Journal of Nematology 25(4):596–602. 1993.

Flow Cytometric Analysis and Sorting of *Heterodera glycines* Eggs[1]

G. L. Tylka,[2] T. L. Niblack,[3] T. C. Walk,[3] K. R. Harkins,[2] L. Barnett,[3] and N. K. Baker[2]

Abstract: A nondestructive technique was developed to characterize and separate eggs of soybean cyst nematode, *Heterodera glycines*, by developmental stage using flow cytometry. Eggs from cysts cultured on susceptible soybean roots were suspended in 0.1% xanthan gum or 59% sucrose and loaded into either a Coulter EPICS 752 or EPICS 753 flow cytometer. Eggs were analyzed and sorted according to forward angle and 90° light scatter, flow cytometric parameters that are relative measures of object size and granularity, respectively. Mature eggs containing vermiform juveniles were less granular and slightly larger than eggs in earlier stages of embryogeny, allowing for separation of mature eggs from immature eggs. The effectiveness of flow cytometric sorting was evaluated by comparing the developmental stages of subpopulations of unsorted and sorted eggs. Of a subpopulation of unsorted eggs, 62% contained vermiform juveniles, whereas 85 to 95% of sorted subpopulations of larger, less granular eggs contained vermiform juveniles. Suspending *H. glycines* eggs in 0.1% xanthan gum or 59% sucrose for flow cytometric analysis had no effect on subsequent egg hatch in vitro. This technique is an efficient and effective means to collect large, relatively homogeneous quantities of *H. glycines* eggs in early or late embryogeny, and would likely be useful for analyzing and sorting eggs of other nematode species for use in developmental, genetic, or physiological research, or for identification and collection of parasitized eggs.

Key words: egg development, embryogeny, flow cytometry, *Heterodera glycines*, method, nematode, nematode egg sorting, soybean cyst nematode.

Eggs extracted from adult females and cysts of soybean cyst nematode, *Heterodera glycines* Ichinohe, are of varying ages and developmental stages because eggs are produced sequentially and embryogeny within populations of eggs is asynchronous. Currently, no efficient technique exists to collect quantities of eggs in a specific embryonic stage. Researchers have relied on tedious microscopic observation and manual selection to obtain homogeneous populations of eggs in specific stages of development (1,4,22). Sucrose density gradient centrifugation was reported to separate eggs of different developmental stages (2), but the resulting subpopulation of eggs was not homogeneous.

Flow cytometry is a rapid and precise technique for simultaneously measuring multiple characteristics of individual biological particles such as cells, organelles, or cell aggregates. Biological particles flow in a laminar manner within a hydrodynamic system through the flow cytometer (13). Hydrodynamic forces orient the long axis of nonspherical particles, such as nematode eggs, parallel with the axis of flow (16). Each particle then intersects a laser beam, and the light energy scattered by the particle is converted into electrical impulses and quantified on photomultiplier detectors and a photodiode (13). The degree of reflection and refraction of the light by the particle determines the pattern of scattered laser light (17) and affects the electrical impulses generated by the scattered light. Thus, differences in qualities such as size or granularity are translated into differences in the electrical impulses, which subsequently can be discriminated, stored, displayed, quantified, and manipulated.

Some flow cytometers also have the capacity to sort or isolate subpopulations of biological particles from within a larger heterogeneous population based on differ-

Received for publication 24 February 1993.

[1] Journal Paper No. J-15255 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011, Project 2285. Research was supported in part by state and Hatch funds.

[2] Assistant Professor, Department of Plant Pathology, Assistant Scientist, Cell Facility, and Research Associate, Department of Plant Pathology, Iowa State University, Ames, IA 50011.

[3] Assistant Professor and Graduate Research Assistant, Department of Plant Pathology, and Senior Research Specialist, Molecular Biology Program, University of Missouri, Columbia, MO 65211.

