ANALYSIS & SORTING OF ACRIDINE ORANGE STAINED *C. ELEGANS* WITH INCREASED GERMLINE APOPTOSIS

OBJECTIVE

The objective of this experiment was to test if acridine orange stained apoptotic corpses in *C. elegans* gonad could be detected, analyzed, and positive worms could be sorted on the COPAS™ BIOSORT instrument.

INTRODUCTION

The COPAS BIOSORT instrument is able to analyze and sort large objects (40-200 microns) at a high rate (up to 50 objects per second) on the basis of the physical characteristics of size, density, and fluorescence signals. A red diode laser (670 nm) is used to measure both the size and optical density of the worms, and a multi-line (488, 514 nm) argon laser is used to excite the acridine orange fluorophore. In this experiment using the COPAS BIOSORT instrument, a mixed population of stained and wild-type *C. elegans* was analyzed and sorted. Non-fluorescent worms were used as a negative control. Once analyzed, the target population was dispensed into Petri dishes and the results were visually inspected for accuracy.

ADDITIONAL INFORMATION

Acridine orange (AO) is a commonly used dye in flow cytometry. AO differentially stains double-stranded DNA to a green fluorescence (around 520 nm) and single-stranded DNA and RNA to an orange fluorescence (around 610 nm). This metachromatic fluorescence is sensitive to DNA conformation, making it a useful probe for detecting apoptotic cells. In mammalian cell culture, apoptotic cells stained by AO show reduced green fluorescence and enhanced orange fluorescence in comparison to normal cells. This is believed to result from the breakdown of DNA to fragments that can more easily denature and bind AO as single-stranded nucleic acid (Darzynkiewicz Z, et al., 1992)

AO can also be used as a vital stain to identify apoptotic corpses in the *C. elegans* germ line (Gumienny et al, 1999). In *C. elegans* engulfed apoptotic germ cells show a much more intense green flourescence tan their living neighboring cells. The fact that screening can take place in living worms has several advantages over other staining methods. First, it is simple and rapid. Second, stained worms can be recovered and propagated, making this stain ideal for genetic screening. Third, normal morphology is maintained in the worms as no permeabilization or fixation is required.

MATERIALS AND METHODS

Acridine orange, Molecular Probes Inc, A3568

Sample 1: *C. elegans* N2 mixed population

Sample 2: *C. elegans* adult population of WT and *-ced-9(n1653)mutants* stained with AO according to protocol

Acridine orange staining protocol (courtesy of the Hengartner lab)

Use 2µl of AO (acridine orange) stock (10mg/ml, Molecular Probes Inc. A3568) per ml of M9 buffer as the staining solution.

Add 500µl of this solution to a plate of non-starved worms (20–24 post L4/Adult molt)

Rotate the plates to be sure that the solution is spread over the whole surface of the plate and is distributed evenly, and store the plates in the dark at room temperature for 1 hour (AO is light sensitive)

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Add 250µl of M9 buffer to the plate and transfer the worms to a new plate. Alternatively, if there are only a few worms, you can pick them to a new plate.

Keep in the dark to de-stain for approximately 45–60 minutes.

The worms are now ready for microscopic or COPAS analysis.

RESULTS:

Sample 1:

An unstained mixed *C. elegans* N2 population was run on the COPAS BIOSORT as a control to optimize the instrument settings. Four physical characteristics of the animals were measured: Time Of Flight (TOF), which is a length measurement; Extinction (EXT), which is a measurement of optical density; and two fluorescence parameters, FLU1 and FLU2, where FLU1 represents the green emission (515 nm) and FLU2 represents the orange emission (585 nm). Figures 1 and 2 illustrate the results of this control group.

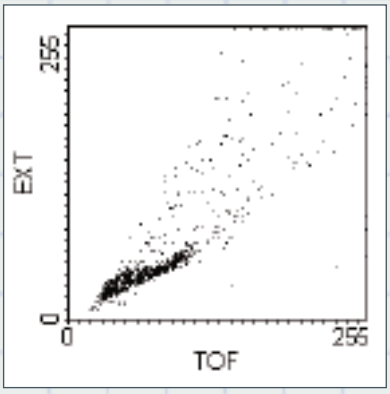

Figure 1: TOF/EXT for N2 C. elegans.

