Elevated levels of fluorescence were detected in *w; TH-GAL4/UAS-lacZ* brains and seems to be due to the expression of *TH-GAL4*. The average greyscale value and standard error of the *w; TH-GAL4/UAS-lacZ* brains was much higher (51.6948 ± 3.4347) than that of the control (36.7924 ± 3.1791). To compare, *w; TH-GAL4/UAS-p35* brains exhibited lower greyscale values (36.4599 ± 4.4692) than that of the *w; TH-GAL4/UAS-lacZ* brains. A contrast test was conducted in order to determine if there existed a significant difference between the fluorescence levels of both groups. Since the p-value obtained (0.0072) was much lower than the adjusted α, it can be concluded that *p35* reduces that amount of *GAL4*-induced cell death occurring the brains of flies possessing the *TH-GAL4* gene.

The differences between the *w; TH-GAL4/UAS-lacZ* and *w; TH-GAL4/UAS-p35* fly brains demonstrate the effectiveness of this protocol. The comparison between *TH-GAL4* homozygotes and *w; TH-GAL4/UAS-lacZ* flies ($p = 0.9961$) suggest that *TH-GAL4*-induced apoptosis may not increase in a dosage-dependent manner. As expected, the *lacZ* gene did not cause an increase or decrease in the amount of fluorescence detected in brains of flies. The reduction in fluorescence observed in the brains of flies expressing the anti-apoptotic *p35*gene can be attributed to caspase inhibition. In summary, the protocol proposed in this study appears to be effective at detecting levels of apoptosis in adult *Drosophila* brains and may be quite useful in studying models of human neurodegenerative diseases in fruit flies.

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A method for collecting dead flies from a vial containing live flies, without anesthetization.

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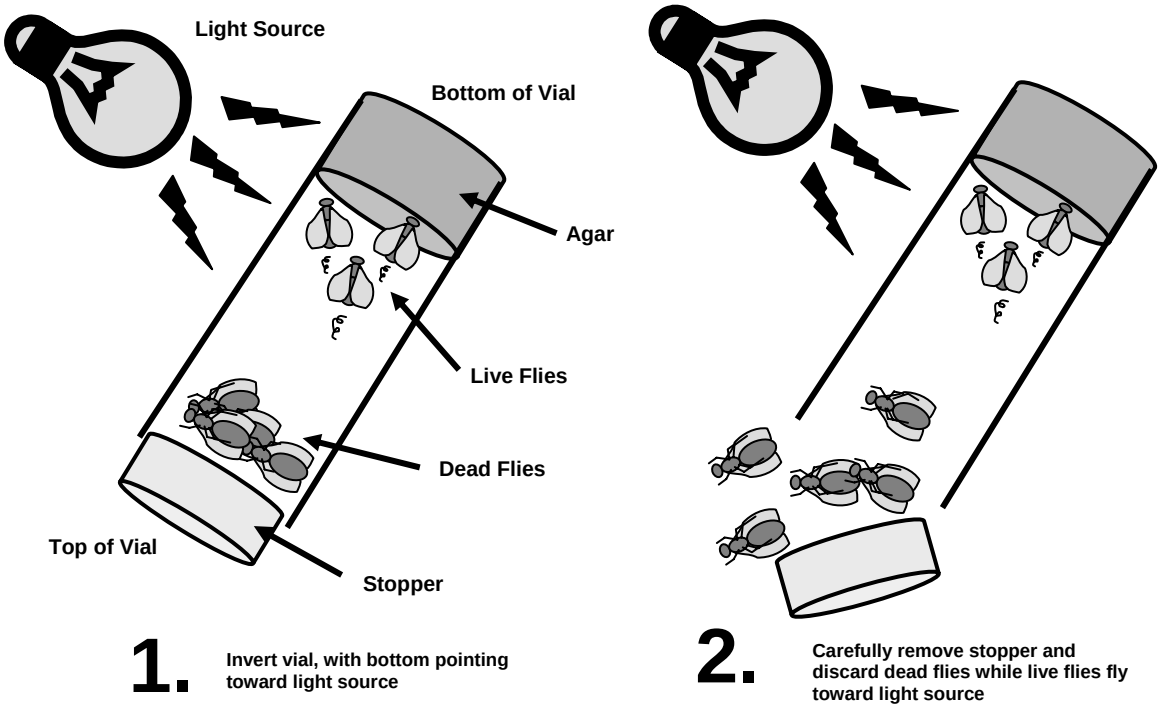
When conducting *Drosophila* experiments, like aging or starvation, sometimes it is necessary to collect dead flies without the anesthetization of the remaining live flies, for further analysis. During our experiments, flies were maintained on a medium consisting of agar and water, and were

transferred, and the dead collected every 6 hours. Collecting the dead flies was an unexpected challenge, because the flies did not become affixed to the agar medium at the bottom of the vials. Thus, when we transferred the live flies to fresh vials, the dead were also transferred. In order to complete the collections as quickly as possible, without anesthetization, and while also preventing the escape of the remaining flies, we devised the following technique:



First, we invert the vial and tap the side gently to knock the dead flies down to the stopper. Then, aim a bright light toward the top of the inverted vial. This will attract the live flies to that end, making it possible to remove the stopper of the vial slightly and collect the dead, while preventing escape of live flies. As the starvation proceeds and the flies become less active, it will be even more important to tap the vial very gently in order to prevent the live flies from falling with the dead to the stopper. If you are careful enough, the live flies should remain on the walls of the vial closer to the light source.

