



Rapid detection of nanoplastics and small microplastics by Nile-Red staining and flow cytometry

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Received: 20 July 2022 / Accepted: 10 November 2022 / Published online: 2 December 2022
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Abstract

Microplastics are of rising health concerns because they have been detected even in remote and pristine environments, from the Arctic snow to the Marianne Trench. The occurrence and impact of nanoplastics in ecosystems is almost unknown, in particular due to analytical limitations such as very small sizes that fall below detection limits of current techniques. Here we take advantage of a common interference in analytical flow cytometry to develop a method for the quantification of the number of plastic particles in the 0.6–15 μm size range. Plastic particles are stained with the lipophilic dye Nile-Red then detected by flow cytometry, a method regularly used in biology for rapid quantification of fluorescent cells. We found that sample analysis lasts 90 s, which is hundreds of times faster than the analysis of filter portions by micro-Raman and other spectroscopic techniques. Our method is highly efficient in detecting polyethylene, with staining efficiency higher than 70% and signal linearity with concentration. Staining efficiency up to 96% was observed for polyvinylchloride and for polystyrene.

Keywords Flow cytometry · Nanoplastics · Microplastics · Nile-Red

Introduction

Production of synthetic plastics derived from fossil fuels continues rising, while the mismanagement of plastic waste has recently caused serious pollution problems (Padervand et al. 2020). Plastics undergo a slow process of erosion in the environment, leading to the breakdown of large plastic debris into microplastics, less than 1 mm-sized (Thompson et al. 2004) and even nanoplastics, less than 1000 nm-sized (Gigault et al. 2018; Dawson et al. 2018; González-Pleiter et al. 2019). The occurrence and impact of nanoplastics in

the environment is almost unknown, because their size is below the instrumental detection limit of the techniques currently used for microplastics, such as micro Fourier transform infrared ($\mu\text{FT-IR}$) and micro-Raman spectroscopy (μRaman), yet nanoplastics are most probably more dangerous than microplastics due to the properties of nanomaterials (Sharma et al. 2022).

Nanoplastics have been detected in water and in soil by pyrolysis–gas chromatography–mass spectrometry, which gives the concentration in mass of plastic polymers (Hurley and Nizzetto 2018; Ter Halle et al. 2017; Wang et al. 2019; Xu et al. 2022; Materić et al. 2022). However, to the best of our knowledge, there is not yet any study that reports on nanoplastics number concentration in a specific environment. To analyze micro- and nanoplastics in the environment, there are several steps to follow: sampling, sample treatment, concentration and/or separation, identification, and quantification (Razeghi et al. 2021; Huang et al. 2022). After sampling, a purification step is needed for the analysis of plastic particles, which usually consists in the digestion of the organic matrix. However, nanoplastics are not inert to the digestion treatments (Bianco et al. 2020); therefore, more research is needed in this field (Cai et al. 2021).

Quantification is the final step of the analysis. $\mu\text{FT-IR}$ and μRaman , scanning electron microscope (SEM) and

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transmission electron microscope (TEM) are widely used to measure the sizes or concentrations in studies into micro and nanoplastics (Sarau et al. 2020; Lê et al. 2021; Zhou et al. 2021; Li et al. 2022). Dynamic light scattering and nanoparticle tracking analysis can also characterize the size of nanoparticles, by measuring fluctuations in scattered light intensity due to the particle Brownian movement (Friskén 2001; Hernandez et al. 2019). However, dynamic light scattering and nanoparticle tracking analysis have considerable limitations when analyzing polydisperse and non-spherical nanoplastics in field samples, and have been mostly used for high-concentration and monodisperse nanoplastics.

The detection and quantification of nanoplastics and small microplastics is still a challenging task, and research is needed to improve the actual methods and develop new ones. Interestingly, nanoplastics and small microplastics are well known to give interferences in bacterial cell counting carried out by dye staining and flow cytometry, because they interact with lipophilic dyes. In this work we take advantage of this characteristic, by testing the detection and quantification of plastic particles of different polymers, in the size range of 200–20,000 nm, by Nile-Red staining and flow cytometry detection. This technique is routinely used in biology for single cell analysis, and it enables a quick and cheap quantification of fluorescent particles. This work has the goal of showing that flow cytometry is suitable for the final steps of nanoplastics and small microplastics analysis, i.e., detection and quantification. It also provides insight into the limits of the method, and the associated need to still develop suitable sampling and purification protocols.

