A simple whole mount technique for looking at *Tribolium* embryos

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Introduction, Results and Discussion

Tribolium castaneum is becoming an important model organism for comparative investigations in insect development. In addition to gene expression studies, the species is especially well suited for genetic analysis of development (Beeman et al. 1993; Sulston and Anderson 1996; Maderspacher et al. 1998). The cuticles of first instar larvae can be studied like in *Drosophila*. However, often it is necessary to scrutinize earlier stages of development in order to correctly interpret mutant phenotypes. *Tribolium* eggs contain large spherical yolk granules of high optic density. This severely hinders the microscopic analysis of whole mount specimen, because the granules diffract light such that one cannot focus through the whole depth of an egg or embryo. Therefore, morphological details of internal organs only can be visualized after sectioning, or after the yolk has been manually removed from the embryo proper. Both methods are impractical for genetic analysis where only a minority of embryos in an egg-lay expresses the mutant phenotype.

We found that embedding of *Tribolium* embryos in "benz mix", a mixture of benzyl benzoate and benzyl alcohol (Westerfield 1993), makes the yolk sufficiently transparent to allow whole mount analysis (see Fig.1). Depending on the fixation method, the optimal composition of this mixture varies between 3.0:1 to 4.3:1 (we usually use 4:1). This embedding medium can be utilized for embryos stained by immuno-histochemistry. Because the embryonic tissues become fully transparent as well, a general tissue stain is necessary for visualization of all cells. Since fluorescent dyes that intercalate into DNA (Shippy et al. 1997) are washed out by the hydrophobic benz mix, we use a modified alcoholic fuchsin staining (Kiernan 1990). This method gives more intense staining than aqueous fuchsin staining or the classical Feulgen procedure and can be applied either alone or after immuno-histochemical staining. In brief, the procedure consists of (1)˚washing flour off the eggs with bleach, (2)˚fixation, (3) cracking vitellin membranes by osmotic shock, (4) alcoholic fuchsin staining and (5) embedding.

For the alcoholic fuchsin staining it is not necessary to completely remove the vitellin membranes from the embryos, which is difficult to achieve for embryos in later developmental stages, when the serosa has become attached to the vitellin membrane. Also in these older embryos, osmotic shock with methanol usually ruptures the egg case ventrally along the amnion cavity, and this slit is sufficient for penetration of staining and embedding solutions. Upon completion of the procedure, preparations can be permanently stored in standard 1.5 ml eppendorf vials, from where they are transferred to depression slides for inspection with the stereo microscope. Alternatively, they can be mounted on standard microscope slides, either in benz mix solution or in a modified medium which is made by dissolution of 9 g powdered Canada balsam in 5 ml of benz mix solution.

Materials and Methods

Fixation solution A: 3 ml PBS + 0,45 ml 37% formaldehyde + 5 ml heptane (this amount is for one batch of embryos; prepare fresh, and shake vigorously before addition of embryos to saturate the heptane phase with fixative). **Fixation solution B**: 4 ml 95% ethanol + 0,5 ml 100% acetic acid + 0,2 ml 37% formaldehyde. **Alcoholic fuchsin solution**: 100 mg pararosaniline (C.I. 42500, Sigma P-7632; there may be batch to batch variation in quality) + 16 ml 100% ethanol + 4 ml destilled water. This solution can be kept at -20¡C. Immediately before use, add 0,2 ml of concentrated hydrochloric acid. **Benz mix:** 4:1 mixture of benzyl benzoate and benzyl alcohol.

Flour removal, fixation and osmotic shock:
Wash eggs twice for 4 minutes in commercial bleach (Chlorox). Wash thoroughly with water and transfer to a glass vial with fixation solution A. After 45 minutes, remove the lower phase with a Pasteur pipette. Add 10 ml of methanol and shake vigorously (osmotic shock). Remove residual heptane and add another 5 ml of methanol such that all embryos sink to the bottom. Transfer embryos to an eppendorf tube using a cut-off blue eppendorf tip. To remove all remnants of heptane, wash three times with methanol. Embryos now can be kept at -20¡C, or used immediately.

Alcoholic fuchsin staining:
Fix embryos again for 1 h in 1 ml of solution B (room temperature) and then wash four times (10 minutes each) with 70% ethanol (roll on wheel). Incubate for 10 minutes in 2N HCl in a 60¡C water bath to hydrolyze RNAs and to modify DNA for Feulgen reaction. Wash once with water and twice with 70% ethanol. Remove supernatant, add 1 ml of alcoholic fuchsin solution and incubate for 30 minutes (roll on wheel). Wash repeatedly in 95% ethanol until no more red color is released by the embryos. Wash two times in 100% ethanol to dehydrate.

Embedding:
Wash once with a 1:1 mixture of benz mix and ethanol. Let embryos settle down, remove supernatant, and add 1 ml benz mix. Mix by rolling and store in the dark at room temperature.

