A High throughput method for egg size measurement in *Drosophila*

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Abstract

Life-history traits are used as proxies of fitness in insects including *Drosophila*. Egg size is an adaptive and ecologically important trait potentially with genetic variation across different populations. However, the low throughput of manual measurement of egg size has hampered the widespread use of this trait in evolutionary biology and population genetics. We established a method for accurate and high throughput measurement of *Drosophila* egg size using large particle flow cytometry (LPFC). The size estimates using LPFC are accurate and highly correlated with the manual measurements. The measurement of egg size is high throughput (average of 214 eggs measured per minute) and viable eggs of a specific size can be sorted rapidly (average of 70 eggs per minute). Sorting by LPFC does not reduce the survival of eggs making it a suitable approach for sorting eggs for downstream analyses. This protocol can be applied to any organism within the detectable size range (10-1500 μm) of the large particle flow cytometers. We discuss the potential applications of this method and provide recommendations for optimizing the protocol for other organisms.

Introduction

Egg size is an important trait in insects that have evolved in response to developmental and ecological pressures (Church et al., 2019). Egg size and other life-history traits (e.g. survival, longevity, and female fecundity) are proxies of fitness in insects. In *Drosophila*, egg size affects other life-history traits e.g. embryonic viability, hatching rate, and embryonic development (Azevedo et al., 1997) and morphological features such as the embryonic anterior-posterior patterning (Huang et al., 2020; Lott et al., 2007). Larval crowding (Venkitachalam et al., 2022) and environmental factors such as temperature influence egg size (Avelar, 1993; Azevedo et al., 1996; Imai, 1932). *Drosophila* egg size increases with latitude in Australia and South America (Azevedo et al., 1996) suggesting that it is an adaptive trait under stabilizing selection (Azevedo et al., 1997). *Drosophila* is one of the most widely used sexual multicellular organisms in evolutionary biology. This popularity is partly due to *Drosophila*'s short generation time and ease of maintenance in laboratory conditions. Despite the widespread use of *Drosophila*, egg size is rarely studied in evolutionary and population biology studies partly due to the low throughput of the manual measurement of egg size.

Traditionally, the size of eggs was measured when viewed under a microscope or stereoscope (Azevedo et al., 1996, 1997; Imai, 1932; Markow et al., 2009; Warren, 1924). Recently, the eggs are photographed under a microscope and their sizes are manually measured from the images (Huang et al., 2020; Jha et al., 2015; Lott et al., 2007; Miles et al., 2011). The methods that rely on manual measurements are time-consuming and tedious because the eggs should be manually transferred and arranged in proper orientation before images are taken. Measuring the egg size manually is also error-prone

due to the experimenter's bias. Moreover, manual measurements usually cannot ensure the recovery of viable eggs that can be used for downstream analyses.

Flow cytometry allows fast and accurate sorting and size measurement of small objects up to 200 μm. But the average size of eggs in *Drosophila* ranges from 400 to 600 μm (Imai, 1932; Lott et al., 2007; Markow et al., 2009; Venkitachalam et al., 2022; Warren, 1924) which is too large to be processed with the traditional flow cytometers. The advent of large particle flow cytometry (LPFC) has made the measurement of live objects up to 1500 μm possible. The LPFC systems operate at a slower flow rate and lower pressures compared to the traditional flow cytometers to avoid disruptive shear forces. LPFC has been used for the analysis of fluorescent-labeled or transgenic organisms e.g. egg of nematodes (Pillai & Dandurand, 2021), *D. melanogaster* (Furlong et al., 2001) and *Anopheles gambiae* (Marois et al., 2012), and coral larvae (Randall et al., 2020). Non-fluorescent labeled objects can also be analyzed and sorted on LPFC solely based on their size. However, in the absence of fluorescence labeling, the shape of objects affects the accuracy of size estimation. For example, the shape of *Arabidopsis* seeds is prolate spheroid or cardioid (Cervantes et al., 2010) thus the size measurement of *Arabidopsis* seeds is almost independent of the orientation of seeds (Morales et al., 2020). However, for objects with oblate spheroid or ellipsoid shapes such as *Drosophila* eggs the estimated size may vary depending on the orientation of the objects in the flow cytometer. The size of the object is properly measured only when the objects are oriented along their long axis but may be underestimated if the objects don't pass along their long axis (Fig. 1A-B).

In this study, we developed a method to measure the size of *Drosophila* eggs using LPFC. In this method, we filter out the eggs that are not oriented properly along their long

axes in-silico and accurately measure the size distribution of *Drosophila* eggs. The measurement of egg size is accurate, fast, and has high throughput. Eggs of a specific size range can be sorted accurately and developed into adults with no reduction in viability. We discuss the potential of using this approach to investigate the natural variation and the genetic basis of egg size in *Drosophila.* LPFC can be used to measure the size of any organism within the detectable range (10-1500 μm) of this instrument. We provide recommendations for optimizing the use of LPFC for other organisms in particular when their shape deviates from the prolate spheroid.

Materials and Methods

Egg length as a proxy of egg size

Studies on *Drosophila* egg size have used the length (Huang et al., 2020; Imai, 1932; Lott et al., 2007; Markow et al., 2009; Warren, 1924) and volume (Avelar, 1993; Azevedo et al., 1996, 1997; Jha et al., 2015; Miles et al., 2011; Schwarzkopf et al., 1999) of eggs as proxies of the size. Using egg volume as a proxy of egg size does not specify if the change in egg volume is due to a change in the egg length or width, or both. In this study, we use egg length as a proxy of egg size for the following reasons. In artificially selected populations for higher egg volume, eggs are longer (Miles et al., 2011). Functional validation of a few candidate genes identified in these populations has also shown that these genes affected egg length (Jha et al., 2015). This suggests that higher egg volume more likely corresponds to an increase in egg length. While it is not reported whether the eggs with higher volume are also wider we speculate that an increase in egg length would probably cause less selection pressure on the morphological changes of ovipositors of the flies.