Experimental

Chemicals

Nile-Red (CAS 7385–67-3, technical grade), H₂O₂ (30% in water), and methanol were purchased from Sigma Aldrich; polystyrene particles in water were purchased from Thermo Fischer Scientific, with diameter of 600 nm, 700 nm, and 1, 2, 3, 4, 5, 6, 10, and 15 µm, with uncertainty on the size below 3%; polyethylene particles (740–4990 nm) were purchased from Cospheric; polytetrafluoroethylene, with average particle dimensions of 3 µm, was purchased from Polysciences Inc; polyvinylchloride H850D high expansion rate spheres, with particles smaller than 36 µm, was purchased from Kureha. All reagents were used as received, without further purification. Water used was of Milli-Q quality. Further polystyrene particles were produced, by grinding polystyrene macroplastics in an agate ball mill for successive cycles (6 × 30 min), with interruptions to avoid temperature increase. Particles with diameter smaller than 25 µm were separated by suspension in methanol, and filtration on filter

paper (Whatman). The use of plastic labware was avoided, and glassware was carefully rinsed with Milli-Q water. Experiments were performed under a bench hood.

Instruments

Dimensional and shape characterization was performed with dynamic light scattering. These measurements were carried out on Nile-Red suspensions in methanol, using an ALV NIBS (not invasive back scattering) apparatus (ALV, Langen, Germany), equipped with a correlator mod ALV5000, and measuring scattered light for at least 20 s at 298 K. Fluorescence excitation–emission matrix spectra were taken with a Varian Cary Eclipse spectrofluorimeter, using a fluorescence quartz cuvette with 1.000 cm optical path length. Excitation–emission matrix spectra were obtained with 600 nm min⁻¹ scan rate, and the slit width on both excitation and emission (5 or 10 nm) was a compromise between spectral resolution and signal intensity.

Flow cytometry analysis was performed with a BD Accuri C6 flow cytometer (BD Biosciences), using 35 µL min⁻¹ flow rate and volume of analysis of 100 µL. Analysis of each sample requires 90 s and is rapid compared to the analysis of filters by µRaman and µFT-IR, which take hours for the analysis of a small portion of a filter. The 488 nm laser of the cytometer was used for excitation, and the emitted fluorescence signal was detected at 530/540 nm. These wavelengths were chosen on the basis of the experiments performed by Dutta et al. (1996).

Results and discussion

Determination of the concentration of Nile-Red

Dyes, and in particular Nile-Red, are used for staining microplastics (Dutta et al. 1996; Erni-Cassola et al. 2017; Costa et al. 2021), and for helping in discrimination between plastic debris and non-plastic particles and fragments. The standard procedure in the use of Nile-Red foresees the dissolution of the dye in acetone or methanol, followed by addition of few drops of the dye solution, to cover each filter containing microplastics. The analysis of the filters is performed after a contact time, ranging from 30 to 60 min (Rumin et al. 2015; Erni-Cassola et al. 2017). In this work we aimed to analyze aqueous nanoplastics suspensions, thus the staining protocol had to be adapted. Previous works report that the solubility of Nile-Red in water is around 100–200 mg L⁻¹. However, preliminary flow cytometry measurements on Nile-Red solutions in Milli-Q water, with concentration below the solubility limit, showed that Nile-Red forms aggregates (Figure S1). Fortunately, these aggregates are not fluorescent, as shown in Figure S2a for an

excitation–emission matrix fluorescence spectrum of Nile-Red alone. Furthermore, aggregates dissolve upon addition of plastic particles, presumably because the dye molecules are preferably adsorbed on plastics. Figure S2b depicts the excitation–emission matrix spectrum of Nile-Red with polyvinylchloride, showing much lower Rayleigh scattering signal compared to Nile-Red alone. Rayleigh scattering is the main linear feature in these spectra, due to light scattering by suspended particles. The same result was confirmed by flow cytometry (Figure S3). A Nile-Red concentration of 10 mg L^{-1} was chosen for the following experiments, as a good compromise between staining efficiency and reduction in aggregate formation.