Sorting and collecting females from males at high speed.

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Abstract

One major limit to analysis of developmental processes is the ability to isolate enough material of a specific age, genotype, or other relevant characteristic. Flow cytometry-based cell sorting instruments are capable of isolating near pure populations of individual cells based on size

and fluorescence signals. The COPAS Select and Express flow cytometry instruments are able to analyze and sort intact *Drosophila* embryos or larva based on similar characteristics. By combining this technology with an appropriate GFP transgene or other fluorescent marker, one can isolate nearly pure populations based on a variety of characteristics, including sex.

Introduction

Collection of large numbers of virgin females for subsequent genetic crosses is a labor-intensive process. COPAS Select and Express flow cytometry instruments are able to analyze and sort embryos or larvae quickly and accurately on the basis of fluorescence signals. Using this technology and an appropriate fluorescent marker, one can separate large numbers of organisms accurately and quickly on the basis of a variety of characteristics including sex, genotype, or age. Such sorting will allow isolation of pure populations for further studies. As one example, male and female *Drosophila* embryos can be separated from each other at the embryonic stage, thereby simplifying the isolation of virgin females for subsequent genetic crosses.

Materials and Methods

Plasmid construction

For this construct, the EGFP coding sequences were amplified from pP{GS[v^+,EGFP]} (a gift from Gunnar Schotta), with primers containing EcoR1 and Spe1 cut sites (EGFP5 Eco, 5'-CCTTCCTTGAATTCCCGCATGGTGAGCAAGGGCG -3' and EGFP3, 5'-TTCCTTCCACTAGTGGTATGCTAGCGACGTCGTCG -3'), then cloned into the multiple cloning site of plasmid 5-1. Plasmid 5-1 contains the Sxl Pe sequences with EcoR1 and Spe1 sites downstream (Paul Schedl, unpublished). The SxlPe/EGFP fusion product was subsequently cloned into the CaSpeR vector for injection into *Drosophila* embryos using standard transformation methods.

Embryo collection and sorting

Approximately 4500 young flies (four days after eclosion) were transferred to 1.2L cylindrical cages containing yeast coated apple juice agar trays (100 × 15 cm). Embryos were washed from the apple juice plate into a 70 µm strainer and rinsed with dH_2O, then dechorionated with 50% bleach for approximately 2 minutes. Dechorionated embryos were washed with Ringer's solution, then rinsed with ESS sheath, and transferred to a sample cup containing ESS sheath.

COPAS Select was optimized for dispensing embryos using sort delay and width values to obtain a single embryo per sorted event. Gate and sort regions are determined for each sample based on the fluorescence signature displayed on the gate and sort windows. The embryos were processed at a speed of 10-40 embryos/second and sorted in bulk (up to 100,000 embryos per day) alternating between the collection of non-fluorescent and fluorescent embryos for each bulk sorting. Sorted embryos were collected onto mesh cloth and screened briefly using a fluorescence, dissecting microscope. Sorted embryos were placed on a vacuum filtration cup, and multiple sorts of like embryos were combined and washed with 0.12M NaCl. The embryos were transferred to 15 ml conical tubes to which liquid nitrogen was added. Samples were frozen at -80^oC.

Nuclear Extract

Sorted embryos were thawed on ice, then homogenized in buffer A (15 mM Hepes-KOH pH 7.6,10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 0.35 M sucrose) containing 1 µl/ml 1 M DTT, 1 µl/ml protease inhibitors (1 mg/ml pepstatin A, 10 mg/ml aprotinin, 1 mg/ml leupeptin in

DMSO), 1 µl/ml benzamidine, 1 µl/ml 1 M $Na_2S_2O_4$, 4 µl/ml 250 mM PMSF in 95% EtOH. Four ml of buffer A was used for each ml of embryos. The homogenate was filtered through three layers of Mira-cloth into a centrifuge tube and centrifuged at 2000×g 10 minutes at 4°C. The pellet was resuspended in 1 ml buffer A per ml original embryos and sonicated.