Moreover, the average egg width is comparable among different species of melanogaster subgroups (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, and *D. erecta*) while the average egg length is different (Markow et al., 2009). Furthermore, our results show a higher variance in egg length (SD = 15.63 μm) than in egg width (SD = 7.11 μm) in an outbred population (Fig. S1) suggesting that egg length is more suitable to investigate the intra-specific variation.

Maintenance of *Drosophila* strains

All the assays were performed using an outbred population that was established from 97 *D. simulans* inbred lines (Signor et al., 2018) using a round-robin crossing scheme. We maintained the crosses for 15 generations at 25°C and mixed an equal number of crosses to set up the outbred population. The population was maintained on standard *Drosophila* medium at low density (400 eggs/bottle) at 25°C with 12hrs light and 12hrs dark daily and 50-60 % humidity. Larval crowding (Venkitachalam et al., 2022) and temperature (Avelar, 1993; Azevedo et al., 1996; Imai, 1932) affect *Drosophila* egg size. To reduce the environmental effects we maintained the cultures at low density (400 eggs/bottle) using a pipetting method (Nouhaud et al., 2018) for at least two generations before the egg size measurement.

Preparation of *Drosophila* eggs for flow cytometry

Around 500 4-6 days old individuals were transferred to an embryo collection cage (Cat. #59-101, Genesee Scientific) with an agar plate (4% agar and 4% sucrose) topped with yeast paste to facilitate oviposition (Becher et al., 2012). Although the age of females

doesn't affect the egg size (Imai, 1932; Lott et al., 2007; Warren, 1924) we restricted the measurements to eggs laid by 4-6 days old females. We limited the oviposition period to 17-19 hours to prevent the hatching of the eggs. A few drops of lukewarm water were added to the agar plate to dissolve the yeast paste, and the solution of yeast and water together with the eggs was transferred to a fine net using a brush. Eggs were washed with water until all yeast grains were dissolved, the eggs were then transferred to a 1.5 ml tube containing 600 µl 1x PBS.

Size measurement and sorting using large particle flow cytometry

We ran all our samples on a Biosorter® (Union Biometrica) machine with fluidics and optics core assembly units (FOCA) 1000 µm using 1x PBS as a sheath solution. To prevent clogging the flow cell, we re-suspended the eggs in 1x PBS such that the final density of ~400 eggs/ml was reached. Samples were stirred continuously using a mechanical stirrer in a 50 ml sample cup and traveled to the flow cell surrounded by the sheath solution (1x PBS) that focused them into the center of the stream where they were interrogated by multiple lasers. We ran Biosorter® using FlowPilot™ software using a pressure sample cup 0.60-0.65 and pressure diverter 1.2. If samples were to be sorted, a droplet containing fluid and the sorted object was generated which dropped into the collecting container. We sorted samples using the following parameters: drop width of 22 milliseconds (ms) and sort delay of 28 ms. We cleaned the mechanical stirrer with distilled water and the system with 1x PBS after running each sample to avoid cross-contamination.

Large particle flow cytometer measures two optical characteristics of the objects: time of flight (TOF) which is the time each object blocks the light, and extinction (EXT)

which is determined by the total integrated signal of the blocked light (Fig. S2). We measured the TOF of polystyrene reference beads (200 and 430 μm) and fitted a linear regression model using TOF as the response variable and size as the explanatory variable. Using the estimated intercept and slope, TOF measurements of all samples were converted to size (size = 5.94 TOF - 491.78). FlowPilot™ software allows real-time visualization of EXT and TOF of objects as scatter plots and regions of interest, i.e. gates, can be chosen on this plot for sorting objects (Fig. S2). Based on the estimated size using TOF measurements, multiple gates were set up that corresponded to different size ranges (Fig. S2). These gates were used for sorting eggs which were dispensed in 1x PBS.

In silico filtering of debris particles

The size distribution of eggs in all analyzed samples was bimodal ('No filtering' curve in Fig. 1C). Manual inspection of the objects in the first mode revealed that the objects are mostly debris and yeast particles while the particles in the second mode were all *Drosophila* eggs. We thus filtered out the objects that corresponded to the first mode by first fitting a kernel density distribution to the size distribution and then identifying the border between the two modes. In brief, we fitted kernel density estimation that is controlled by a smoothing parameter, i.e. bandwidth, to the size distribution. To identify a suitable bandwidth for smoothing the density distribution we performed an exhaustive search over a range of bandwidths by fitting the kernel density function with a Gaussian shape. We performed K-Folds cross-validation by splitting the dataset into train and test sets. We split data into five folds; four folds formed the training set and one fold was used as a validation set. The best bandwidth giving the highest score was chosen as the