Experiments on synthetic plastics

Figure S4 shows good linearity of the flow cytometry signal (scattered light), when calibrated with unstained polystyrene beads with size ranging from 0.6 to $15 \mu\text{m}$. Then, an aqueous suspension of polyethylene nanoplastics, with average dimension of $\sim 3 \mu\text{m}$, was spiked with Nile-Red to a final dye concentration of 10 mg L^{-1} . After 30 min, the suspension was analyzed by flow cytometry. Figure 1a shows the scatter plot, reporting the side scatter, which is the light scattered by suspended particles at right angles with respect to the laser beam, as a function of the forward scatter, which is light scattered in a forward direction. The scatter plot identifies two distinct regions, and the red dots highlight the fluorescent particles that make up 73.4% of the total, as shown in Fig. 1b. These particles range in size between 0.5 and $5 \mu\text{m}$, as reported by the blue dots in Fig. 1a, obtained by

polystyrene beads calibration. The size range thus obtained is in excellent agreement with the commercial product information ($0.74\text{--}4.99 \mu\text{m}$), which suggests that the polyethylene particles were well stained and fluorescent. In the volume analyzed ($100 \mu\text{L}$) there are 1.12×10^5 particles, of which $(8.23 \pm 0.16) \times 10^4$ are fluorescent. It is worth noting that the extrapolation of these values gives a concentration of fluorescent particles of the order of 8×10^5 fluorescent particles mL^{-1} , which is not far from the known initial concentration in the sample (1×10^6 part mL^{-1}).

To better test the repeatability of the analysis, and the relationship between the theoretical and measured amount of particles, suspensions with different concentrations of polyethylene nanoparticles were analyzed in triplicate. The results are presented in Figure S5, clearly showing a linear relationship with $R^2 = 0.96$, and coefficient of variation of triplicates lower than 10%. This calibration shows that the method can be applied for a quantitative analysis of small microplastics and nanoplastics, in the number concentration range of $10^4\text{--}10^6$ particles mL^{-1} .

Commercially available and monodisperse polystyrene micro- and nanoparticles of 1, 3, and $5 \mu\text{m}$ in diameter were stained with Nile-Red, using the same procedure explained before but with two different concentrations of Nile-Red, i.e., 10 and 90 mg L^{-1} . As a result, polystyrene particles of 1 and $3 \mu\text{m}$ were not stained by the dye, while $5 \mu\text{m}$ beads were stained only at the highest Nile-Red concentration. This finding could be a consequence of the functionalization of polystyrene particles, which is required to maintain them in stable suspension in water. For this reason, we also obtained polystyrene particles from commercial objects. A

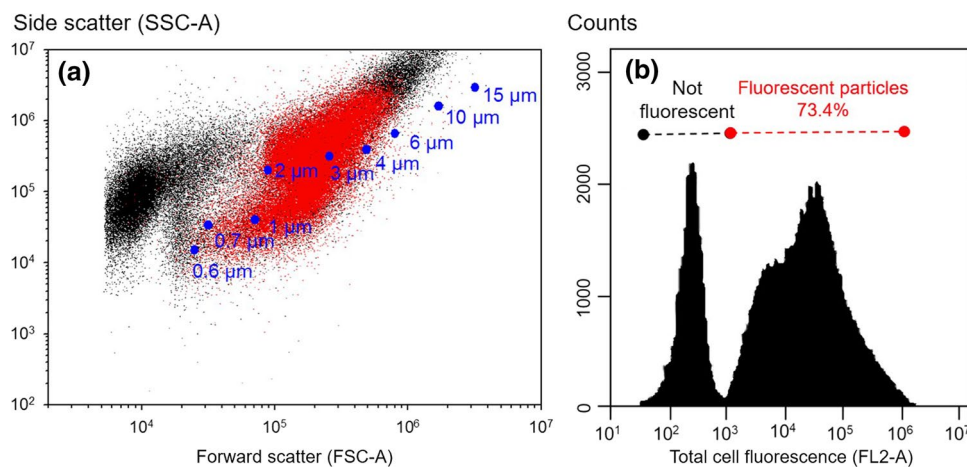


Fig. 1 **a** Side scatter (SSC-A), which is the light scattered by suspended particles at right angles with respect to the laser beam, as a function of the forward scatter (FSC-A), which is light scattered in a forward direction, for all the particles detected in the sample (black), or for fluorescent polyethylene particles (red). The blue dots are obtained by calibration with polystyrene beads. The size range

