

Drosophila Fluorescent-Imaginal disc Analysis and sorting by COPAS Flow Cytometry Technique

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Introduction

The COPAS™ family of instrumentation comprises a collection of large particle cytometers utilizing at least 1 excitation laser and up to 8 channels of fluorescence collection. Large objects can be analyzed for size, density, and fluorescence characteristics before being gently dispensed into a multi-well plate or other collection container for further investigation or reuse. In this experiment, COPAS SELECT and EXPRESS flow cytometry instruments (500µm flow channel) were able to analyze and sort *Drosophila* embryos on the bases of size and fluorescence signals. Another potential application of interest is to use the COPAS systems to reliably analyze and sort fluorescent tissues from *Drosophila* larvae. For example, the ability to isolate imaginal discs would be beneficial. Currently, this is most often done manually and is a tedious task. We have set out to test whether we can create a simpler way of harvesting a relatively pure sample of fluorescent discs. We have tested two protocols for disrupting larvae for the extraction of intact imaginal discs. We have used the samples prepared by these methods and analyzed them using COPAS flow analysis instruments to accurately isolate relatively pure populations of imaginal discs.

Sample Preparation

Sample preparation using Douncer and Pestle: All preparations were carried out on ice and with solution at 4°C. Third instar larvae were washed from culture bottles (100ml culture flasks) with 10% HaCl. The larvae were transferred onto a 600µm mesh and washed thoroughly with waters, the BSS (buffered salt solution). The larvae were resuspended in 30ml BSS and split into two 50ml Falcon tubes. The larvae from the first tube were ground in the dounce tube with the pestle for 2-3 minutes. The grindate (disrupted larval tissues) was filtered over a 350 µm mesh into a large beaker on ice. The larvae from the second tube were then processed in the same manner and two samples pooled. At each step, a forceful stream of cold BSS was used to wash the sample, forcing small larvae parts (discs) through the mesh.

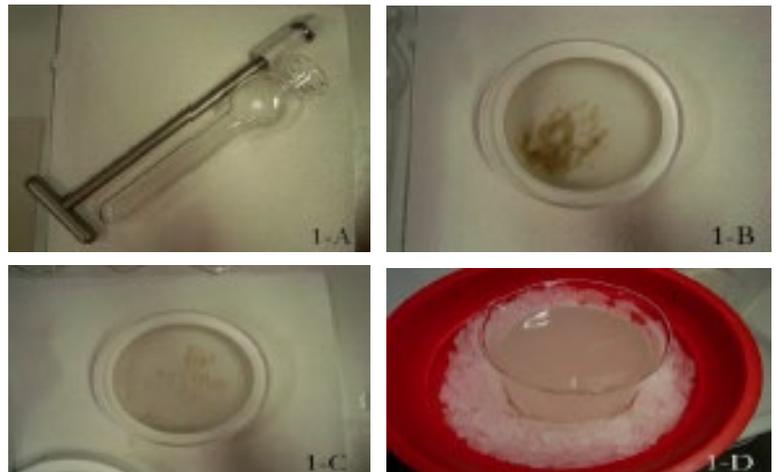


Figure 1: Tools used to carry out preparation using dounce tube and pestle.

Since the samples were still turbid, half of the sample was further cleaned by the following series of washing and settling steps: 120ml of the larval tissue sample was diluted to 1 liter and allowed to settle on ice for 20 minutes. The liquid was aspirated to 500ml and made up again to 1 liter with BSS. The sample was stirred and allowed to settle for 15 minutes. The sample was aspirated to 200ml, transferred to a 600ml beaker, and made up to 500ml. The sample was allowed to settle for 10 minutes. Sample was aspirated to 100ml, transferred to a 250ml beaker, and made up to 200ml. The sample was allowed to settle for 10 minutes. The sample was aspirated to 50ml and brought to 200ml with BSS, settled again for 10 minutes. Finally, the samples were aspirated to 30ml.



Figure 2: Tools used to carry our preparations using chopper or pasta machine.

Both Samples were then analyzed on the COPAS EXPRESS instrument.

Sample Preparation Using “Chopping” and “Pasta” Machine: For this test of sorting imaginal discs prepared by a combination of chopping and a “pasta machine”, the strain we used contained GFP-labeled eye discs (SoGFP # 46, 2nd chromosome and SoGFP # 43, 3rd chromosome, provided by Lydia Michaut). Third instar larvae were collected on a 279µm screen to wash away food and other debris. The larvae were then

transferred using cold Ringer’s to a Petri dish. The sample was cleaned of further contaminants (pupae or carcasses) and the larvae were roughly sliced, with either a razor or “chopper machine”, to break open the cuticle. The broken larvae were then processed twice through the pasta machine on a very fine setting (using a feeler gauge the separation between rollers was between about 0.152 mm to 0.254 mm) to separate the internal tissues from the cuticle. The Wolfner pasta machine was provided by Steve Russell, University of Cambridge, UK. This sample was collected in a beaker and filtered, in sequence, first through a 279µm and then through a 198µm mesh. At each step, a forceful stream of cold Ringer’s was used to wash the sample forcing small larvae parts (discs) through the mesh. The tissue containing flow-through was then collected on 70µm cell strainers and transferred to a Ringer’s soaked 50 ml falcon tube. Using cold Ringer’s the volume was brought up to 40 ml and the disc tissues were allowed to settle at the bottom for twenty minutes at 4°C. The top portion was removed and discarded and once or two more times the volume was brought up and the discs were allowed to settle. After the final removal of floating debris, the sample was ready for sorting and analysis by the COPAS SELECT (500µm flow channel).