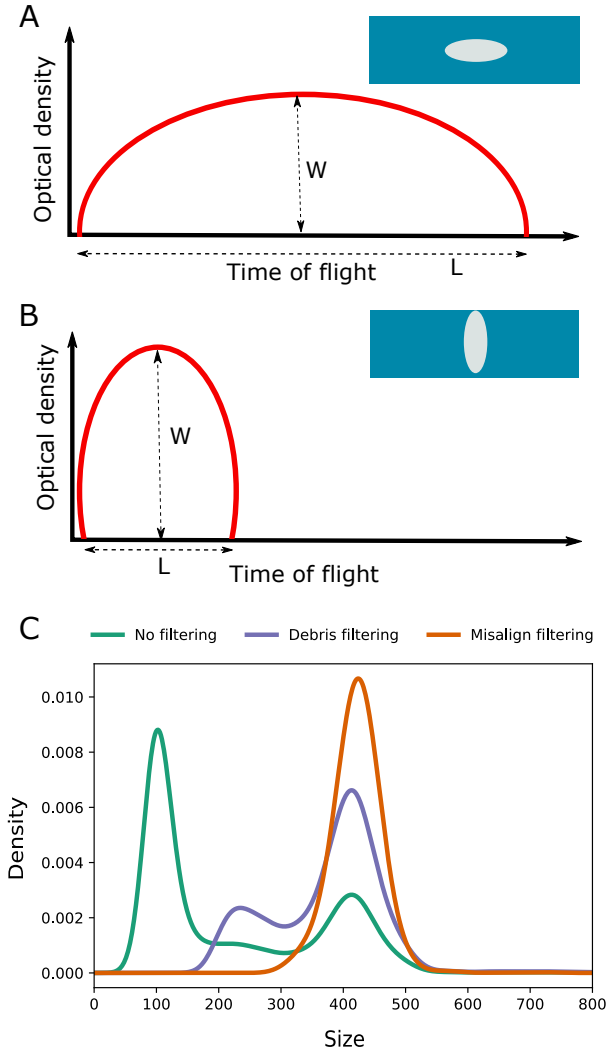

Fig. 1 In silico filtering of debris and misaligned objects using the optical density profiles. A) *Drosophila* eggs are elliptical, and accurate size measurement based on time of flight (TOF) is obtained when an egg is aligned on its long axis. B) The size of a misaligned egg, i.e. aligned on its short axis, is underestimated. The misaligned eggs can be identified based on the ellipticalness index (EI) and W/L ratio where W is the maximum optical density of an object and L is the number of extinction measurements along an object's time of flight. C) The initial size distribution is bimodal (No filtering). The 1st mode, around 92 µm, corresponds to debris and yeast particles, and 'Debris filtering' remove these small objects. Further filtering steps based on EI and W/L parameters (Misalign filtering) removed the misaligned eggs. Size is in µm units.

bandwidth to smooth the kernel density distribution. We then computed the log-likelihood of each dataset under the fitted kernel density distribution and identified the border between the two modes as the first valley in the log-likelihood. The border between the two modes was used as the empirical size threshold for filtering yeast particles for each sample ('Debris filtering' curve in Fig. 1C).

In silico filtering of misaligned eggs

The size of a fraction of eggs was estimated to be 200-400 μm ('Debris filtering' curve in Fig. 1C) by LPFC in all the samples which is smaller than the range of *Drosophila* egg size, i.e. 400 to 600 μm (Imai, 1932; Lott et al., 2007; Markow et al., 2009; Venkitachalam et al., 2022; Warren, 1924). Manual measurement of these eggs showed that their size was underestimated by LPFC likely due to the orientation of eggs during measurement. When an ellipsoid object passes through the laser path along its long axis the recorded TOF corresponds to its size (Fig. 1A). But if the object is misaligned, i.e. the object doesn't pass along the long axis, TOF and subsequently size will be underestimated (Fig. 1B). Using a more viscous sheath solution, e.g. 1x PBS with 4% methyl cellulose, will reduce the flow rate and facilitate the alignment of objects along their long axis when passing through the laser path (Fig. S3). However, the viscosity of the sheath solution dramatically decreases the speed of analysis.

Thus, in addition to the debris filtering, we used the optical density information to filter out misaligned eggs that were not aligned on their long axis. The optical density which measures the extinction of objects varies as a function of the structure of objects (Fig. 1A-B). We extracted the maximum optical density recorded for an object (W) and the

total number of extinction measurements along each object's time of flight (L) and computed the ellipticalness index ($EI = \frac{Area}{\pi/4LW}$) which is the ratio of the area under the optical density profile of an object to the area of the closest elliptical profile (Li et al., 2022; Morales et al., 2020). Due to the elliptical shape of the *Drosophila* egg, an egg aligned on the long axis has a smaller W/L ratio than eggs aligned on the short axis (Fig. 1A-B). For each sample, we computed EI and W/L for all objects and retained objects distributed within half of the standard deviation around the median of EI and W/L. Lastly, after filtering objects based on EI and W/L, the first mode of the distribution of *Drosophila* egg size, which corresponds to the misaligned eggs, was filtered out similarly to the debris filtering step described above.

Manual measurement of egg size

To validate the accuracy of the estimated egg size using LPFC, we measured the size of eggs manually and automatically using LPFC. Around 2000 individuals from the outbred *D. simulans* population were transferred to an embryo collection cage with agar plates topped with yeast paste and laid eggs for 17-19 hours. We prepared eggs for flow cytometry as described above, and sorted 74 eggs from 6 gates corresponding to 400-600 μm (with intervals of 50 μm) in a 96-well plate. We then manually measured the size of the sorted eggs. We transferred the eggs to a petri-dish containing a dark medium (4% agar and 0.5% charcoal), positioned the eggs on their dorsal side, and took high-resolution photos using a Leica stereomicroscope (M205 FA). We measured the egg size manually using ImageJ (Schneider et al., 2012). The scale of images was transformed to μm and the egg size was measured by drawing a line along the longitudinal axis of each egg. The

concordance between the manual size measurements and the automated size estimates by LPFC was tested using Spearman's rank correlation.

