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Easy and fast detection of vacuoles in cell death committed prepupal salivary glands of *Drosophila melanogaster* by acridine orange.

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The processes of programmed cell death and its most frequent form, apoptosis, are a critical feature of the regulated development of multicellular organisms. *Drosophila* significantly contributed to the understanding of cell death by genetic screens for cell death activators (White *et al.*, 1994; Green and Beere, 2000), caspases (Kumar and Doumanis, 2000), caspase inhibitors (Hay *et al.*, 1994, 1995), and genetic basis of engulfment (Bangs *et al.*, 2000). Besides research on cell death function in the pattern formation and embryogenesis, there is increasing interest in the studies involving their fundamental role in adult tissue homeostasis and during metamorphosis.

At the onset of metamorphosis, most of the obsolete larval tissues and organs like salivary glands become committed to programmed cell death, while imaginal discs and histoblasts will undergo proliferation and differentiation into imaginal structures. Salivary glands display several features of apoptosis (Jiang *et al.*, 1997; Farka_ and Mechler, 2000), some features of autophagy (Lee and Baehrecke, 2001), and interestingly also several unambiguous signs of necrosis (Farka_ and Mechler, 2000).

In salivary glands, vacuolization of the cytoplasm precedes execution phase of a cell death program during which caspases become activated. Although these two phases can genetically be dissected as shown by Farka_ and Mechler (2000), in wild types they are tightly associated each other. We have also shown that there is a positive correlation between degree of salivary gland vacuolization and progression in number of dying cells. For these reasons, convenient and reproducible methods to monitor vacuolization can be important. Except for classical electron microscopy (Lane *et al.*, 1972; von Gaudecker, 1972; Farka_ and _u_áková, 1998), most recently used techniques to detect vacuoles in the salivary glands were antibody staining in combination with laser confocal microscopy (Farka_ and Mechler, 2000) and histological sections of LR-White (plastic)-embedded tissue (Lee and Baehrecke, 2001). In the course of the work for different purposes, it was found that staining with acridine orange can be very useful and versatile method to detect vacuoles in prepupal salivary glands.

Acridine orange, a dual-fluorescence dye that interacts with DNA and RNA by intercalation or electrostatic attractions, was successfully applied to detect also apoptosis in living *Drosophila* imaginal discs and embryos (Spreij, 1971; Abrams *et al.*, 1993). In this case acridine orange is used in the same way as classical trypan blue in dye exclusion assay to assess cell viability (Broman *et al.*, 1965; Holl, 1965; Harris, 1966; Phillips, 1973). Although this technique works to some extent also in such a polytenized tissue as salivary glands, we found that if glands are first fixed, and then stained with acridine orange, the dye will specifically be taken up by huge vacuoles that appear after pupariation. Staining of vacuoles becomes overwhelming (Figure 1b) in comparison to nuclear DNA staining which is most abundant in unvacuolized late larval period before or during secretory phase, all prior to pupariation (Figure 1a). This vacuole staining by acridine orange is not applicable to living tissue and requires fixation. As shown in Figure 2, paraformaldehyde fixation in combination with glutaraldehyde gives a better staining signal than paraformaldehyde alone.

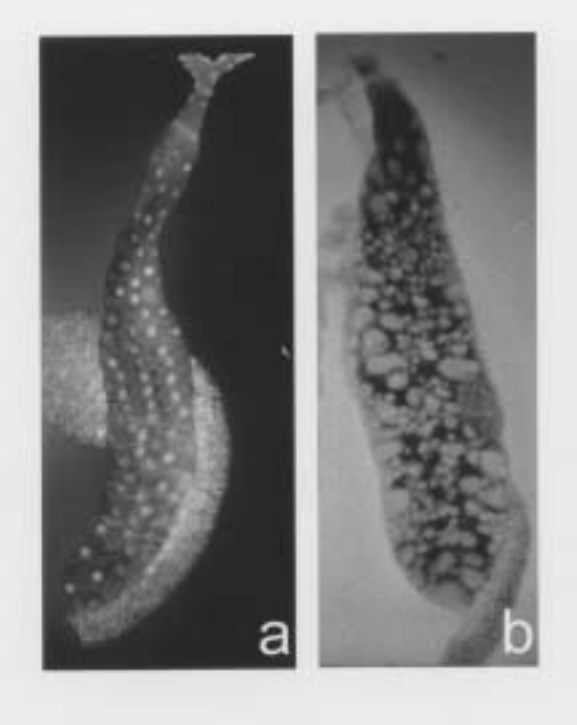

Figure 1. Staining of fixed salivary glands with acridine orange. (a) Wild type late larval salivary gland showing clear signal in nuclei. (b) Prepupal highly vacuolized salivary gland with strong acridine orange staining in these vacuoles.