The authors thank Janet Edwards for technical assistance.

ences in particle size, granularity, and fluorescence, which result in differences in light scatter. After the biological particles are analyzed, sorting is accomplished by establishment of a small electrostatic charge on droplets of carrier medium containing particles with preselected characteristics. The charged droplets are diverted towards an oppositely charged electrical deflection plate and accumulate in a collection container separate from the noncharged droplets (13).

Other apparatus have been used to enumerate nematodes suspended in liquid carrier media. A nephelometer was developed to estimate numbers of *Caenorhabditis briggsae* based on changes in light transmitted through a suspension of the nematodes (23). Changes in the electrical resistance of an electrolyte solution when nematodes pass through a detector provided the basis for enumerating *Neoaplectana* sp. in the Coulter counter, a predecessor to the flow cytometer (18). Later, a modified Coulter counter was developed to enumerate and determine the relative size of *Caenorhabditis elegans* juveniles and adults in suspension (8). The aforementioned apparatus effectively estimated numbers of nematodes in suspension but were not capable of distinguishing subtle qualitative differences in the nematodes.

Flow cytometry has been used to analyze and sort plant protoplasts (6,19), animal spermatozoa (15), plant and animal chromosomes (3,10), and plant microspores (11). Our objectives were to determine whether flow cytometry could be used to detect differences in the developmental stages of *H. glycines* eggs and to sort or recover subpopulations of eggs at selected stages of development. A preliminary report of this work has been published (21).

Materials and Methods

Populations of *H. glycines* were reared on the susceptible soybean (*Glycine max* (L.) Merr.) cultivars Corsoy 79, Essex, or Williams 82 in the greenhouse. Eggs were extracted from adult *H. glycines* females and cysts (5) collected from ca. 30-day-old soybean roots. The extracted eggs were subsequently separated from debris by sucrose centrifugation (14).

Effects of xanthan gum and sucrose on hatch: To minimize settling and facilitate proper flow of relatively large biological particles through the flow cytometer, the particles must be suspended in a buoyant carrier medium such as 0.1% xanthan gum or 59% sucrose. Consequently, *H. glycines* eggs were suspended in these carrier media for several hours to determine whether incubation in the carrier media affected subsequent egg hatch. Eggs were collected as described, then surface disinfested in 0.5% chlorhexidine diacetate for 15 minutes and rinsed several times in sterile deionized water (2). Subsequently, eggs were incubated for 3 hours at 25 C in 0.1% xanthan gum, 59% sucrose, or deionized water.

Following incubation, eggs were concentrated on a 25-μm-pore sieve, rinsed thoroughly with sterile deionized water, and dispensed onto 38-μm-pore microsieves. Microsieves were constructed of cylinders made from 18-mm-d and 20-mm-d polypropylene test tube caps with the tops removed. A 33-mm-d circle of 38-μm-pore nylon monofilament was suspended between the two cylinders to form the bottom of the microsieve. The microsieves with eggs were placed in 32-mm-wide × 72-mm-long × 14-mm-deep rectangular polystyrene trays, which were filled with 12 ml of a hatch solution of either deionized water or 3.1 mM zinc sulfate. Hatching trays with microsieves and eggs were incubated at 25 C in complete darkness in 20-cm-wide × 27-cm-long × 9.5-cm-deep polystyrene boxes. The microsieves were transferred to new rectangular hatching trays filled with fresh solution every 3 to 4 days, and the number of hatched second-stage juveniles (J2) in the old hatch solution was determined after each transfer.

Treatments comprised combinations of three incubation solutions and two hatch solutions in a complete factorial design arranged in five randomized complete

blocks. After approximately 40 days, the remaining unhatched eggs in the microsieves were enumerated, and daily counts of hatched J2 were converted into daily percentages based on the total number of eggs added to each microsieve. Cumulative percentage of hatch at each day of observation was analyzed by a two-factor analysis of variance and subsequent Fisher's least significant difference test when a main effect of incubation solution was detected (20). The experiment was repeated once.