obtained, between 0.5 and $5 \mu\text{m}$, is in excellent agreement with the commercial product information ($0.74\text{--}4.99 \mu\text{m}$). **b** Histogram of counts *versus* total cell fluorescence FL2-A, depicting the percentage of particles that show fluorescence, which confirms that the polyethylene particles were well stained and fluorescent

compact-disk cover was firstly manually fragmented into small pieces, and then grinded into a ball mill, following the procedure described in Sect. 2.1. The resulting methanol suspension of polystyrene particles, with size lower than 25 μm , was evaporated and resuspended in Milli-Q water, then stained with Nile-Red, and finally analyzed with flow cytometry. Results show that particles had sizes ranging from nanometric to micrometric, with maximum diameter between 2 and 4 μm , and that most of the particles were stained (96.6%) (Fig. 2).

The experiments on polystyrene highlight the limitation of the methodology combining dye staining with flow cytometry: it cannot be applied to plastic particles functionalized with hydrophilic functions, especially in the case of monodisperse polystyrene, where surface functionalization is applied to obtain stable water suspensions. On the other hand, flow cytometry was able to detect polystyrene particles obtained from a daily life object, containing additives and antioxidants, in addition to allowing for the detection of non-functionalized nano and microplastics made of polyethylene (Fig. 1) and polyvinylchloride [data not shown].

The same approach was tested on polytetrafluoroethylene as well, but these particles were not efficiently stained by Nile-Red.

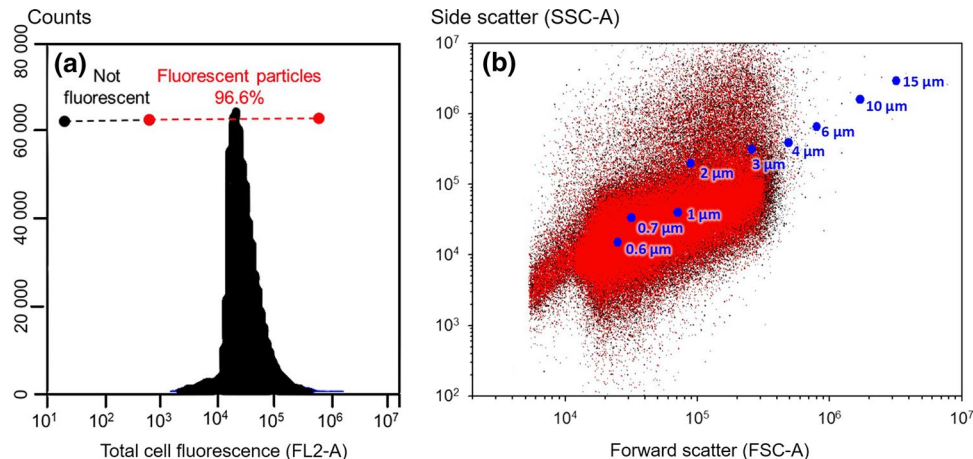
Experiments on natural samples

The main challenge of this work was to test the flow cytometry performance in a complex natural matrix. To this purpose, river water was sampled and spiked with polyethylene particles, to obtain a final concentration of 6×10^6 part mL^{-1} . Vantaa river (Helsinki, Finland) water was filtered on glass fiber filters, and nano and microplastics of polyethylene were deposited on these filters, as explained in SI-S1. As a first approach, the material deposited on the filters was just extracted by ultrasounds in methanol. The suspension thus obtained was evaporated under fume hood, reconstituted

with Milli-Q water, added with Nile-Red, and then analyzed (see Scheme S2). The organic matter present in river water is likely to interact with plastic particles (Boldrini et al. 2021), and thus interfere with the analysis of nano and microplastics. Therefore, we applied a simple purification protocol: organic matter was digested with H_2O_2 (as described in SI-S1), and H_2O_2 residues were eliminated with FeSO_4 or peroxidase. To obtain a reference blank, Milli-Q water was treated in the same way. The full list of samples that were treated and analyzed by flow cytometry is reported in Table S1. The samples in which peroxidase was used to quench residual H_2O_2 are not shown, because the fluorescence signal of peroxidase hid the signal of Nile-Red on plastics. A comparison of the results, obtained by analyzing Vantaa River water with and without polyethylene, is shown in Fig. 3.