Purification of fluorescent imaginal discs on the COPAS SELECT was achieved using a two step procedure – first, enrichment for the fluorescent discs by setting the sort trigger on green fluorescence and a subsequent sort using extinction as the sort trigger to separate disc from non-disc material.

Results

The results from the samples prepared using the douncer and pestle are as follows: The percentage of positive selected events in the first sample was 29% at a speed of processing 1-3 events per second. Sort results showed that 30% of the events were labeled discs. Number of discs and contaminating objects collected were determined using a stereomicroscope. The low purity is due to the fact that fluorescent parts of gut and salivary gland are also present in this strain. In the second sample, the concentration of positive events had increased to 32%. A recovery loss of almost

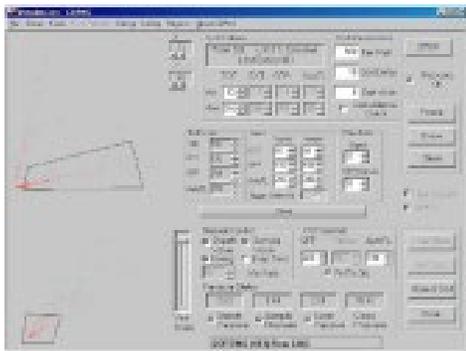


Figure 3: Screen Capture of COPAS software screen showing results of first sample run



Figure 4: The imaginal discs remain intact as shown in the image of a wing-disc after collection. The color is inverted since the GFP on the black background is difficult to see. The darker right side is GFP positive.



Figure 5: Image of imaginal disc from SoGFP #46 expressing GFP

50% of positive events by the settling steps was calculated based on the comparison of the two samples. However, from this sample further purified by a series of settling steps, 80% of the collected events were discs. This indicated that the settling steps eliminated most of the contaminating events sorted in the first sample. The results from the samples prepared using the “Chopping” and “Pasta” Machine are as follows: We performed seven tests involving the collection of 100 objects per run. The number of discs and number of contaminating objects per 100 objects collected were determined using a stereo-microscope. Total number of discs was 554 or about 79% purity (544/700). There was a total of 92 discs counted in the waste container after these sorts. Taken together, 646 imaginal discs passed through the flow cell from the prepared sample contain imaginal discs. This indicated that about 85% of all the imaginal discs

were sorted (554/646). From the original two preparations we estimate that about 12% of all discs were recovered. In two experiments that started with 6000 embryos (two eye discs per embryo or a total of 12,000 discs), we collected 1869 fluorescent objects. We estimate that 79% of these sorted objects are eye discs.

Conclusions:

Using the COPAS SELECT or EXPRESS (500 μ m flow channel) for analysis and sorting on the basis of fluorescence allows for greatly enriched fluorescent imaginal disc preparation from a crude tissue sample from *Drosophila* larva. In one experiment using a dounce tube and pestle, the yield of the sorting was 11% of the total events. The strain

(engel4;UAS::GFPgpi, provided by Daniela Panáková) expressed GFP in the posterior half of 3 pairs of imaginal discs, part of the gut and salivary gland. The purity of the collected events depended on the sample treatment and reached a level of about 80%. The purity and yield were affected by the presence of fluorescent body parts (gut and salivary gland) other than the GFP-fluorescent imaginal discs. Using a separate protocol, we also tested a strain containing labeled eye discs (SoGFP #46, 2nd chromosome and SOGFP #43, 3rd chromosome, provided by Lydia Michaut). The mass isolation of the discs in this experiment was done with a combination of a chopping machine and the Wolfner “pasta-machine” (provided by Steve Russell, University of Cambridge, UK). We used a two-step purification of the fluorescent discs with the COPAS SELECT. First an enrichment, then a purification. The recovery of the imaginal discs from the larval preparation was estimated to be about 12% (essentially the same as the experiment using the dounce and pestle). Following a two-step purification method, the purified samples were determined to be 79% imaginal disc. This data shows that the COPAS instrument can be used for sample preparation of fluorescent imaginal discs from *Drosophila*.

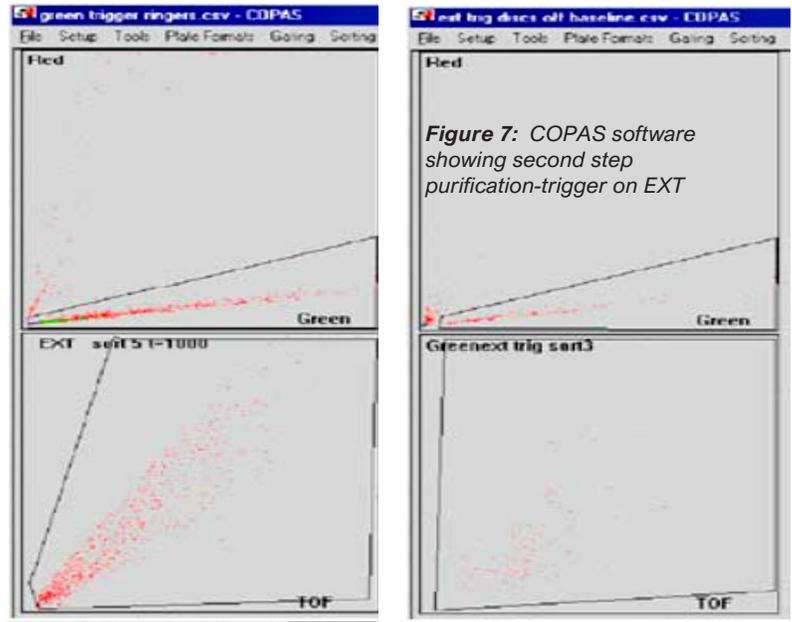


Figure 7: COPAS software showing second step purification-trigger on EXT