Development of sorting technique: For all flow cytometry and sorting purposes, eggs were suspended in 0.1% xanthan gum or 59% sucrose and were agitated manually or with a stream of air to prevent settling. Egg suspensions were loaded onto a Coulter EPICS 752 flow cytometer with a 200-μm flow cell tip or an EPICS 753 flow cytometer with a 150-μm flow cell tip and analyzed at a wavelength of 488 nm. Adjustments of the various parameters of the flow cytometers were made until the following optimal settings were determined: laser power = 10 to 20 mW, sheath pressure = 41.4 kPa, stream velocity = 10 m/s, bimorphic crystal frequency = 8 kHz, flow rate = 100 eggs/s. A 10% neutral density filter was used to reduce laser light intensity of forward light scatter to the photodiode, and a 488 nm dichroic filter was utilized for reflecting laser light scattered at 90° to the photomultiplier tube.

Preliminary experiments revealed that *H. glycines* egg size and granularity varied with stage of egg development. More mature *H. glycines* eggs, containing vermiform juveniles, were slightly larger and less granular than eggs in earlier stages of embryogeny. Forward angle and 90° light scatter are directly proportional to particle size and granularity, respectively. Consequently, larger and less granular eggs were expected and found to be distributed in the lower right portion of a flow cytometer scatter diagram of 90° light scatter plotted on the *Y* axis versus forward angle light scatter plotted on the *X* axis. Five populations of *H. glycines* eggs were analyzed with the flow cytometer, and a consistent scatter diagram distribution was established. Eggs from selected regions of the scatter diagrams were sorted and observed microscopically to assess the stage of egg development.

Sorting effectiveness: Two types of experiments were conducted to quantify the effectiveness of flow cytometric sorting in selecting and segregating subpopulations of eggs in either early or late stages of development. In one set of two experiments, subpopulations of 4,000 to 25,000 eggs were collected from either the entire (100%) scatter diagram distribution or the lower right 50 or 10% of the distribution of a population of *H. glycines* eggs. Six replicate random samples of 100 eggs were collected from each sorted subpopulation and observed at a magnification of 100× or 200× with an inverted compound microscope. Eggs were assigned to one of six categories according to stage of development. Because the treatments were coded during observation, the evaluator categorizing the stage of egg development did not know from which portion of the scatter diagram distribution the eggs were collected. Chi-square analysis was used to test the degree of independence between the proportion of eggs in defined stages of development and the region of the distribution from which the eggs were recovered (20). Additionally, percentages of the sorted egg subpopulations in each of the six categories were subjected to analysis of variance, followed by a Fisher's least significant difference test when significant treatment effects were detected (20).

In another set of three experiments, eggs were either not loaded into the cytometer or were loaded into the cytometer and subpopulations collected from 100% of the scatter diagram distribution, the lower right region of the distribution, or the upper left region of the distribution. Three replicate random subsamples of 100 eggs each were drawn from each subpopulation of eggs. Eggs were observed at a magnification of 25× with a dissecting microscope and assigned to one of three

classes: eggs containing vermiform juveniles, eggs not containing vermiform juveniles, and free vermiform juveniles. The numbers of individuals within each class were analyzed by analysis of variance followed by the Waller-Duncan k-ratio *t*-test (k = 100) to compare means (20).

Results

Effects of xanthan gum and sucrose on hatch: Results of the two experiments were similar; consequently, data presented are from the first experiment. Incubation of *H. glycines* eggs in 0.1% xanthan gum or 59% sucrose had no beneficial or negative effects on subsequent egg hatch relative to hatch of eggs incubated in deionized water. Maximum cumulative hatch ranged from 45 to 73%, and most eggs hatched between days 3 and 12, regardless of treatment (Fig. 1). Eggs incubated in deionized water and subsequently incubated in 3.1 mM zinc sulfate had the most overall hatch. Throughout the experiment there was never a statistically significant main effect of incubation solution detected. However, significantly more eggs hatched in zinc sulfate than in deionized water beginning on day 9 and persisting throughout the remainder of the experiment. Mean maximum cumulative percentage hatch of eggs incubated in deionized water and 3.1 mM zinc sulfate was 48 and 69%, respectively.