RT-PCR

Sorted embryos were extracted with TRI Reagent, and chloroform, and precipitated with polyacryl carrier. Each sample was resuspended in 18 µl DNAse buffer and treated with RNAse free DNAse at 37°C for 30 minutes. The reaction was stopped by adding 2 µl 250 mM EDTA and heating the samples to 65°C for five minutes. Reverse transcription was performed according to the procedure of Frohman *et al*., 1988 using 4 µl of the immunoprecipitated RNA and primer T41a (CGTGTCCAGCTGATCGTC). 1.5-3% of the cDNA was used as template, and primers mes17 (CGCTGCGAGTCCATTTCC) and BelA1 (GTGGTTATCCCCCATATGGC) were used to amplify the alternatively spliced region of Sxl. PCR cycles were 1× 95°C 4 minutes, 30× 95°C 1 minute, 65°C 45 seconds, 72°C 30 seconds, 1× 72°C 10 minutes

Western

20 µl of 50% antibody linked protein AG beads were added to a 150 µl aliquot of sonicate. The mixture was rocked at 4°C overnight, then washed 5 times with co-IP buffer (20 mM Hepes, pH7.5, 150 mM NaCl, 250 mM sucrose, 0.05% (w/v) Tergitol NP-40, 0.5% (v/v) Triton-X 100 plus with 1 mM DTT, 1 mM $Na_2S_2O_5$, protease inhibitors, benzamidine and 1mM PMSF). 10 µl protein sample buffer was added to each sample. The samples were boiled 3 minutes, spun down briefly, then loaded onto a 10% polyacrylamide gel, run out and transferred to Immobilon-P (Millipore Corp. Billerica Mass). Blots were stained with mouse anti-Sxl antibody 114 primary antibody (1/10) and antimouse HRP as the secondary (1/2500) and developed using the ECL kit from Amersham (Amersham Biosciences UK Limited. Little Chalfont, Buckinghamshire).

Results and Discussion

To test the COPAS Select technology we attempted to sort *Drosophila melanogaster* embryos by sex, based upon sex-specific expression of GFP. A transgenic construct containing the *Sxl* early promoter (*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 activated only in fly embryos that have a 1:1 ratio between X-chromosomes and autosomes (Keyes *et al*., 1992). Consequently, female embryos containing the *Sxl* P_E */EGFP* transgene exhibit a green fluorescence, while male embryos do not. Two transgenic lines were used in our analysis. One had the transgene integrated on the X-chromosome (G5b) and the other had the integration on chromosome 3 (G78b).

Sxl P_E is activated in female embryos at the syncytial blastoderm stage and is turned off at the cellular blastoderm stage, approximately a three hour window. Since EGFP will linger after expression ceases, it was anticipated that the actual period of GFP expression would be somewhat longer than 3 hours. To determine the actual period of GFP expression, transgenic embryos were inspected at various times after egg deposition (AED), and single embryos were tracked. We determined that peak fluorescence occurs between 5-8 hours AED. Beyond 8 hours the fluorescence begins to decrease but is still sufficient to allow distinction between GFP positive and GFP negative embryos for the remainder of embryonic development.

After establishing an approximate 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 all members of the fluorescent

population were indeed fluorescent and likewise for the non-fluorescent population. Embryos with the inappropriate fluorescence were noted. These collections of embryos were allowed to continue development to the adult stage, and their sex was determined visually by microscopy.

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 at least six hours old. 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. We collected embryos from broad time-windows (for example, 6-22 hr. collection) and separated fluorescent from non-fluorescent. Table 1 shows a summary of the data for the accuracy of separating males and females. The data indicate that the presence of fluorescence allows for the collection of females, with near 100% accuracy. Likewise, the data show that the absence of fluorescence can be used to collect male embryos with a similar accuracy.

Table 1. Sorting accuracy. Embryos from each strain were collected and allowed to mature until they were at least 6 hours old. They were then dechorionated and sorted into fluorescent and non-fluorescent populations. These populations were manually inspected with a fluorescent microscope to determine the number of misclassifications.

SxlPE-GFP on 3	% male	% female	Number sorted
GFP + sample	0.3%	99.7%	3846
GFP - sample	99.4%	0.6%	3550
SxlPE-GFP on X	% male	% female	Number sorted
GFP + sample	0.1%	99.9%	7480
GFP - sample	99.8%	0.2%	9500

The purity of sorting 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 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 nonfluorescent 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 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 (data not shown).

To validate the use of COPAS Select sorted embryos with molecular and biochemical techniques, we established egg-laying cages of adult flies and collected large numbers of sex-sorted embryos for analysis. The separate samples were processed for either RT-PCR or Western blot analysis of expression of the endogenous Sxl gene.

