SEX-SPECIFIC GFP-EXPRESSION IN *DROSOPHILA* EMBRYOS AND SORTING BY COPAS™ FLOW CYTOMETRY TECHNIQUE

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INTRODUCTION

COPAS Select and Express flow cytometry instruments are able to analyze and sort *Drosophila* embryos on the basis of size and fluorescence signals. One application of this technology is to sort embryos that have sex-specific GFP-expression. The benefit of this technique is that males and females can be separated from each other at the embryonic stage and thereby simplify the isolation of virgin females for subsequent genetic crosses. Furthermore, this allows for the collection of embryonic samples for biochemical studies of early sex-specific differences, at a stage before morphological differences between the males and females become evident. Sex-specific expression occurs from certain promoters as a result of the assessment of the X chromosome to autosome ratio by mechanisms not completely understood. However, this regulation could be exploited for the process of distinguishing males from females at an early developmental stage. We tested two different transgenic *Drosophila* strains that carried EGFP under the control of *Sxl* P_E promoter. One strain has the transgene integrated on the X-chromosome and one has the integration on Chromosome 3. We show that using either of these strains it is possible to sort embryos on the basis of green fluorescence and separate males from females.

METHODS

Expression vector and transgenics

A construct containing the *Sxl* P_E and the coding sequence for EGFP was designed so that the X-chromosome to autosome ratio would regulate transcription and translation of GFP. The *Sxl* P_E is active only in fly embryos that have a 1:1 ratio between X-chromosomes and autosomes. Consequently, female embryos containing the *Sxl* P_E/EGFP fusion construct exhibit a green fluorescence, while male embryos do not. To create this construct, EGFP was cloned into a vector containing the *Sxl* P_E and this fusion product was subsequently cloned into the CaSpeR vector for injection into *Drosophila* embryos using standard transformation methods (Graham and Schedl, personal communication). Two transgenic lines were used in our analysis of sexing embryos. One had the transgene integrated on the X-chromosome (G5b) and the other had the integration on chromosome 3 (G78b).

Embryo collection and analysis

Embryos were collected from egg-plates and were dechorionated prior to analysis on the COPAS Select. Dechorionated embryos were allowed to recover on apple juice agar plates. Embryos were inspected at various ages (hours post-egg laying) and single embryos were tracked to determine when expression begins and a time course of expression. After establishing the most appropriate time for analysis, we then separated fluorescent and non-fluorescent embryos from each other using the COPAS Select sorter. We analyzed these separate populations with a dissecting microscope to determine whether the fluorescent population was indeed all fluorescent and likewise for the nonfluorescent population. Embryos with the inappropriate fluorescence (non-fluorescent embryos among the fluorescent embryos and vice versa) were noted. These collections of embryos were allowed to continue development (fluorescent separated from the non-fluorescent) to the adult stage and their sex was determined visually by microscopy.

WORK PERFORMED BY:

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To further validate the use of these *Drosophila* strains in studies of early embryonic sex differences we established egg-laying cages of adult flies and collected large numbers for biochemical analysis. These were collected and matured to an age where GFP-expression was known to occur and then sorted into separate male and female samples. The separate samples were then processed for RT-PCR with sample RNA, as well as, Western blot analysis of protein samples.

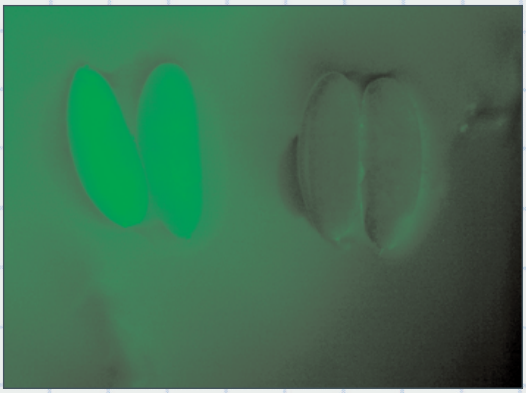

Figure 1. Dechorionated embryos from G78b (3rd chromosome) viewed by fluorescent microscopy.