Development of sorting technique: The scatter diagram distribution of 90° light scatter plotted on the *Y* axis versus forward angle light scatter plotted on the *X* axis revealed a consistent inverse relationship (Fig. 2). Eggs collected from the upper left area of the scatter diagram were smaller than the other eggs, were more granular or opaque, and contained nematode embryos in the early stages of embryogeny (Fig. 3A). Conversely, eggs from the lower right region of the scatter diagram were larger and more translucent than the other eggs, and a majority of the eggs contained vermiform juveniles (Fig. 3B). Subpopulations of eggs collected from opposite ends of the scatter diagram distribution were not completely exclusive of eggs in other stages of embryogeny. Flow cytometric analysis and sorting of subpopulations of 4,000 to 20,000 eggs took from 2 to 4 hours to complete.

Sorting effectiveness: The results obtained when the two types of experiments were repeated were similar to the results of the initial experiments; thus, results from the first of each type of experiment are pre-

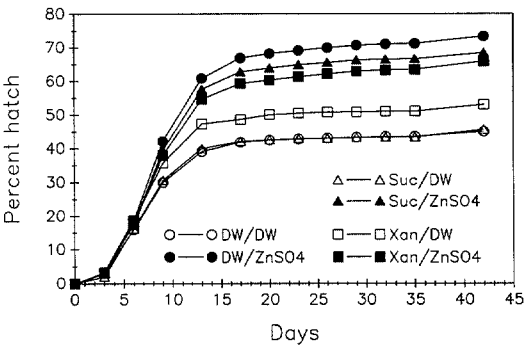

FIG. 1. Effects of incubation of *Heterodera glycines* eggs in deionized water (DW), 59% sucrose (Suc), and 0.1% xanthan gum (Xan) on cumulative percentage hatch of eggs in DW and 3.1 mM zinc sulfate (ZnSO4). Designation before "/" represents egg incubation treatment and designation after "/" indicates the solution in which the eggs were incubated for hatch.

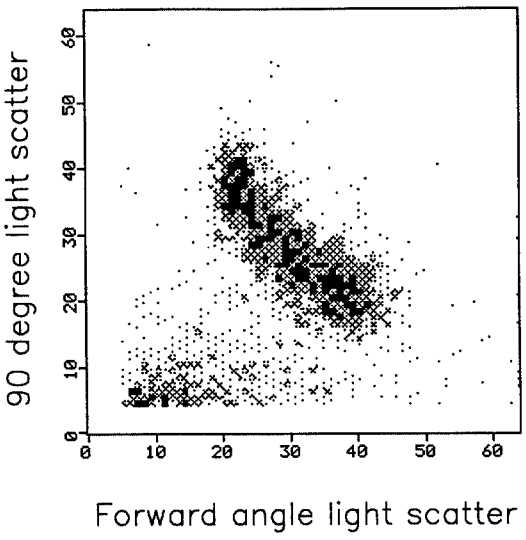

FIG. 2. Scatter diagram of 90° light scatter (*Y* axis) versus forward angle light scatter (*X* axis) of *Heterodera glycines* eggs.