Moreover, we tested whether submersion in PBS influences the egg size measurement. We positioned 46 eggs on their dorsal side on a petri-dish containing a dark medium and took high-resolution photos. We submerged the eggs in a 96-well plate for 2.5 hours, then transferred the eggs to a dark medium and took high-resolution photos. We measured the egg size manually using ImageJ as described above. The concordance between the egg size measurements before and after submersion in PBS was tested using Pearson's correlation.

Egg-to-adult viability assay

During the egg size measurement and sorting using LPFC eggs might be immersed in PBS for up to several hours. Moreover, while LPFC employs a slower flow rate and lower pressure than traditional flow cytometry to ensure that the sorted objects are viable, passing through the flow cytometer may affect the survival of the eggs. We performed an egg-to-adult viability assay to determine the effect of submersion in PBS and passing through the flow cell on the development of eggs into adults. We collected eggs laid by an outbred *D. simulans* population for 17-19 hours, as described above in the Manual measurement of egg size. To determine the egg-to-adult viability of the eggs without exposure to PBS, the eggs on the agar plate were not washed with PBS ('no PBS' treatment); 5 independent sets of 50 eggs were manually counted and transferred to 5 vials with a brush. We then washed the eggs laid on the yeast paste and transferred around 30 µl eggs to three 1.5 ml tubes containing 600 µl 1x PBS; 2 tubes corresponded to

'0-hour' and '4-hour' treatments and 1 tube to 'sorted 4-hour' treatment. The eggs in 0-hour and 4-hour treatments were maintained at room temperature (25 °C) for only 20 minutes and 4 hours, respectively. For each treatment, 5 independent sets of 50 eggs were manually counted under a stereoscope and each set was transferred to a vial containing standard *Drosophila* medium. We ran the eggs in the 'sorted 4-hour' treatment on LPFC and sorted 7 independent sets of 50 eggs from gates corresponding to 400-600 μm, i.e. the whole range of egg size distribution. Each set was transferred to a vial. The number of eclosed adults from each vial for all the treatments was counted on 4 consecutive days until no more flies were closed.

To test for the effect of PBS submersion and sorting by LPFC on the egg-to-adult viability we fitted a generalized linear mixed model (using a binomial distribution) with treatment, i.e. submersion time and sort status, as a fixed categorical effect with 4 levels (no PBS, 0-hour, 4-hour, and sorted 4-hour). The used model is as follows: $success_{ij} \sim \mu + treatment_i + error_{ij}$, where success is a matrix with the number of survived and dead eggs. All treatments have a sample size of 5 replicates except for the sorted 4-hour treatment which has 7 replicates. The replicates were included as a random effect. The significance of the fixed effect was tested using ANOVA F-tests and we used Tukey's HSD to correct for multiple testing.

Results

Egg size distribution after in-silico filtering of debris particles and misaligned eggs

The size distribution of all samples was bimodal (Fig. 1C) and the average size of the 1st and 2nd modes were 92.38 and 412.21 μm (n = 16 samples, Table S1). The objects in the

first mode were mostly debris and yeast particles upon manual inspection. We used the computed border between the two modes as an empirical size threshold to remove the objects in the 1st mode. These sample-specific empirical thresholds ranged from 92.9 to 212.21 μm with an average of 184.29 μm (n = 16 samples, Table S1).

The 2nd mode with an average size of 412.21 μm (n = 16 samples) corresponds to the size of *Drosophila* eggs (Imai, 1932; Lott et al., 2007; Markow et al., 2009; Venkitachalam et al., 2022; Warren, 1924) but also contained some misaligned eggs (Fig. 1A-B). The size of misaligned eggs was estimated between 200 and 400 μm by LPFC (first peak in the 'Debris filtering' curve in Fig. 1C) but was underestimated likely due to the improper orientation of eggs during measurement. To filter the misaligned eggs, we computed the ellipticalness index (EI) and the ratio of the maximum optical density of an object to the number of extinction measurements along an object's time of flight (W/L) values using the optical density data for each sample. Each dataset was further filtered by removing eggs that were not aligned on the long axis (Fig. 1B) by retaining objects with EI and W/L values within half of the standard deviation around the median. As the final step for filtering the misaligned eggs, we removed the objects corresponding to the 1st peak of the egg distribution (first peak in 'Debris filtering' curve in Fig. 1C). These filtering steps did not remove objects with the large size showing that EI and W/L parameters properly identify misaligned eggs where the size in underestimated (Fig. 1C). The computed sample-specific empirical threshold for filtering these objects ranged from 163.36 to 317.12 μm, with an average of 317.12 μm (n = 16 samples, Table S2).

Accuracy and throughput of egg size measurement and sorting

We validated the accuracy of egg size measurements and sorting using LPFC by comparing the estimated egg size by LPFC with the manual measurements. We sorted eggs from several gates corresponding to 400 to 600 μm and measured the size of the eggs manually. We observed a high correlation between the manual measurements and the estimated size using LPFC (Fig. 2A). We further assessed the effect of submersion in PBS on the egg size. The manually measured egg size before and after 2.5 hours of submersion in PBS was highly correlated (Fig. 2B).