References

Beeman RW, Stuart JJ, Brown SJ, Denell RE (1993) Structure and Function of the Homeotic Gene Complex (HOM-C) in the Beetle, Tribolium-Castaneum. Bioessays 15: 439-444

Kiernan J. (1990) Histological & Histochemical Methods: Theory & Practice. 2nd edition, Pergamon Press, p. 128-129

Maderspacher F, Bucher G, Klingler M (1998) Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*. Dev Genes Evol, in press

Shippy T, Brown SJ, Denell RE (1996) Confocal imaging of *Tribolium castaneum* embryos. Tribolium Information Bulletin 36, 80-82

Sulston IA, Anderson KV (1996) Embryonic patterning mutants in *Tribolium castaneum*. Development 122: 805-814

Westerfield M (1993) The zebrafish book. Eugene, Univ. of Oregon Press

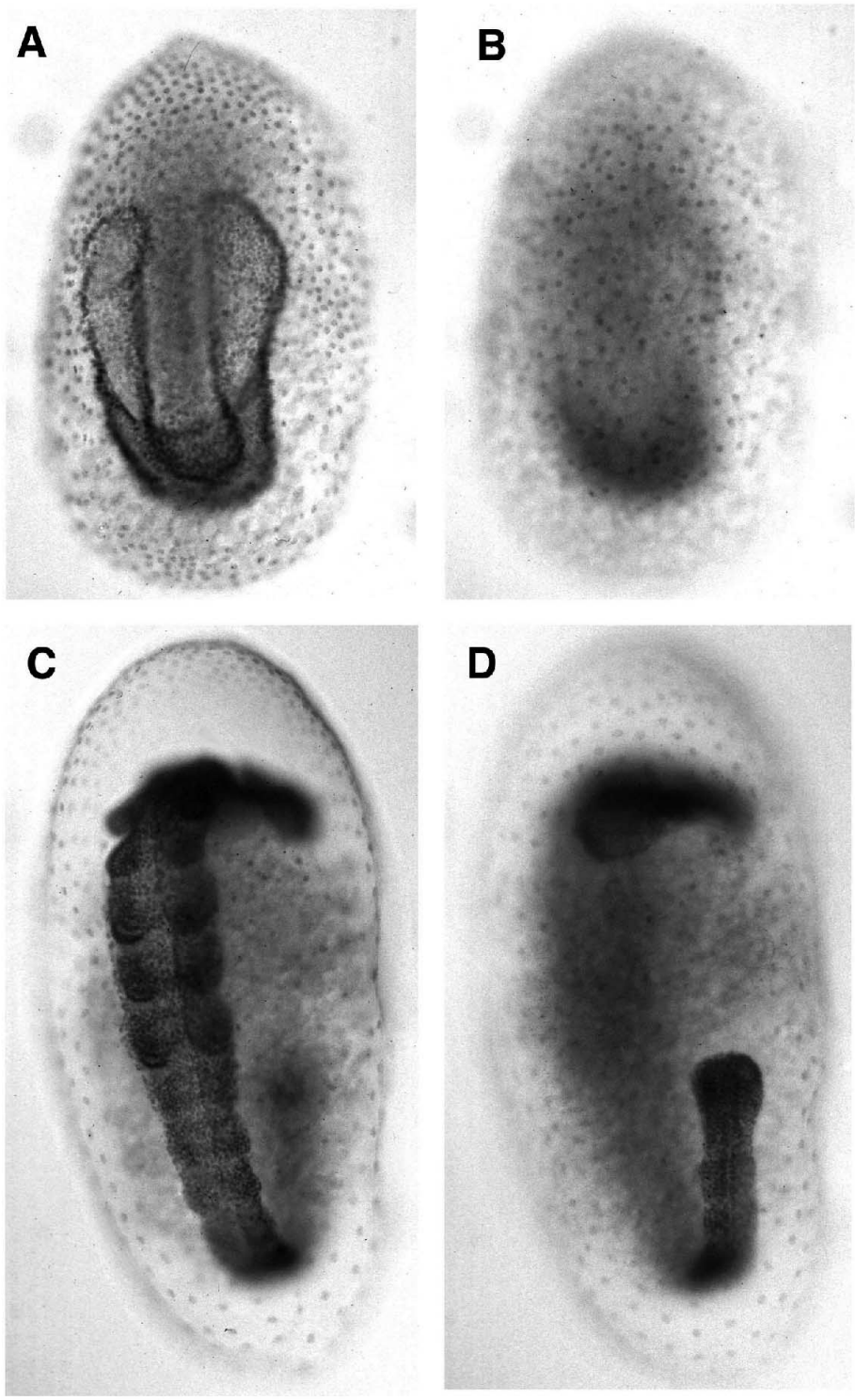

Fig. 1.
Tribolium embryos after alcoholic fuchsin staining, embedded in benz mix. (A, B) and (C, D) each are two focal planes of the same embryos. Embryos are oriented with anterior up. (A) Focus on the germ rudiment of an gastrulating embryo. Serosal cells are visible around the embryo proper. (B) Same embryo as in A, with focus on the serosa cells at the dorsal side of the egg. (C) Ventral view of an embryo near completion of germ band growth. Thoracic segments with developing appendages are in focus. (D) Same embryo as in C with focus on the terminal segments (lower part) and head lobes.