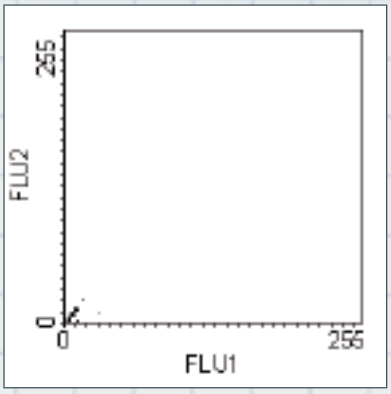

Figure 2: FLU1/FLU2 for N2 C. elegans.

Sample 2:

An adult *C.elegans* population of WT and *ced-9(n1653)* mutants stained with AO was then run on the COPAS BIOSORT to determine if the instrument can be used to detect and sort worms with increased germline apoptosis as indicated by increased AO staining. A region (R1) representing the adult worms in the sample was selected on a dot plot for sorting (Figure 3). The fluorescence properties of this gated region from Figure 3 are displayed in Figure 4. The fluorescence of this adult population ranges from near the origin of the X-Y axis, similar to the unstained wild type animals (Figure 2), to values across the 256 channels of the dot-plot.

A region (R2) representing the brightest objects (FLU1, green emission vs. FLU2, orange emission) in the sample was selected on a dot plot for sorting (Figure 4). These were dispensed and visual inspection confirmed that all sorted *C. elegans* from Region 2 had brightly AO stained apoptotic germ cell corpses.

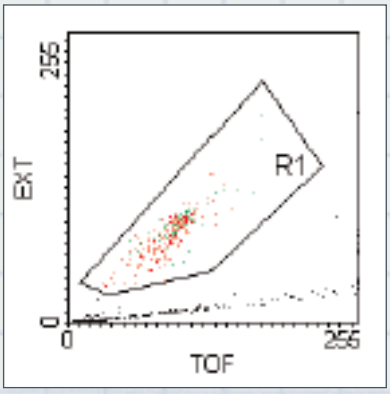

Figure 3: TOF/EXT for AO stained C. elegans.

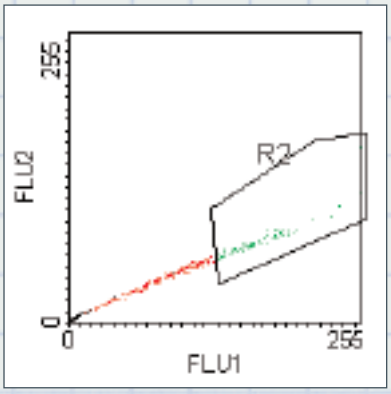

Figure 4: FLU1/FLU2 for AO stained C. elegans.

VERIFICATION UNDER A MICROSCOPE

WT

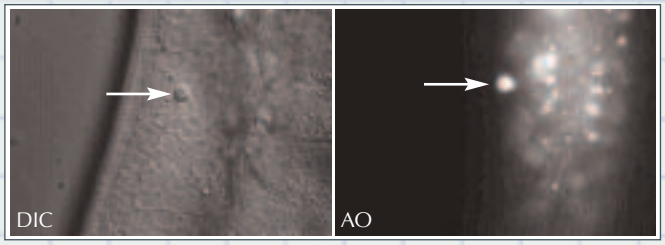

Figure 5: Wild type animal observed by differential interference confocal microscopy (DIC) and showing one apoptotic corpse (left), and the same animal stained with AO (right).

ced-9(n1653)

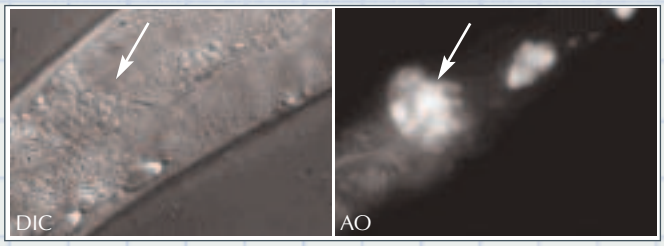

Figure 6: ced-9(n1653) adult mutants observed by DIC (left) and the same animal stained with AO (right). ced-9 provides a very strong "survival" signal and the loss of function mutation results in increased number of germline apoptosis, as observed by DIC and Nomarski.

CONCLUSION

This experiment demonstrates that the COPAS BIOSORT may be used, in the standard configuration using the 488 excitation wavelength and green and orange emission filters, to analyse and sort acridine orange stained *C. elegans* with increased germ cell apoptosis.

References:

Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F. Cytometry (1992) 13, 795-808.

Gumienny TL, Lambie E, Hartwieg E, Horvitz HR and Hengartner MO (1999) Development, Vol 126, Issue 5 1011-1022.

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