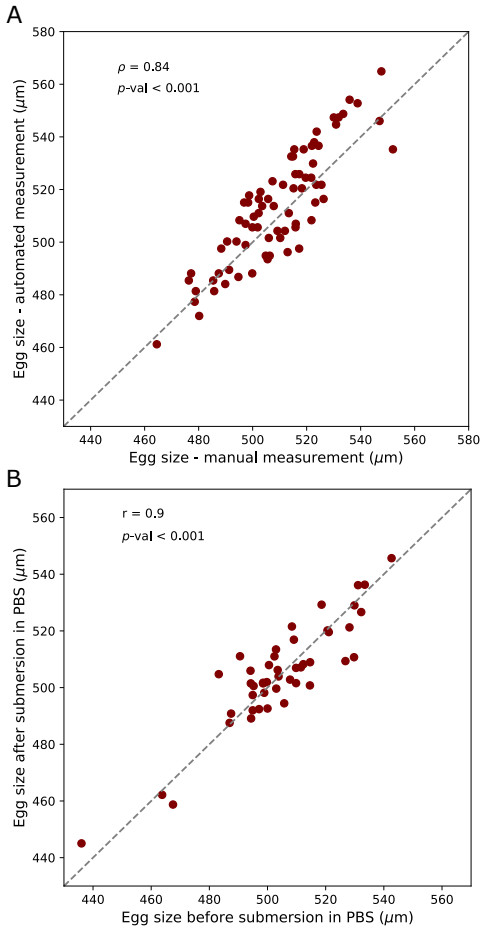

Fig. 2 Accuracy of the estimated egg sizes using large particle flow cytometry. A) The estimated sizes through manual measurement and large particle flow cytometry are highly correlated (Spearman's rank correlation **ρ** = 0.84, *p*-value < 0.001). B) Submersion in PBS does not affect the egg size as the measured egg size is highly correlated before and after submersion in PBS (Pearson's correlation **r** = 0.9, *p*-value < 0.001). The slope of the dashed lines in A and B is 1.

The measurement of egg size is high throughput and fast. We measured an average of 316 eggs/min which corresponds to 214 eggs/min (n = 13 samples) after filtering out the misaligned eggs (Fig. 3A). We evaluated the efficiency of sorting as sort recovery which is the percentage of objects that were sorted and dispensed as a fraction of all the objects fulfilling sorting criteria, i.e. size. In our hands, the median sort recovery was 63.54% (n = 28 samples, Fig. 3B) and the median speed of sorting was 69 eggs/min (n = 28 samples, Fig. 3C).

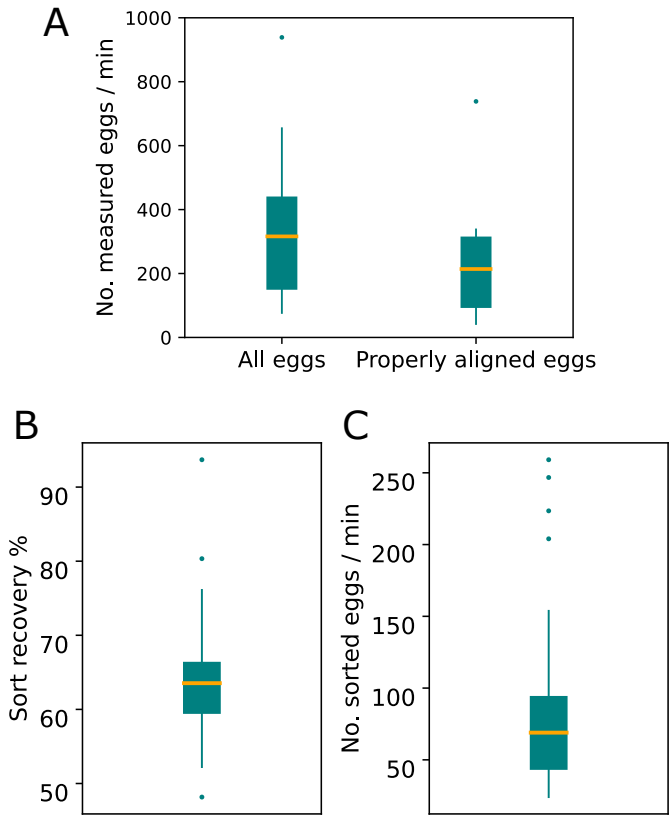

Fig. 3 Throughput of the size measurement and sorting of eggs using large particle flow cytometry. A) The median speed of egg size measurement is 316 eggs/min after filtering for debris ('Debris filtering' curve in Fig. 1C) and 214 eggs/min (n = 13 samples) after filtering the misaligned eggs ('Misaligned filtering' curve in Fig. 1C). B) The median sort recovery as an estimate of the efficiency of sorting is 63.54% (n = 28 samples). C) The median speed of sorting is 69 eggs/mins (n = 28 samples).

The effect of handling and sorting by large particle flow cytometer on egg viability

To demonstrate the suitability of using LPFC for the measurement and sorting of viable eggs, we tested whether the sorted eggs develop normally in adults. We observed no significant difference in egg-to-adult viability between eggs that were not washed with PBS (42.4±3.36 out of 50) and those that were submerged in PBS briefly for 20 minutes (40.74±6.08 out of 50) and 4 hours (43.2±5.12 out of 50) (Fig. 4, a generalized linear mixed model followed by Tukey's HSD test with correction for multiple testing, $P_{\text{no-PBS-0hour}}$ = 0.7307, $P_{\text{no-PBS-4hour}}$ = 0.9567, $P_{\text{0hour-4hour}}$ = 0.4405). Moreover, eggs that passed through LPFC showed no decrease in egg-to-adult viability (42.57±3.6 out of 50) compared to the eggs submerged in 1x PBS for the same duration of time (43.2±5.12 out of 50) (Fig. 4, $P_{\text{no-PBS-sorted 4hour}}$ = 1.0000, $P_{\text{0hour-sorted 4hour}}$ = 0.6673, $P_{\text{4hour-sorted 4hour}}$ = 0.9540).