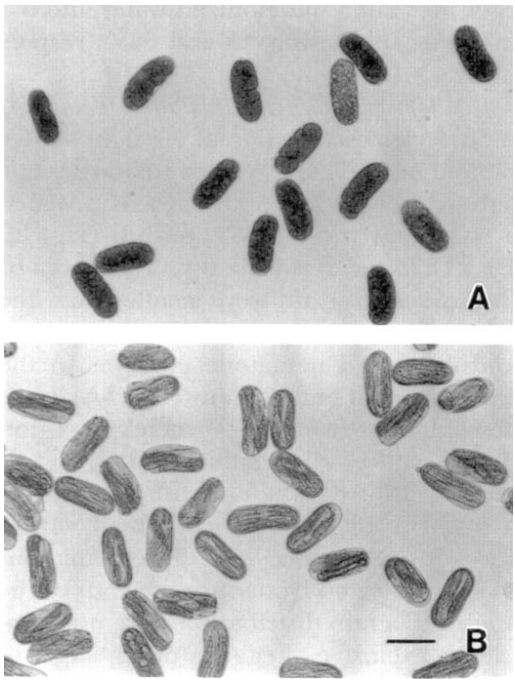

FIG. 3. Subpopulations of *Heterodera glycines* eggs analyzed and sorted by flow cytometry. A) Eggs collected from upper left region of the scatter diagram distribution. B) Eggs collected from lower right region of the scatter diagram distribution. Scale bar = 100 μm.

sented, unless otherwise stated. In both types of experiments, eggs collected from the lower right region of the scatter diagram distributions contained significantly larger proportions of nematodes in later stages of embryogeny than eggs collected from the upper left region of the scatter diagram distributions. In the experiment where sorted eggs were assigned to one of six categories, 62% of the eggs collected from the entire scatter diagram distribution, 85% of eggs collected from the lower right 50% of the distribution, and 95% of the eggs collected from the lower right 10% of the distribution contained vermiform juveniles (Table 1). There was a consistent relationship between stage of embryogeny and the flow cytometric characteristics of the eggs as indicated by a highly significant ($P < 0.001$) chi-square value.

In experiments where subpopulations of eggs were assigned to one of three categories, few of the eggs run through the cytometer and collected from the upper left region of the scatter diagram distribution contained vermiform juveniles, but nearly 80% of eggs collected from the lower right region of the distribution contained them (Fig. 4). A majority of the eggs in subpopulations not run through the cytometer contained nematode embryos that had not yet developed into vermiform juveniles, and the remaining eggs contained vermiform juveniles. Similar results usually were obtained when subpopulations of eggs were run through the cytometer and collected from the entire scatter diagram distribution. However, in one of three experiments, 10% of the individuals observed from a subpopulation run through the cytometer and collected from the entire scatter diagram distribution were not eggs, but were free vermiform juveniles (Fig. 4).

DISCUSSION

Flow cytometry is an effective and efficient means of collecting large quantities

TABLE 1. Developmental stages of *Heterodera glycines* eggs separated by flow cytometry.

Region of scatter diagram subpopulation collected from	Percentage of eggs in subpopulation					
	1-celled	2-celled	3- to 4-celled	5- to 8-celled	>8-celled, but no vermiform juveniles	Containing vermiform juveniles
100%	3.3 a	0.5 a	1.0 a	5.0 a	27.7 a	62.2 c
Lower right 50%	0.8 a	0.7 a	0.2 b	0.0 b	12.8 b	85.3 b
Lower right 10%	0.3 a	0.0 a	0.0 b	1.0 b	3.5 c	95.2 a

Eggs were collected from different regions of a scatter diagram of 90° light scatter (*Y* axis) versus forward angle light scatter (*X* axis).

Values are means of six replicates. Means within columns followed by the same letter are not significantly different according to a protected Fisher's least significant difference test ($P = 0.05$).

