Automated Analysis and Sorting of 1st instar larvae of the Malaria Mosquito Anopheles sp.

Objective

This report demonstrates the automated sorting of early live stages (1st instar) of mosquito larvae using COPAS SELECT. Sorting male vs. female larvae at early live stages serves many different applications, e.g. developing genetic tools for vector controls or studying sexual differentiation before morphological differences become visible. Furthermore, using COPAS SELECT for sorting allows the user to sort larvae at an even higher speed than using COPAS XL. High throughput male mosquito larvae sorting using COPAS instruments is essential for rearing large numbers of insects for field release. Besides sorting male vs. female larvae, COPAS SELECT is a very useful tool for sorting homozygous larvae that carry a fluorescent labeled transgenes from its heterozygous and non-fluorescent siblings at early larval stages.

Introduction

The COPAS family of research instrumentation comprises a collection of large particle cytometers utilizing at least 1 excitation laser and up to 8 channels of fluorescence collection. Unique to COPAS instrumentation is the Profiling feature which graphically plots the fluorescence intensity changes along the length of the object as it passes through the laser(s). Large objects up to 1.5mm in diameter can be analyzed for physical and fluorescence characteristics and gently dispensed into a multi-well plate or other collection container for further investigation or reuse. The COPAS Vision also has equipped a camera to take an image of the object inside the flow channel. This image accompanies the cytometry data and can be analyzed using Union Biometrica's FlowPilot software or other image analysis tools. In this experiment, the COPAS Select utilizing a 500 micron flow channel was used to analyze and sort mosquito males from females.

Malaria

Malaria is a vector-borne, infectious disease, which is caused by single-cell protozoan parasites of the genus *Plasmodium*. *Anopheles sp.* acts as the vector for malaria. Bites of the female mosquitoes transmit *Plasmodium* to humans. Malaria is widespread in tropical and sub-tropical regions. According to the Robert-Koch Institute, Berlin, Germany, 1.5 to 2.7 million humans die from malaria every year, half of them are children below the age of five years. Each year, approx. 300 to 500 million people get newly infected with malaria. Malaria causes symptoms like high, periodic fever, chills, anemia and flu-like illnesses, in severe cases coma and death. Currently there is no vaccine available. Preventive drugs must be taken to reduce the risk of infections. Malaria can be treated with anti-malaria drugs. Prophylactic and anti-malaria drugs are often too expense for people that live in endemic areas. Additionally, malaria treatment is hampered by the fact that malaria parasites rapidly develop resistance to new drugs.

Vector control

A method of eradication of malaria is by controlling its vector, the mosquito *Anopheles sp*. There are different strategies for mosquito control:

- Control of its habitat, which includes avoidance of stagnant waters and better management of waste water
- Control by insecticides
- Control by larvicides
- Introduction/replacement with malaria-refractory mosquitoes
- Introduction of sterilized male mosquitoes in order to reduce vector breeding rate (Sterile Insect technique (SIT))

SIT has already demonstrated its efficiency in the eradication of the New World screwworm *Cochliomyia hominivorax*. SIT is dependent on the release of large numbers of laboratory produced sterile male-only mosquitoes. The release of female mosquitoes must be avoided as they would contribute to the transmission of the disease. It has been shown earlier that 3rd instar male mosquito larvae can be identified by the expression of GFP in the gonads from a male-specific promoter (Catteruccia et al., 2005). Males can be efficiently and quickly separated from females at the 3rd instar larvae stage using the COPAS XL for automated sorting (Catteruccia et al. 2005).

Materials and Methods

Anopheles gambiae and *Anopheles stephensi* larvae were analyzed and sorted one to two days after hatching (1st instar larvae). Sample was passed through a mesh and cleaned from eggs, egg shells food particles and other debris before larvae were introduced to the COPAS SELECT sample cup. De-mineralized water served as sample and sheath solution. Sample concentration was set to approximately 500 larvae per ml.

Results

Sorting male vs. female larvae

The *A. gambiae* strain 7ax carries a DsRed insertion on the X-chromosome. At this developmental stage no morphological difference is visible between male and female larvae. It was expected that female larvae (XX, homozygote) will express higher levels of DsRed protein than male (XY, heterozygote) larvae. At this developmental stage, it is not possible to observe the expression intensity differences by fluorescent microscope. Larvae were analyzed by size (Time of flight, Tof) and optical density (Extinction, Ext) (Figure 1A) and by red and yellow fluorescent emission (Figure 1B). The parameters Tof vs. Ext were used to separate 1st instar larvae (Gate 1, Figure 1A) from debris. The parameters Red vs. Yellow were used to detect fluorescent emission from the DsRed transgene. Two populations are visible when red fluorescent emission intensities were analyzed: One population with lower (Gate 2, Figure 1B) and one population with higher (Gate 3, Figure 1B) red emission intensities. Larvae from both populations were sorted and allowed to develop to adulthood. As expected, all larvae that appeared less red in the dot plot were male; all larvae with a higher red intensity were female.