The data from RT-PCR and Western blots confirm the accuracy of the sorting technique. Once $SxlP_E$ is silenced, the *Sxl* gene is transcribed in both sexes from the *Sxl* maintenence promoter *(Sxl* P_M, Bell *et al*., 1988; Samuels *et al*., 1991). However, SXL protein is expressed only in females due to a sex-specific alternative splice directed by the SXL protein produced from the early promoter. In females exon 2 is spliced to exon 4, skipping the male specific exon 3. In males the default splice product includes exon 3, which contains several in frame stop codons. Thus male embryos contain a longer *Sxl* mRNA, but produce no SXL protein.

To examine the *Sxl* mRNAs in the sorted population, we reverse transcribed the *Sxl* mRNAs with a primer in exon 6, then performed PCR with primers flanking the alternatively spliced region. Each population of embryos showed the expected PCR product, with the male product being larger due to the inclusion of exon 3 (Figure 1A). 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 (Figure 1B).

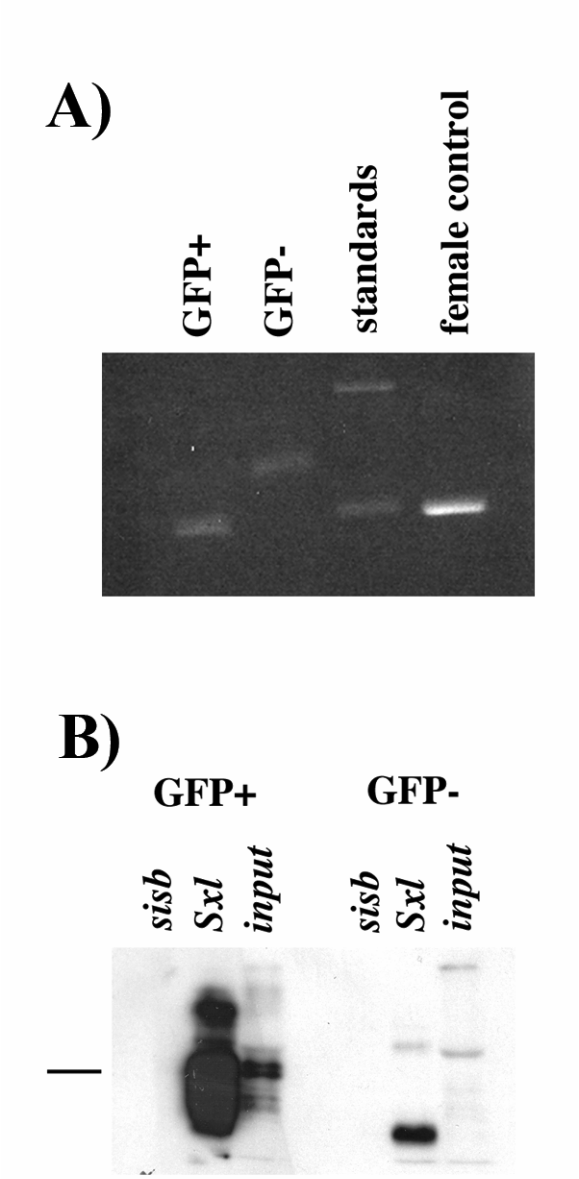

Figure 1. Sex specific mRNAs and proteins can be isolated from sorted embryos. A) RNA was isolated from nuclear extract made from GFP+ or GFP- embryos. Reverse transcription – PCR was performed with primers flanking the alternatively spliced region of *Sxl*. A female *Sxl* cDNA was used as a positive control for the PCR. Male transcripts contain a 180 base pair exon not found in female transcripts. B) Nuclear extract from GFP+ or 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.

Conclusion

When studying a biological process, one frequently needs to accurately isolate large populations of organisms sharing a particular characteristic for analysis. In the past isolation of such populations often involved labor or time intensive manipulations. Therefore, isolation of large numbers of organisms for analysis became impractical. Flow cytometry instruments with cell sorting ability have allowed isolation of near pure populations of individual cells based on size and fluorescence signals. The COPAS Select system allows rapid sorting (50,000- 70,000 embryos per hour) of fluorescent multicellular embryos or larvae from *Drosophila* or other species including *C. elegans* or zebrafish.

In the studies reported here, we described the use of the COPAS Select system with *SxlP_E-GFP* expressing strains to rapidly isolate separate populations of male and female *Drosophila* at the embryonic stage. Samples sorted in this manner can be collected and grown to adult stages with the male and female flies maintained separately insuring virgin populations for mating. Alternatively, the separate male and female samples can be processed for molecular, immunological or biochemical analysis of sex specific differences (Figure 1). During this study almost 2 million embryos were sorted in 13 days, providing ample material for several different types of analysis.

The ability to sort embryos based upon fluorescence can be combined with the UAS GFP expression system to allow collection of embryos based on a variety of different criteria. For example, to obtain a large number of embryos homozygous for a lethal mutation, one can place the mutation of interest over an EGFP balancer chromosomes (Casso *et al*., 2000; Halfon *et al.*, 2002).