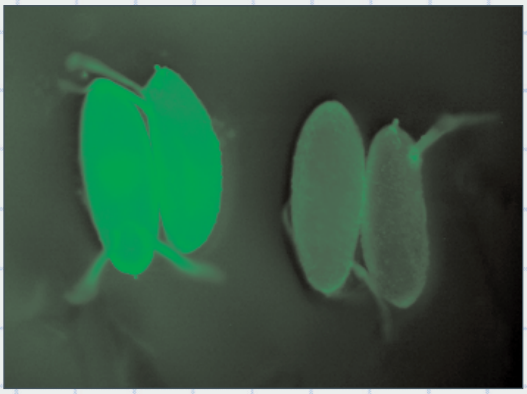

Figure 2. Embryos with intact chorions from G78b (3rd chromosome) viewed by fluorescent microscopy.

RESULTS:

We collected embryos for broad time-windows (for example, 6-22 hr. collection) and separated fluorescent from non-fluorescent. From our aging experiments we determined that our greatest accuracy for correctly separating males from females on the basis of fluorescence occurs when embryos are six hours old and on. Prior to six hours, some of the non-fluorescent embryos that are scored as male develop into fluorescent females at later stages, suggesting that the GFP reporter had not yet turned on in these embryos. Table 1 shows a summary of the data for the accuracy of separating males and females. The data indicates that the presence of fluorescence allows for the accurate collection of females, with near 100% accuracy. Likewise, the data shows that the absence of fluorescence can be used to collect the male embryos with a similar accuracy.

The purity of sorting the two sexes separately is essentially identical for both transgenic strains. Our experiences suggest that there are no obviously noticeable position effects for these two strains. However, overall there appears to be slightly higher levels of GFP expression in the strain with the integration of GFP on chromosome 3. This may reflect a mild position effect or, possibly, dosage compensation. This difference is not great enough to result in a difference in the ability to sort females from a mix of males and females. We also notice that there is a slight bias to greater numbers of non-fluorescent embryos. We believe that this bias can be accounted for by the fraction of unfertilized eggs and dead embryos, although we have not systematically addressed this question. We have determined peak fluorescence occurs between 5-8 hours. Beyond 8 hours the fluorescence begins to decrease but is still sufficient to allow distinction between GFP positive and GFP negative embryos. This difference continues for the remainder of embryonic development.

***sxl*P_E-GFP on 3**	**%M**	**%F**	**n**
GFP +	0.3%	99.7%	3846
GFP -	99.4%	0.6%	3550
***sxl*P_E-GFP on X**	**%M**	**%F**	**n**
GFP +	0.1%	99.9%	7480
GFP -	99.8%	0.2%	9500

Table 1. Sorting accuracy. Embryos from each strain were collected and allowed to mature until they were at least six hours old. They were then dechorionated and sorted into fluorescent and non-fluorescent populations. These populations were manually inspected with a fluorescent microscope and the number of misclassifications were determined.

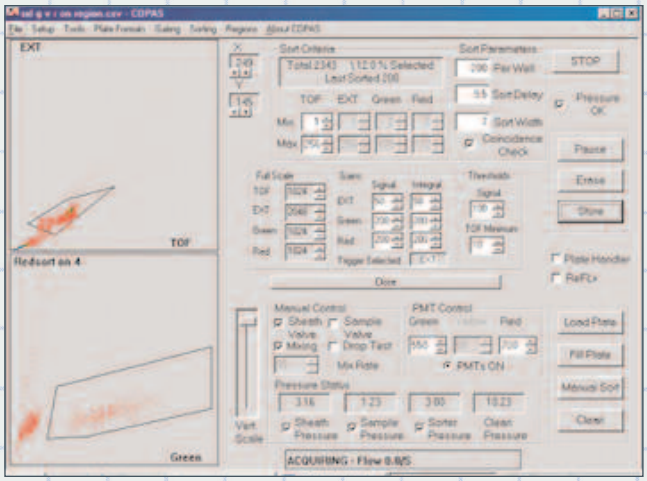

Figure 3. Sample screen capture from COPAS software for the selection and sorting of green embryos (female). These embryos express GFP from the Sxl P_E promoter.