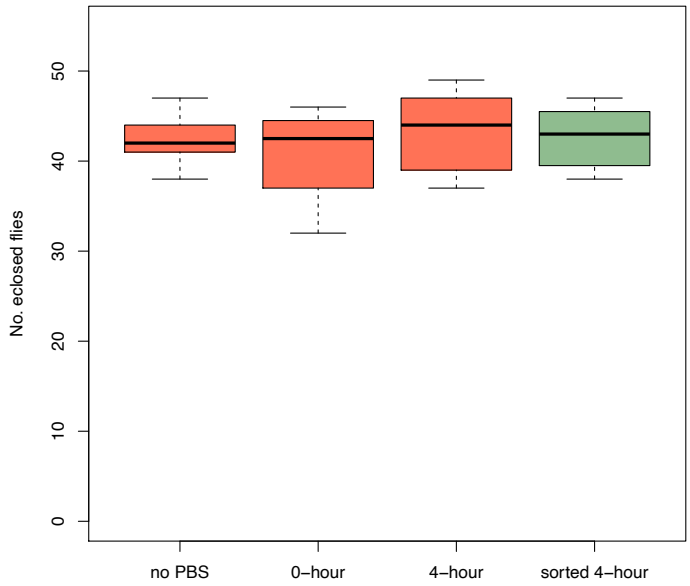

Fig. 4 The viability of eggs is not reduced by submersion in PBS and sorting by large particle flow cytometry. There is no significant difference in the viability of eggs that were not washed with PBS (no PBS), with those that were submerged in PBS for 20 minutes (0-hour), and 4 hours (4-hour). Passing through the flow cytometer (sorted 4-hour) does not reduce the egg-to-adult viability either (a Generalized linear mixed model followed by Tukey's HSD test with correction for multiple testing, $P_{\text{no-PBS-0hour}}$ = 0.7307, $P_{\text{no-PBS-4hour}}$ = 0.9567, $P_{\text{0hour-4hour}}$ = 0.4405, $P_{\text{no-PBS-sorted 4hour}}$ = 1.0000, $P_{\text{0hour-sorted 4hour}}$ = 0.6673, $P_{\text{4hour-sorted 4hour}}$ = 0.9540).

Discussion

Here, we establish a method for accurate and high throughput measurement of *Drosophila* egg size using large particle flow cytometry. The measurement of egg size is highly accurate (Fig. 2), fast and high throughput (Fig. 3A). Viable eggs within a specified size range can be sorted rapidly (Fig. 3C) with no reduction in egg-to-adult viability (Fig. 4).

Applicability of measuring *Drosophila* egg size using LPFC

Obtaining accurate and high throughput size measurements of *Drosophila* eggs has several applications. First, this high throughput method is a suitable approach for the use of *Drosophila* egg size as a life history trait in large-scale studies where many populations should be analyzed in parallel. Second, this approach allows high throughput analysis of the variation in egg size in natural populations of different species of *Drosophila*. Third, the high throughput of egg size measurement using large particle flow cytometry coupled with the available reference panels of *D. melanogaster* (Mackay et al., 2012) and *D. simulans* (Signor et al., 2018) provide an opportunity to investigate the genetic architecture of egg size variation through QTL mapping and GWAS. Forth, this method provides the possibility of sorting viable *Drosophila* eggs from the range of the size distribution and performing selection experiments with large populations size and replicates.

Recommendations for the use of LPFC for size measurement

The opportunities provided by LPFC for the measurement of the organism's size are not limited to *Drosophila* eggs and can be applied to any organism within the detectable range of this instrument (10-1500 μm). Our recommendation is to perform preliminary experiments to optimize the settings of the instrument. The two most important settings

include flow rate which is adjusted by the pressure of the sample cup and determine the number of objects analyzed in a unit of time and the speed of mixing to assure samples are not damaged. The estimated size should be validated manually before the use of the method for large-scale experiments. If viable organisms are required for the downstream analysis, viability assays should be performed as well.

LPFC has been previously used to estimate the size of *Arabidopsis* seeds (Morales et al., 2020) which have a spheroid shape (Cervantes et al., 2010). To our knowledge, this is the first use of LPFC for the measurement of *Drosophila* egg size which has an ellipse shape and the orientation of the object during measurement affects the estimated size. Similarly, additional adjustments and filtering steps are needed for the organisms with shapes that deviate from a sphere. We recommend a similar in-silico filtering step used in this study to remove debris and misaligned objects. Additionally, using a viscous sheath solution to reduce the flow rate will minimize the number of misaligned objects (Fig. S3). However, a lower flow rate will reduce the speed of analysis. Moreover, the average maximum height of optical density (W), which we used for filtering misaligned objects, can also be used in combination with other sorting criteria, e.g. size, to increase the efficiency of sorting objects.

Caveats

Large particle flow cytometers are expensive instruments that may not be easily acquired by small research groups. However, many research facilities house such instruments that can provide access to researchers. Most research facilities have trained personnel that can assist and trait researchers.

Author Contributions

Neda Barghi conceived the ideas and designed the methodology; Neda Barghi and Claudia Ramirez-Lanzas collected the data; Neda Barghi analyzed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data Availability Statement

All the data and code to reproduce the results are deposited in https://github.com/NedaBarghi/EggSizeBiosorterProtocol.

Acknowledgment

The BioSorter® Large Particle Flow Cytometer (Union Biometrica, Holliston, MA) of the Max Perutz Labs BioOptics FACS Facility (https://www.maxperutzlabs.ac.at/research/facilities/biooptics-facs) was used for sorting. We thank Johanna Stranner and Kitti Dora Csalyi for their help in optimizing the sorting protocol. We thank Paula Marconi for her help in the egg-to-adult viability assay. We thank Emmanouil Lirakis for assistance with using the Leica stereomicroscope. This work was supported by the Austrian Science Fund (FWF, P 32672). We thank Sara Signor (North Dakota State University) for providing *D. simulans* inbred lines.