COPAS Select can collect the non-fluorescent embryos, thereby producing a nearly pure population of homozygous embryos. Use of a less stable form of GFP (Xianqiang *et al*., 1998) and a stage specific promoter should allow collection of embryos at a specific developmental stage. These embryos can then be allowed to grow to the developmental stage of interest and examined. COPAS Select technology also allows the use of GFP, YFP or RFP fluorescence markers. These markers can be detected simultaneously, allowing one to select for multiple characteristics in a single sorting run.

Acknowledgments: pP{GS[v^+,EGFP]} DNA was a gift from Gunnar Shotta. Patricia Graham was supported by a grant from the National Institutes of Health to P.D.S.

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A simple method to prepare DNA fibres in the male germ line.

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Cytologic analysis of the male germ line of *Drosophila melanogaster* has been extensively developed in the past years (Lindsley and Tokuyasu, 1980; Cenci *et al.*, 1994). In slides prepared in this way, cellular structures are preserved before and after fixation procedures, and DNA organization in germ cells is largely maintained. A critical step during slide preparation is to avoid excessive spreading of the tissue and the consequent loss of cell identity. For this, testes must be gently squashed between slides and coverslips. To obtain that, usually a small drop (3 to 4 µl) of buffer is put on the coverslip, then testes are carefully transferred inside it after dissection, and finally the slide is put on it upside down; capillarity permits obtaining good squashes in a few seconds. In case of too small a drop, waiting up to 1-2 minutes is still sufficient for a good spreading. However, this kind of preparation does not permit analysis of single DNA fibres, since DNA organization inside nuclei is preserved. Fibres of DNA must be prepared by stressing cells, to break down nucleus organization.

Several attempts using different experimental conditions permitted the conclusion that it is possible to achieve this result by strongly pressing the coverslip over the slide, after the previously described tissue spreading. Best results are obtained by an orthogonal pressure, without moving the coverslip laterally; in this way a sufficient number of DNA fibres become visible. Instead, lateral movements completely destroy the tissue and avoid using it any further. The strength to be used in these preparations should be similar to that used for larval brain squashes. During this preparation, cellular organization is no more visible under a phase contrast microscope, and tissues generally appear like a layer of uniform material. However, most of the nuclei preserve their organization and are comparable to those described by Cenci and coworkers (1994) after DAPI or Hoechst staining (Figure 1 A and B). The most delicate cells in the male germ line are primary spermatocytes, which undergo quite a long maturation stage lasting 90 hours, during which the nuclear volume increases its size 30 times. Inside mature primary spermatocyte nuclei, DNA is less compact that in other cells, and it is organized in three chromatin clumps corresponding to the three couples of the major