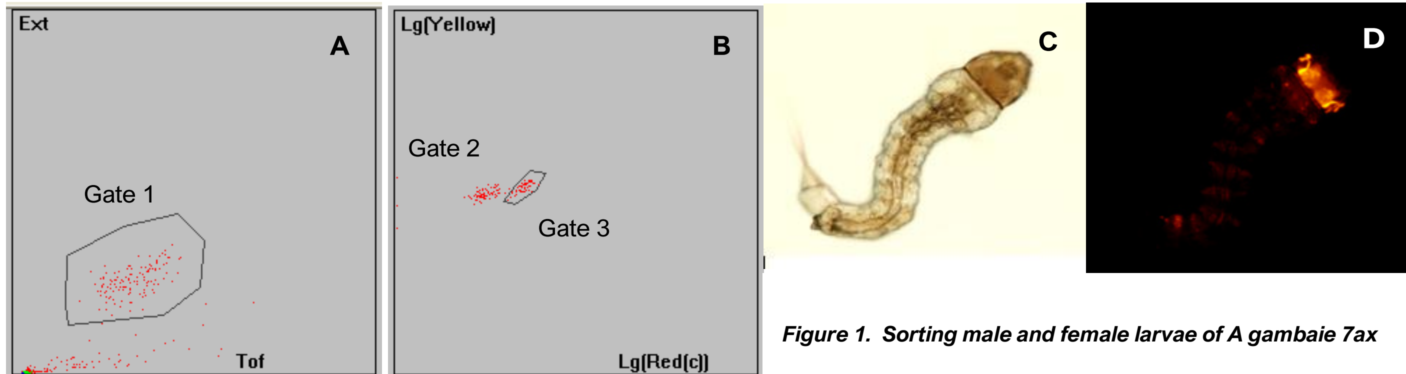

Figure 1. Sorting male and female larvae of A gambaie 7ax

Dot plots of the analysis of A. gambiae 7ax 1st instar larvae that carry a DsRed insertion on the X-chromosome. Larvae were analyzed by size (Time of flight, Tof) and optical density (Extinction, Ext). Larvae were gated in order to discriminate larvae from debris (Gate 1, A). Gate 2 encircles the less red population that corresponds to male larvae (B). Gate 3 marks the population with high red fluorescent emission intensities that correspond to female larvae (B). Each red dot represents one analyzed event. Microscopy pictures (C) showing a transgenic 7aX larva (left panel, bright field; right panel, red fluorescence).

Establishing transgenic strains

In this experiment 1st instar larvae of *A. gambiae* DG1 were analyzed and sorted. *A gambiae* DG1 carries one DsRed transgene. First, heterozygous transgenic mosquitoes were backcrossed to wild-type mosquitoes in order to amplify transgenic mosquitoes. The results of this cross are larvae that are heterozygous for the DsRed transgene and larvae that do not carry the transgene (ratio 1:1). DsRed heterozygous larvae were sorted using COPAS SELECT (Figure 2A, B) and crossed with themselves. The outcome of this cross is larvae that are homozygous for the transgene, heterozygous for the transgene or negative for the transgene (ratio 1:2:1). Larvae with highest DsRed expression were sorted in order to establish a homozygous stock (Figure 3 A, B).

For the first experiment (Figure 2A, B), larvae were analyzed by size (Tof) and optical density (Ext) (Figure 2A) and by red and yellow fluorescent emission (Figure 2B). 1st instar larvae were discriminated from debris (Gate 1, Figure 2A). The parameters Red vs. Yellow were used to detect fluorescent emission from DsRed transgene. The dot plot shows