The number of particles detected in the polyethylene-spiked samples was lower ($\sim 46\%$) compared to the theoretical number of particles added to the samples. Still, these results demonstrate as a proof-of-concept that it is possible to detect small microplastics and nanoplastics also in complex media, by using a simple and fast methodology. On the other hand, there could be several reasons for the observed quantification problems, which are most likely associated with the digestion process and the interference with organic and inorganic matter (Matijaković Mlinarić et al. 2022). Indeed, digestion could degrade some of the smallest plastic particles, which are not inert to the hydroxyl radicals that could be generated by H_2O_2 , in the presence of some river water solutes such as Fe species, occurring naturally or added as FeSO_4 to eliminate H_2O_2 (Bianco et al. 2020; Atugoda et al. 2022). Another effect that could influence the number of detected particles might be their interaction with dissolved organic matter, as shown in previous studies (Chen et al. 2018). Small plastic particles may in fact form aggregates with dissolved organic matter, and facilitate dissolved organic matter assembly (Chen et al. 2018). This

Fig. 2 **a** Histogram of counts versus total cell fluorescence FL2-A, showing that 96.6% of polystyrene particles were stained by Nile-Red and fluorescent. **b** Side scatter (SSC-A) versus forward scatter (FSC-A) of all the particles detected in the sample (black), with polystyrene fluorescent particles in red. The blue dots are obtained by calibration with polystyrene beads. Results show that particles had sizes ranging from nanometric to micrometric, with maximum diameter between 2 and 4 μm



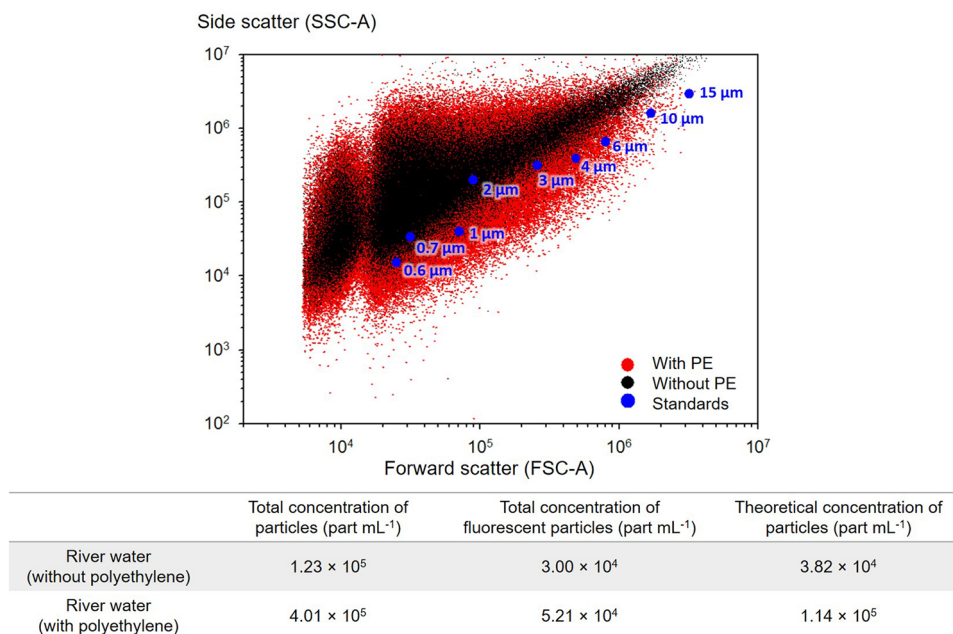


Fig. 3 Side scatter (SSC-A) versus forward scatter (FSC-A) plot of all the particles detected in Vantaa River water, with (red) and without (black) addition of polyethylene (PE) particles, after digestion with H₂O₂. Polystyrene standards are reported in blue. This plot demonstrates that the detection method can be applied also to environmental samples. The table presents the values of the detected particles for

both samples, as well as the theoretical concentration of polyethylene particles, calculated with the linear regression presented in Figure S5. The number of particles detected in the polyethylene-spiked samples was lower than the theoretical number of particles added to the samples, because of aggregation of particles or loss during the digestion process