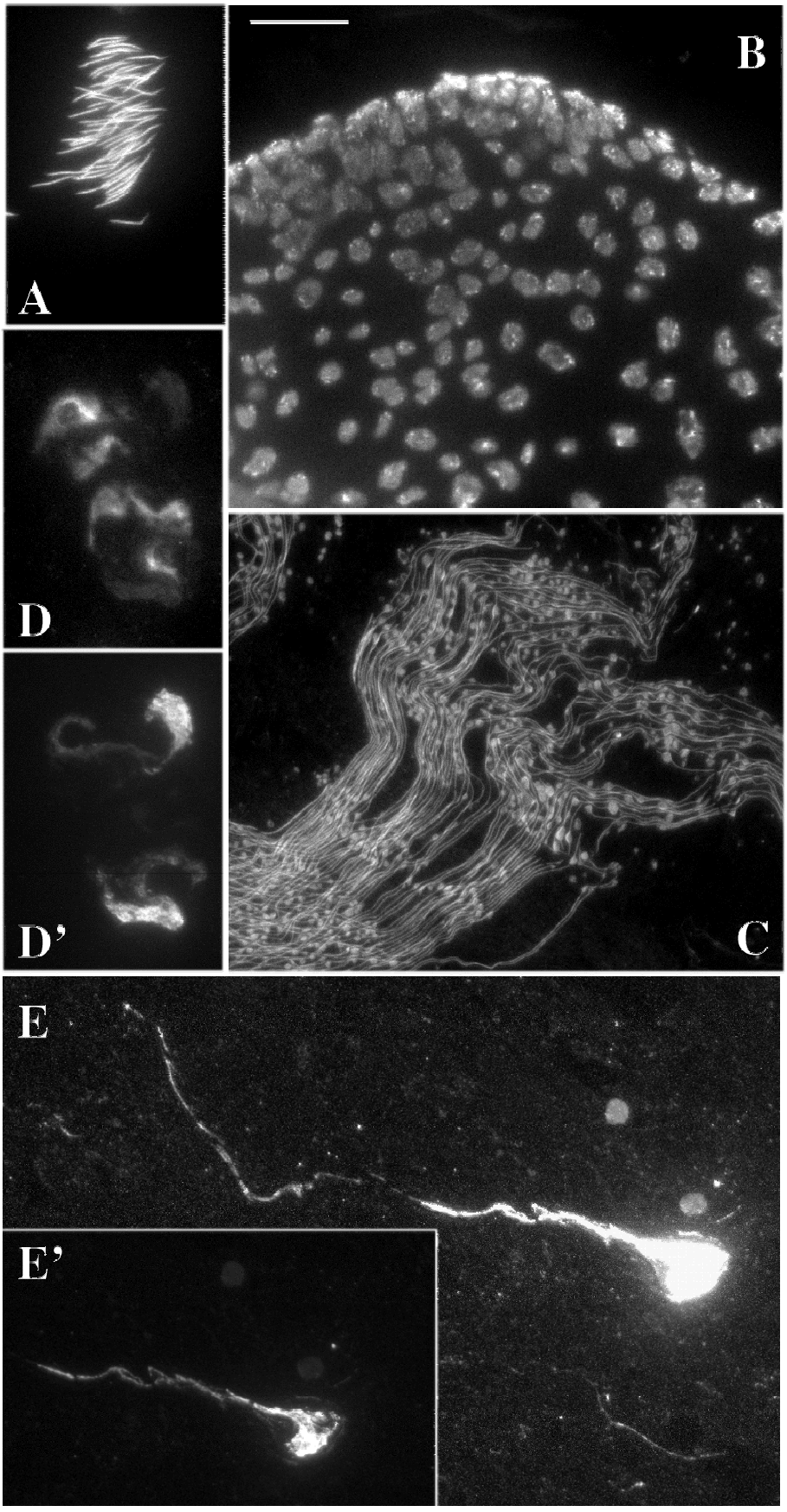

Figure 1. Microphotographs of slides prepared for the analysis of DNA fibres. Most cells of the germ line, such as sperm heads (A), spermatogonia and young primary spermatocytes (the tip of a testis is shown in B) are not altered under these experimental conditions. T53-1 antibody is still able to decorate kl-3 loop (data not shown) as well as sperm tails (C). On the contrary, DNA of mature primary spermatocytes is easily spread, although it is still possible to find almost normal nuclei (D). Also S5 staining remains normal, permitting sometimes to discriminate between kl-5 and ks-1 loops (D'). In E a kl-5 loop is shown, from which a DNA fibre is released; note that in order to evidentiate the fibre, the microphotograph was over-exposed (in E' the normal exposure is shown). All pictures were taken at the same magnification. Bar represents 20 µm.

chromosomes. Spaces among the three clumps are filled by the nucleolus and by the three lampbrush like loops, namely kl-5, kl-3 and ks-1, which are the cytological evidence of the activity of the corresponding fertility factors mapping on the Y chromosome (Bonaccorsi *et al.*, 1988). Slides for fibres usually show completely crushed primary spermatocyte nuclei and in many cases it is possible to observe isolated DNA fibres even at low magnification (Figure 1 D and E).

In order to evaluate the possibility of performing immunostaining of these fibres, two different antibodies were used, T53-1 (Pisano *et al.*, 1993) and S5 (Saumweber *et al.*, 1980; Risau *et al.*, 1983). T53-1 specifically decorates sperm tails and the kl-3 loop, while S5 strongly stains kl-5 and, more faintly, ks-1 loops. There are four reasons these two antibodies were chosen: (i) they recognize DNA binding proteins, which (ii) are very abundant during this phase of germ line development, but (iii) they react with a specific portion of DNA, so it is possible to see if the organization of this subset of chromatin is still preserved during fibres preparation; besides (iv) it is also possible to evaluate if cross-reactions occur after this mechanical stress. As shown in the picture, the conclusions of this study are that (i) both T53-1 (data not shown) and S5 (Figure 1 D' and E-E') antibodies are still able to bind to Y loops fibres, and (ii) there is no cross reaction with other DNA fibres (Figure 1 D-D'), indicating that this method is able to preserve the DNA-proteins interaction and its specificity. Moreover (iii) in many cases also intact loops are preserved, and S5 is still able to discriminate between kl-5 and ks-1 (Figure 1 D').