Upon dissection in Ringers or PBS, salivary glands are fixed for 5 - 10 minutes in 4% paraformaldehyde or 4% paraformaldehyde + 2% glutaraldehyde in PBS or in 100 mM Pipes pH 7.2, rinsed three times for 5 minutes, and then stained in 8 μM acridine orange in PBS for 2-3 minutes. To remove extensive dye, tissue was rinsed with PBS intensely for 2 minutes, immediately mounted, and viewed under epifluorescent light with fluorescein filter. Other fluorescent filters allow examination as well, but the signal from acridine orange positive areas under UV or rhodamine illumination produces much less intensity.

In contrast to the use of acridine orange for cell death detection in unfixed tissues, which is based on its free penetration through dead membranes, there is no straightforward explanation for strong and specific uptake of this dye by vacuoles in fixed material. One can speculate that it can be due to differences in pH of vacuoles in contrast to surrounding cytoplasm. In this case it would be logical to expect highly acidic environment in the vacuoles, which are made quickly after secretion of glue granules as a consequence of apical membrane recycling. It was found that acridine orange can be used as a cell-permeant probe for yeast, *Paramecium*, or mammalian acidic vacuoles (Allen and Fok, 1983; Orci *et al.*, 1987; Anderson and Orci, 1988; Baintner, 1994). In yeasts or mammals, acridine orange can be detected in acidic organelles of living cells, but we failed to obtain any positive signal from unfixed highly vacuolized salivary glands of *Drosophila*, even under slightly acidic conditions of culture media. Similarly, application of LysoTracker or other commercially available lyso sensor dyes (Molecular Probes, Inc.) failed to co-localize the acridine orange signal within these vacuoles. Thus, it appears that if highly acidic environment of vacuoles is the reason for strong acridine orange staining, then fixation might be a necessary prerequisite to preserve or make this environment attractive for dye binding. This result also means that acidic vacuoles, which are mostly 2 - 10 μm in diameter, are not identical with lysosomes. On the contrary, numerous, albeit smaller, autophagic vacuoles within pupal fat body are not stained by this technique, altough they intensely pick up acridine orange upon vital staining, especially if stained under acidic pH conditions. Nevertheless, acridine orange staining of fixed salivary gland vacuoles due to its simplicity and versatility can be used as practical tool to monitor and study vacuolization and acidification processes asso-ciated with programmed cell death.

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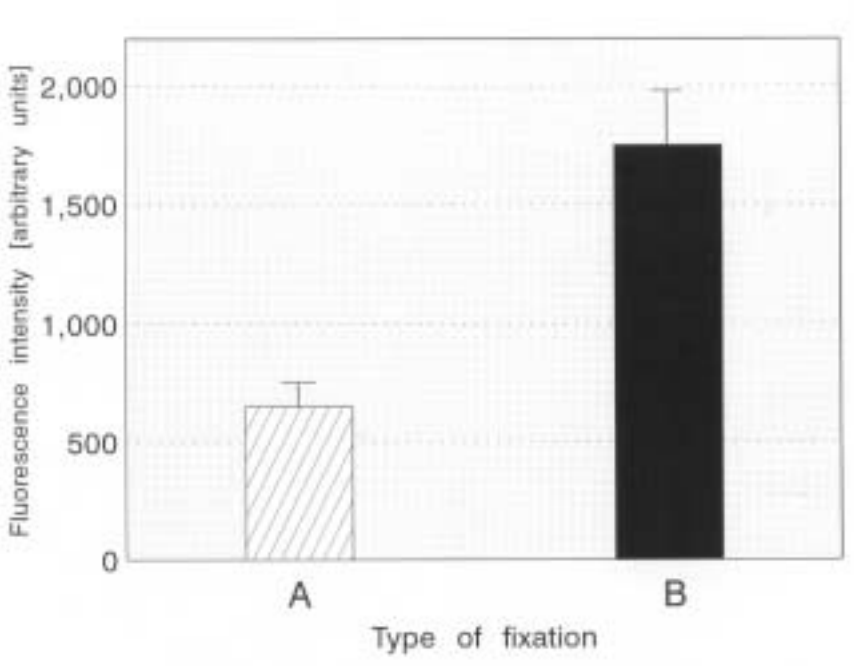

Figure 2. Graphical comparison of fluorescence intensity obtained by integrated CCD camera from acridine orange-stained vacuoles of paraformaldehyde (a) and paraformaldehyde plus glutaraldehyde fixed prepupal salivary glands. (b) presence of glutaraldehyde apparently helps to increase fluorescence signal from vacuoles.