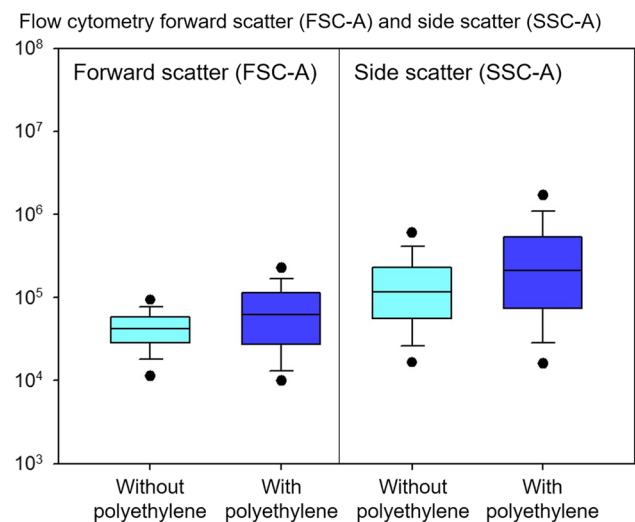


Fig. 4 Box plots of the forward scatter and side scatter parameters, calculated for river water samples with and without polyethylene particles. The bottom and top lines of the boxes correspond to the 25th and 75th percentiles, respectively. The middle line represents the median. The ends of the whiskers represent the 10th and 90th percentiles. Results indicate that polyethylene potentially induces the aggregation of organic matter and the formation of larger particles

phenomenon is confirmed by the trends observed in Fig. 4, which reports the forward scatter and the side scatter values

for river water in the absence and in the presence of polyethylene particles, indicating that polyethylene potentially induces the aggregation of organic matter and the formation of larger particles.

The results presented in this article clearly show that the new methodology consisting in staining with Nile-Red and detection by flow cytometry is rapid and efficient for the analysis of pure plastic particles (polyethylene), plastic particles produced from a daily life object (polystyrene compact-disk cover), and plastic particles in environmental samples, such as river water.

Conclusion

We have designed a new methodology for the detection of small microplastics and nanoplastics. Plastic particles were stained with the lipophilic dye Nile-Red and analyzed by flow cytometry. The detection efficiency was tested on polyethylene, which is the most common polymer and accounts for more than 30% of all produced plastics (Plastics—The Facts 2021). Good results were also obtained with polyvinylchloride, and with plastic fragments derived by a commercial polystyrene object. Unfortunately, Nile-Red could not efficiently stain either polytetrafluoroethylene, or polystyrene nanobeads bearing a functionalized hydrophilic

surface. The Nile-Red—flow cytometry technique enables the detection of polyethylene particles spiked to a natural (river) water sample, as a proof-of-concept of the applicability of the methodology. Unfortunately, particle quantification was problematic, because of issues that are likely connected with the occurrence of organic matter, which was not eliminated well enough in the purification (H₂O₂ digestion) step used in this work. The new method we tested is promising, and it could be a rapid and competitive alternative to more expensive and complex methods for analyzing small microplastics and nanoplastics. However, further optimization is required in the purification and separation steps of plastic particles occurring in natural samples, to allow for standard routine analysis.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10311-022-01545-3>.

Acknowledgments We would like to thank M. Joly and A.M. Delort from ICCF (Institut de Chimie de Clermont Ferrand) for providing Nile-Red for the preliminary analysis, and Noora Aarnio at Biomedicum Flow cytometry unit for helping with the measurements.

Author's contributions AB: contributed to conceptualization, investigation, writing; LC contributed to investigation; NP contributed to instrumental development; FS contributed to investigation; DV contributed to supervision, writing; MP contributed to conceptualization, funding acquisition, supervision. All authors reviewed and edited the draft.

Funding Open Access funding provided by University of Helsinki including Helsinki University Central Hospital. This work is part of a project that has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program, grant agreement No 948666 – ERC-2020-StG NaPuE and from University of Helsinki (Three-Year Research Grant).

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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