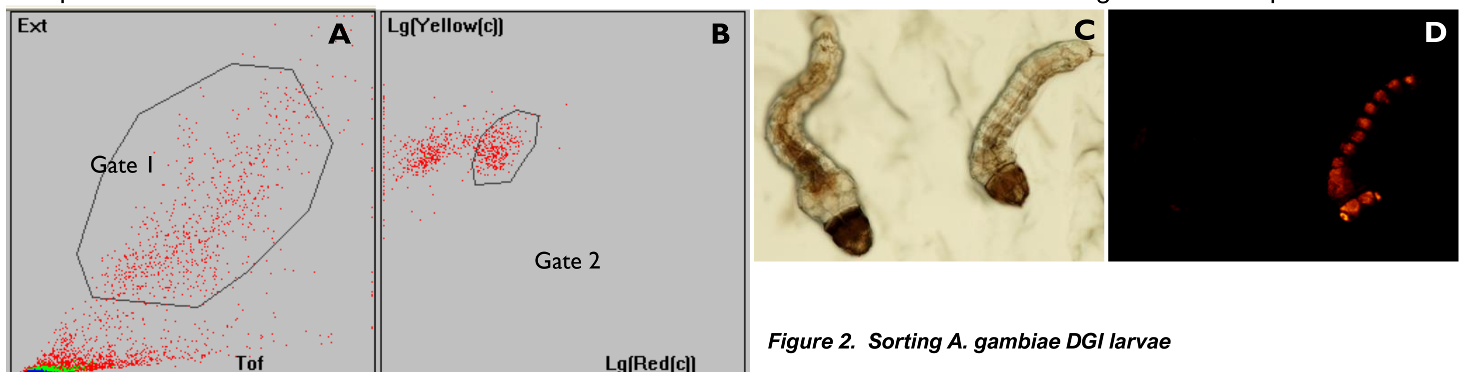

Figure 2. Sorting A. gambiae DGI larvae

Dot plots of the analysis of A. gambiae DG1 1st instar larvae carrying a DsRed insertion. Two sorting experiments were preformed: In the first experiment (A, B), DsRed heterozygous larvae were separated from dsRed negative larvae. Larvae were analyzed by size (Time of flight, Tof) and optical density (Extinction, Ext). Larvae were gated in order to discriminate larvae from debris (Gate 1, A). Larvae with strongest DsRed expression were sorted (Gate 2, B). Microscopy pictures (C) showing a non-fluorescent, wild-type larva next to a transgenic DG1 larva (left panel, bright field; right panel, red fluorescence)

two populations: one with low fluorescent emission (auto-fluorescence) and one with high fluorescent emission. Larvae with high red emission were sorted (Gate 2, Figure 2B). Larvae were grown to adulthood and crossed with themselves. Progeny of this cross were used for second experiment.

In the second experiment, larvae were again analyzed by size (Tof) and optical density (Ext) (Figure 3A) and by red and yellow fluorescent emission (Figure 3B). 1st instar larvae were discriminated from debris (Gate 1, Figure 3A). The analysis of DsRed expression showed three distinct populations in the dot plot (Figure 3B), corresponding to DsRed homozygous negative larvae (most left, Figure 3B), DsRed heterozygous (middle, Figure 3B) and DsRed homozygous positive larvae (Gate 2, Figure 3B). DsRed homozygous positive larvae were sorted fast and precisely to establish homozygous stock.

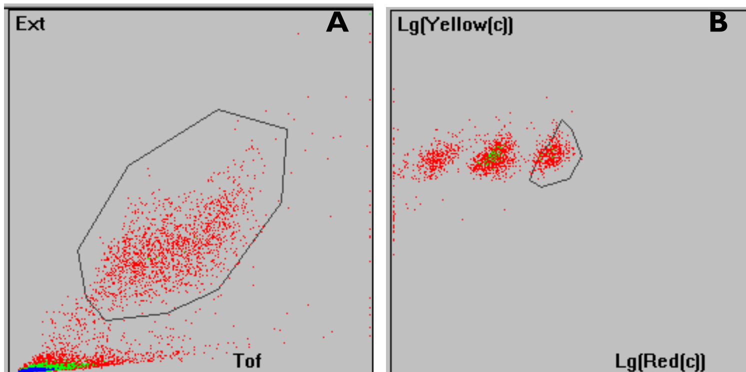

Figure 3. Sorting A gambiae DGI larvae

In a second experiment, the progeny of the first experiment, described in Figure 2, were analyzed and DsRed homozygous larvae were sorted. Larvae were analyzed by size (Time of flight, Tof) and optical density (Extinction, Ext). Larvae were gated in order to discriminate larvae from debris (Gate 1, Figure 3A). Larvae with strongest DsRed expression were sorted (Gate 2, Figure 3B).

Similarly, sorting transgenic *A. stephensi* larvae revealed that the transgene insertion was on the X chromosome and allowed to separate males and females. Therefore, the technique is applicable to several mosquito species.

Conclusions

These experiments clearly demonstrate that COPAS SELECT is suitable to sort A. gambiae and *A. stephensi* 1st instar larvae fast and accurate. It is a reliable and easy-to-use technique to sort young mosquito larvae. It does not affect viability of larvae. The COPAS SELECT provides possibilities that cannot be done using manual techniques: Sorting is much faster than any kind of manual sort. Larvae are sorted at an analysis rate of 10-20 larvae per second. Additionally, fluorescence intensities can be clearly quantified. Therefore, COPAS SELECT is an essential tool for sorting huge numbers of male larvae for future SIT field releases. Furthermore, this report shows that COPAS SELECT is much more sensitive than the human eye. Using the COPAS SELECT it is possible to differentiate between populations that express different levels of a DsRed transgene, a difference that is not visible by microscopic observation.

Work performed in 2004, QTN reissued 2024:

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References:

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