It is noteworthy that, in the described situation, this method also adds some new knowledge about the molecular organization of the Y loops. In fact it is known that the kl-3 loop has a filamentous aspect while the other two, after S5 immunostaining, show a more compact appearance. Preparation of DNA fibres indicates that also the last two loops have a filamentous organization (Figure 1 E-E'), and that their compactness in standard preparations is probably due to their higher order, three-dimensional organization, which is lost after the stress induced by squashing. Sometimes, as illustrated in Figure 1 E-E', the kl-5/ks-1 loops show a variable thickness of the fibre, which size increases from one extremity to the other. At the moment it is not possible to assess if this increasing thickness is a chance or if it reflects a real organization of the fibre. However, it would be intriguing to argue that the accumulation of the protein bound to the loop (a protein which recognizes nascent RNAs; Saumweber *et al.*, 1980) increases towards the 3' end of the filament, resembling the ultrastructural organization of the amphibian oocytes' lampbrush-like loops from which they take their name.

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Submissions to Drosophila Information Service are welcome at any time. The annual issue now contains articles submitted during the calendar year of issue. Typically, we would like to have submissions by 15 December to insure their inclusion in the regular annual issue. but articles can be accepted for this volume until 31 December. Submissions in Microsoft Word, which is now the

program we use for our page setup, are especially helpful. Submissions by email is preferred, with text and figures submitted electronically as attached files. Pictures and line drawings should be as sharp and high contrast as possible. Where large or complex tables are concerned, we may request that authors send a paper copy to facilitate accurate formatting. But submit them electronically first. Details are given in the Guide to Authors.

A commercial phospho-Smad antibody detects endogenous BMP signaling in *Drosophila* tissues.

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Introduction

The bone morphogenetic protein (BMP) homolog Dpp has numerous functions in embryonic and larval development of *Drosophila melanogaster* (reviewed in Parker *et al*., 2004). BMP signaling in *Drosophila* leads to the phosphorylation and nuclear accumulation of Mad, which is orthologous to mammalian Smads 1 and 5 (reviewed in Raftery and Sutherland, 1999). In particular, Mad shares identical C-terminal sequences with mammalian Smads 1 and 5, including two serines that are phosphorylated by activated BMP type I receptors. For the past seven years, the most common reagent used to detect endogenous phosphorylated Mad (pMad, Tanimoto *et al*., 2000) has been a rabbit polyclonal anti-phospho-Smad1 antibody (PS1), which was raised against a phosphorylated peptide in the laboratory of Dr. Peter ten Dijke (Persson *et al*., 1998). However, a polyclonal antiserum is a limited resource. Hence, we have sought an alternative antibody to detect endogenous BMP activity in *Drosophila*. Here we document the results for a commercial rabbit monoclonal antibody preparation, anti-phospho-Smad1/5 (pSmad1/5) from Cell Signaling. This monoclonal pSmad1/5 antibody replicates previously reported immunofluorescence results from PS1 in embryos as well as wing, eye, and antennal imaginal discs, and therefore is an acceptable reagent for detecting Dpp signaling in these tissues.

Materials and Methods

Drosophila *stocks*

For embryo and wing imaginal disc analysis: y^1 w^{67c53} was used for wild type; *Mad Df(2L) C28/CyO, Kr-GFP* was used to obtain embryos deficient for the *Mad* gene (Sekelsky *et al.*, 1995). Eye-antennal imaginal discs were obtained from late third instar larvae of the genotypes *y w eyFLP*/Y or + ; *Ubi-GFP FRT40A*/*FRT40A* or *y w eyFLP*/Y or +; *FRT82B Ubi-GFP*/*FRT82B* (Xu and Rubin, 1993). All of the tissue in these discs is wild type tissue; these mosaic eye-antennal discs were generated as controls for unrelated experiments.

Immunostaining

Freshly collected embryos were fixed at the interface of 4% formaldehyde in PBS pH 7.4 with heptane, for 20 minutes at room temperature (~22ºC). Embryos were devitellinized in methanol, and then serially rehydrated in 90%, 60%, and 30% methanol in PBS pH 7.4. Imaginal discs were