Competing interests Statement

The authors have no conflicts of interest to declare.

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Supplementary figures and Tables

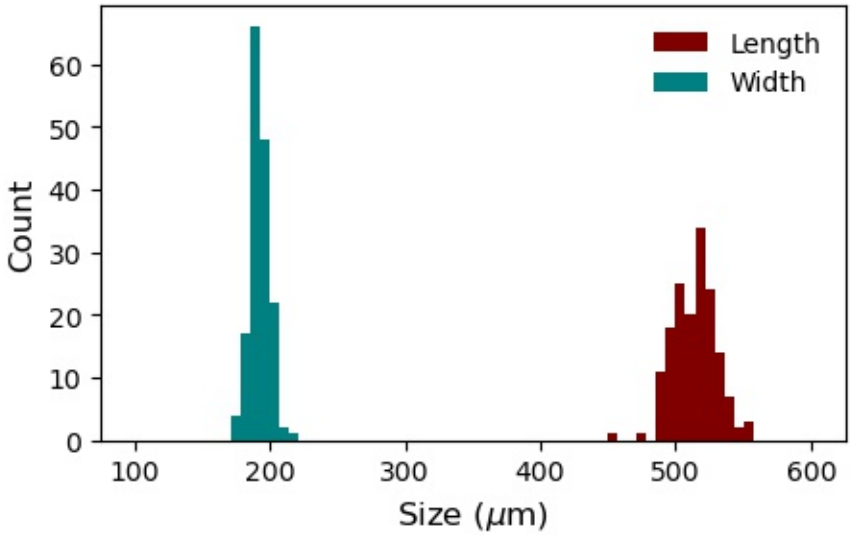

Fig. S1 Variation of egg length and width in an outbred population of *Drosophila*. Collection of eggs and manual measurement of eggs is described in Materials and methods, section Manual measurement of egg size. In brief, a total of 160 eggs were transferred to a petri-dish containing a dark medium, and high-resolution photos were taken using a Leica stereomicroscope (M205 FA). We measured the egg size manually using ImageJ. Egg length has higher standard deviation (SD = 15.63 μm) than egg width (SD = 7.11 μm).

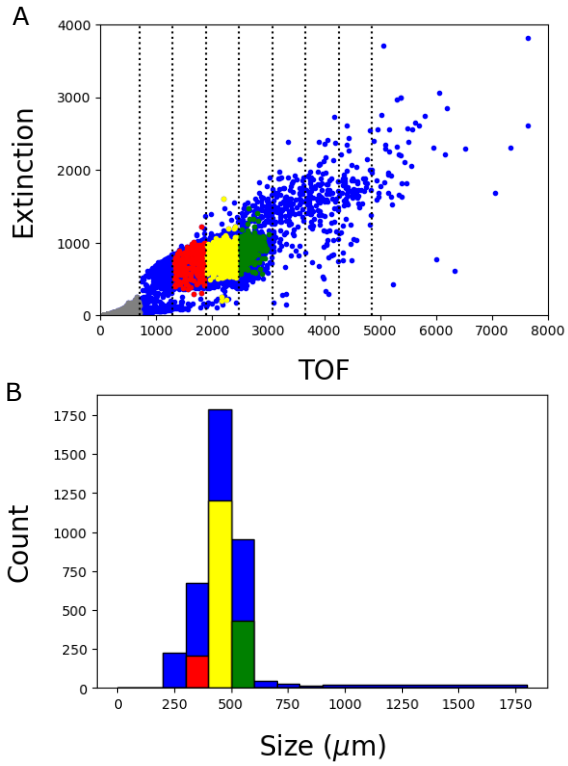

Fig. S2 Time of flight (TOF), the measured time an object blocks the light, is used to estimate the object's size. *Drosophila* eggs are washed and dispensed in 1x PBS buffer and run on large particle flow cytometry. TOF and extinction are measured (A) and TOF is converted to size (μm) using the TOF data of reference beads. Based on the size distribution, user-defined sorting gates are set up (depicted as dotted lines in (A), these gates correspond to size 200-900 μm with intervals of 100μm), and the required number of eggs from each gate can be sorted. Red, yellow and green bars in (B) show the number of sorted eggs in these 3 size gates in (A).