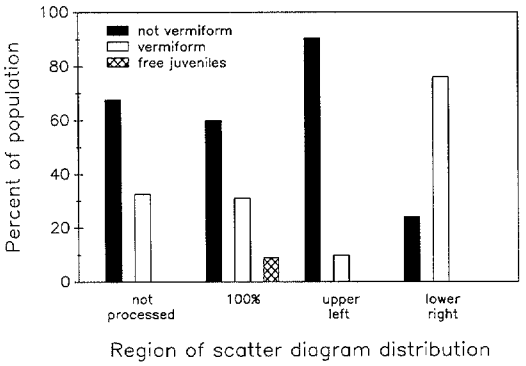

FIG. 4. Embryonic stage of subpopulations of *Heterodera glycines* eggs that were either unanalyzed or flow cytometrically analyzed and sorted. Eggs were either not processed through the flow cytometer (not processed), collected from the entire scatter diagram distribution (100%), or collected from the upper left (upper left) or lower right (lower right) regions of the scatter diagram distribution. Individuals from randomly selected subpopulations were classified as not containing vermiform juveniles (not vermiform), containing vermiform juveniles (vermiform), or as free vermiform juveniles (free juveniles).

of subpopulations of *H. glycines* eggs in early or late stages of embryogeny. Although we did not attempt to collect subpopulations of eggs in intermediate stages of embryogeny, the technique should serve this purpose as well. Flow cytometric sorting is more rapid than observation and manual selection of nematode eggs (1,4, 22) and results in collection of more homogeneous subpopulations than those obtained with sucrose density gradient centrifugation (2). The technique does not exclude all eggs in unwanted stages of development, primarily because more than one egg can be contained within a single sorted droplet of carrier medium. If greater purity is needed, several options exist. Unwanted eggs could be removed manually from a sorted subpopulation, or a second cytometric analysis and sort could be performed on a previously sorted subpopulation of eggs using more stringent sort selection criteria. However, the latter option would require millions of eggs and would take days to perform. Alternatively, one could increase the purity of a single sort by narrowing the sort selection criteria; however, efficiency of the sort will simultaneously decrease (9).

Sorted eggs may be adversely affected by physical forces during passage through the flow cytometer, by intersection with the argon laser beam, or by the establishment of an electrostatic charge on the droplets of carrier medium during sorting. In one of our experiments, 10% of the individuals in a subpopulation analyzed by the cytometer and collected from the entire scatter diagram distribution were free vermiform juveniles that were not encased in egg shells, but free vermiform juveniles were not observed in a subpopulation that was not analyzed by the cytometer. The free juveniles may have been prematurely released from eggs due to passage through the flow cytometer. However, there are no published reports of adverse effects of flow cytometric analysis and sorting on the sorted biological particles. Likewise, we have conducted hatch experiments in vitro using sorted subpopulations of eggs collected with the flow cytometer and have not observed any changes in egg viability or hatching activity.

Flow cytometric analysis and sorting of nematode eggs has many potential uses in nematological research. The technique we describe would likely be directly applicable for sorting eggs of *Meloidogyne* species or other nematodes that produce large quantities of eggs. Sorted subpopulations of nematode eggs would be useful for developmental studies, genetic engineering of nematode embryos, physiological research, and studies on the effects of pesticides or other chemicals.

The maturity of populations of *H. glycines* eggs obtained from adult females and cysts varied considerably, with 30 to 60% of the eggs containing vermiform juveniles. This variation was noticeable when 90° light scatter of the eggs was plotted on the *Y* axis against forward angle light scatter on the *X* axis in a scatter diagram. Such scatter diagrams are representations of the overall maturity of the egg populations and, consequently, may be useful in research on factors affecting embryogeny and diapause in *H. glycines* or other nematode species.

Parasitized nematode eggs often appear distorted, enlarged, discolored, or unusually opaque (7,12). Such qualitative differences are readily detected and easily quantified using flow cytometry technology. Consequently, the technique we describe may represent an automated and efficient approach to identifying and selecting parasitized nematode eggs, which would expedite the search for new organisms with potential as biological control agents. Alternatively, flow cytometric analysis might be useful in determining the success of introductions of biological control agents by assessing the degree of parasitism in a population of nematode eggs previously inoculated with a known nematode egg parasite.

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