We have recently tested embryos with intact chorions and see results that are similar to what we obtained from the dechorionated embryos. The contamination of the samples with the incorrect sex is slightly greater for chorion-intact embryos than for the dechorionated embryos. No data is presented at this time.

Samples of fluorescent and non-fluorescent embryos were collected and processed for RNA and protein. RNA samples were used in an RT-PCR reaction to identify sex-appropriate mRNA. Protein samples were also collected and analyzed by Western blotting.

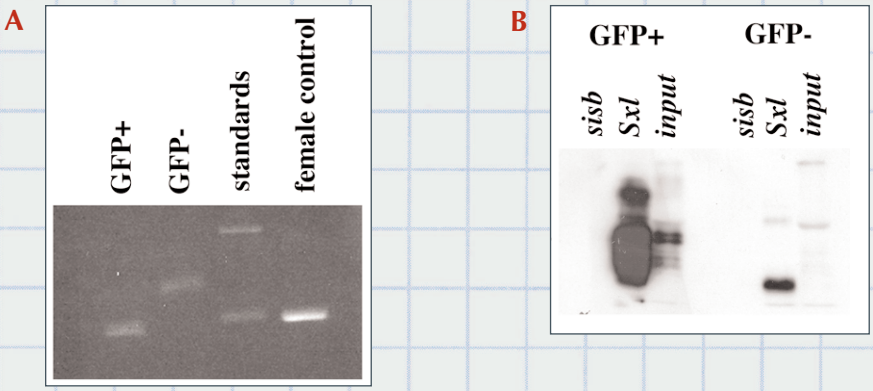

Figure 4. Sex specific mRNAs and proteins can be isolated from sorted embryos.

A) *RNA was isolated from nuclear extract made from GFP+ and GFP- embryos. Reverse transcription – PCR was performed with primers flanking the alternatively spliced region of Sxl. A female Sxl cDNA was used as a positive control for the PCR. Male transcripts contain a 180 base pair exon not found in female transcripts.*

B) *Nuclear extract from GFP+ and GFP- embryos was incubated with no beads, with anti-sisb beads or with anti-Sxl beads. The proteins isolated from each sample were analyzed on a Western blot probed with antibodies to Sxl.*

The data from RT-PCR and Western blots supported the notion that samples from embryos expressing GFP are female and those not expressing GFP are male. The GFP+ embryos contained the female specific RT-PCR product and the GFP- embryos did not. However, their RNA did yield a different, (larger) male-specific transcript from RT-PCR. Likewise, the Western blots revealed the expected sex-specific differences. GFP+ embryos express abundant levels of *Sxl*-protein while the GFP- embryos do not produce *Sxl*-protein of the expected size.

CONCLUSION

We used the COPAS Select to isolate separate populations of female and male embryos by taking advantage of a sex-specific promoter driving the expression of GFP in females but not in males. We determined that if the embryo populations are aged to about 6.0 hours, better than 99% of sorted embryos that develop to adulthood are in fact sorted correctly. Approximately one in a thousand embryos is sorted to the wrong sample. Our attempts of separating female from male embryos with intact chorions shows that these sorts are not as accurate as what we are able to achieve for the dechorionated embryos.

The COPAS Select can be used with these two transgenic strains to rapidly isolate separate populations of males and females at the embryonic stage. Samples sorted by the COPAS can be collected and grown to adult stages and the male and females can be kept separately, insuring virgin populations for mating. The separate male and female samples can also be collected and processed for biochemical analysis.

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