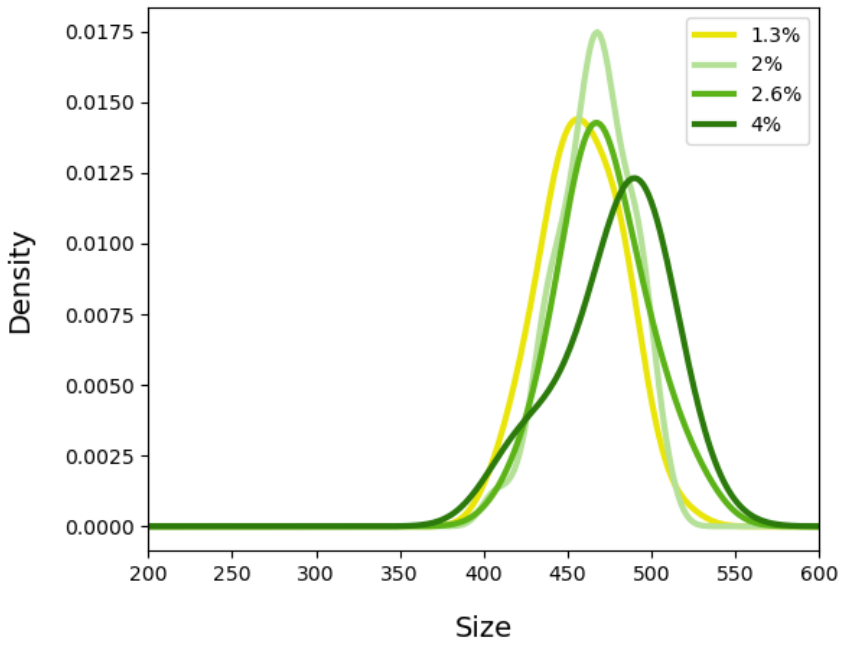

Fig. S3 Distribution of the egg size estimated using different concentrations of methyl cellulose (MC) in 1x PBS as sheath buffer. As the concentration of MC increases the sheath buffer becomes more viscous which will reduce the flow rate. The slower flow rate facilitates the alignment of objects along the long axis while passing through the laser path. However, the increased viscosity of the sheath buffer dramatically reduces the speed of analysis. The same sample has been used for all runs shown in the plot.

Table S1 The statistics of egg size distribution. Size threshold is used to remove 1st peak containing egg debris from the 2nd mode in the first filtering step. Dsim#, #: stock number (*D. simulans*), Lausanne5 (*D. melanogaster*), Dere01 (D. *erecta*), Dsan01 (D. *santomea*) and Dmau151 (*D. mauritiana*).

Sample	1st peak mode (μm)	size threshold (μm)	2nd peak mode (μm)
Dsim1	94.903	212.212	449.078
Dsim4	92.209	176.977	459.851
Dsim5	92.209	196.196	427.531
Dsim11	92.209	177.778	426.184
Dsim75	92.209	207.407	450.425
Dsim90	92.209	173.774	427.531
Dsim91	92.209	92.893	93.556
Dsim146	92.209	180.981	450.425
Dsim166	92.209	198.599	436.958
Dsim185	92.209	201.802	426.184
Dsim196	92.209	194.595	423.491
Dsim237	92.209	182.583	431.571
Lausanne5	92.209	146.547	420.798
Dere01	92.209	207.407	431.571
Dsan01	92.209	211.411	384.438
Dmau151	92.209	187.387	455.811

Table S2 The statistics of egg size distribution filtered using sample-specific threshold. Each dataset is filtered as described in Fig. 1. The 1st filtering step is based on the size threshold in Table S1. The filtered objects at this step are mostly egg debris. # nonegg: the number of small objects filtered in the first filtering stage. The mode, mean and median of these objects are Mode nonegg, Mean nonegg, and Median nonegg respectively. The second filtering step involved removing the misaligned eggs. The number, mean and median of these misaligned eggs are shown as #misaligned egg, Mean misaligned egg and Median misaligned egg. The last filtering step is to remove the objects in the first peak of distribution after removal of the misaligned eggs. Size threshold (μm) is used for this filtering step. #egg, Mode egg, Mean egg and Median egg are the number, mode, mean and median of the eggs in the final dataset. Dsim#, #: stock number (*D. simulans*), Lausanne5 (*D. melanogaster*), Dere01 (*D. erecta*), Dsan01 (*D. santomea*) and Dmau151 (*D. mauritiana*).

Sample	size threshold (μm)	#egg	#nonegg	#misaligned egg	Mode nonegg	Mean nonegg	Median nonegg	Mode egg	Mean egg	Median egg	Mean misaligned egg	Median misaligned egg
Dsim1	269.069	361	2710	849	94.903	110.546	102.983	440.998	438.457	442.345	382.667	391.171
Dsim4	283.483	1980	6707	4572	92.209	111.721	105.676	458.505	453.336	457.158	373.373	387.131
Dsim5	248.248	265	917	699	92.209	108.428	102.983	427.531	443.051	447.731	384.705	389.824
Dsim11	241.041	458	1530	1211	92.209	108.814	102.983	420.798	426.752	431.571	377.862	385.784
Dsim75	263.463	955	3678	2396	92.209	109.350	101.636	457.158	454.088	457.158	393.318	399.251
Dsim90	221.822	941	3075	2119	92.209	106.286	100.289	427.531	434.046	438.304	384.049	397.904
Dsim91	217.818	1913	1707	14055	92.209	92.209	92.209	438.304	416.989	426.184	128.336	102.983
Dsim146	256.256	1229	6138	2957	92.209	106.108	100.289	455.811	454.377	459.851	389.055	404.638
Dsim166	274.675	906	4005	2263	92.209	110.152	102.983	453.118	461.060	462.545	391.385	389.824
Dsim185	249.049	1535	12413	3878	92.209	110.019	101.636	443.691	437.533	440.998	376.696	385.784
Dsim196	298.699	1630	10442	6186	92.209	111.171	101.636	423.491	421.893	422.144	362.492	380.398
Dsim237	237.838	725	3422	2147	92.209	106.550	100.289	427.531	429.827	431.571	396.969	388.478
Lausanne5	163.363	689	4069	2401	92.209	107.065	102.983	420.798	363.069	404.638	312.924	315.757
Dere01	221.021	2056	1453	4757	92.209	114.704	104.329	431.571	433.829	434.264	392.111	403.291
Dsan01	317.117	250	2169	929	92.209	113.170	104.329	403.291	411.872	410.024	364.281	365.584
Dmau151	277.878	3079	15325	6214	92.209	108.169	101.636	466.585	464.235	465.238	381.830	388.478