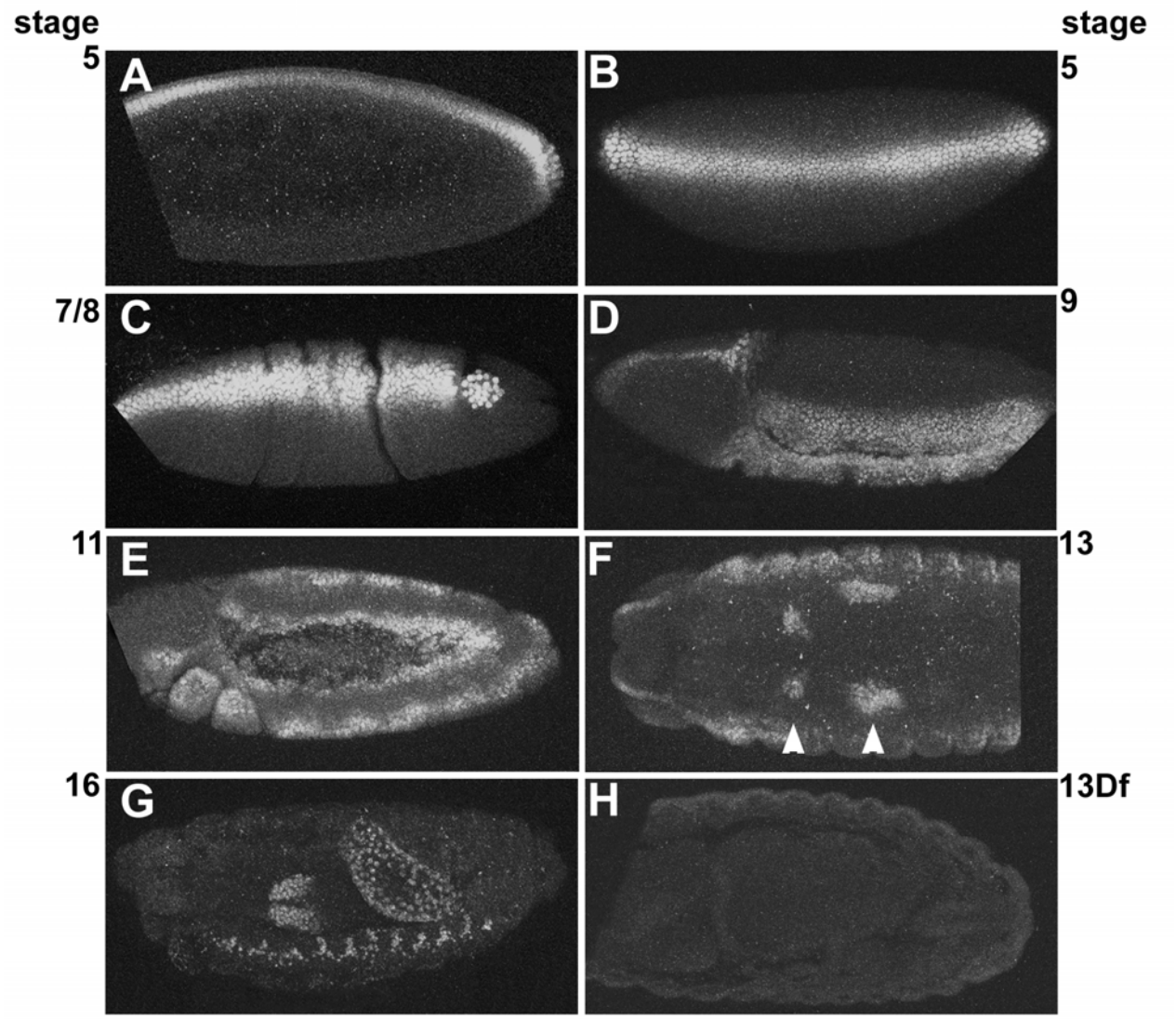

Figure 1. Monoclonal pSmad antibody staining patterns during embryogenesis. Confocal projections of embryos stained with the Cell Signaling monoclonal pSmad1/5 antibody. All panels are anterior to left; panels A, E, and H are side views; B, F, and G are dorsal views; C and D are dorso-lateral. At all stages, monoclonal pSmad1/5 staining was similar to PS1 staining and produced staining patterns consistent with regions of Dpp expression. (A and B) wild type stage 5 embryos. (C) wild type stage 7 embryo. (D) wild type stage 9 embryo. (E) wild type stage 11 embryo. (F) wild type stage 13 embryo. Arrowheads indicate parasegment 3 and parasegment 7 of visceral mesoderm. (G) wild type stage 16 embryo. (H) pMad staining is absent from *Mad Df(2L) C28* homozygous embryos at stage 13.

dissected from late third instar larvae, fixed in 4% formaldehyde in PBS pH 7.4 for 20 minutes at room temperature. All washes were performed in 0.1% Triton X-100 in PBS pH 7.4. Prior to antibody staining, embryos and imaginal discs were permeabilized for 20 minutes in 0.1% or 0.3% Triton X-100 in PBS pH 7.4, respectively.

All antibody incubations were performed in 5-10% normal goat serum / 0.1% Triton X-100 in PBS pH 7.4. Primary antibodies (overnight incubation at 4ºC) were used at the following dilutions: Commercial rabbit monoclonal anti-phospho-Smad1/5 (Ser463/465, Catalog #9516, Cell Signaling) was used at a dilution of 1:20 for embryos and 1:100 for imaginal discs. Rabbit PS1, a gift of P. ten Dijke (Persson *et al*., 1998), was used at 1:200 for eye-imaginal discs. For all experiments, Alexa 568-conjugated goat-anti-rabbit secondary antibody (Molecular Probes) was used (1:400 for eye discs, 1:200 for all others, with a two hour incubation at room temperature). Embryos were staged according to morphology as described by Campos-Ortega and Hartenstein (1985).

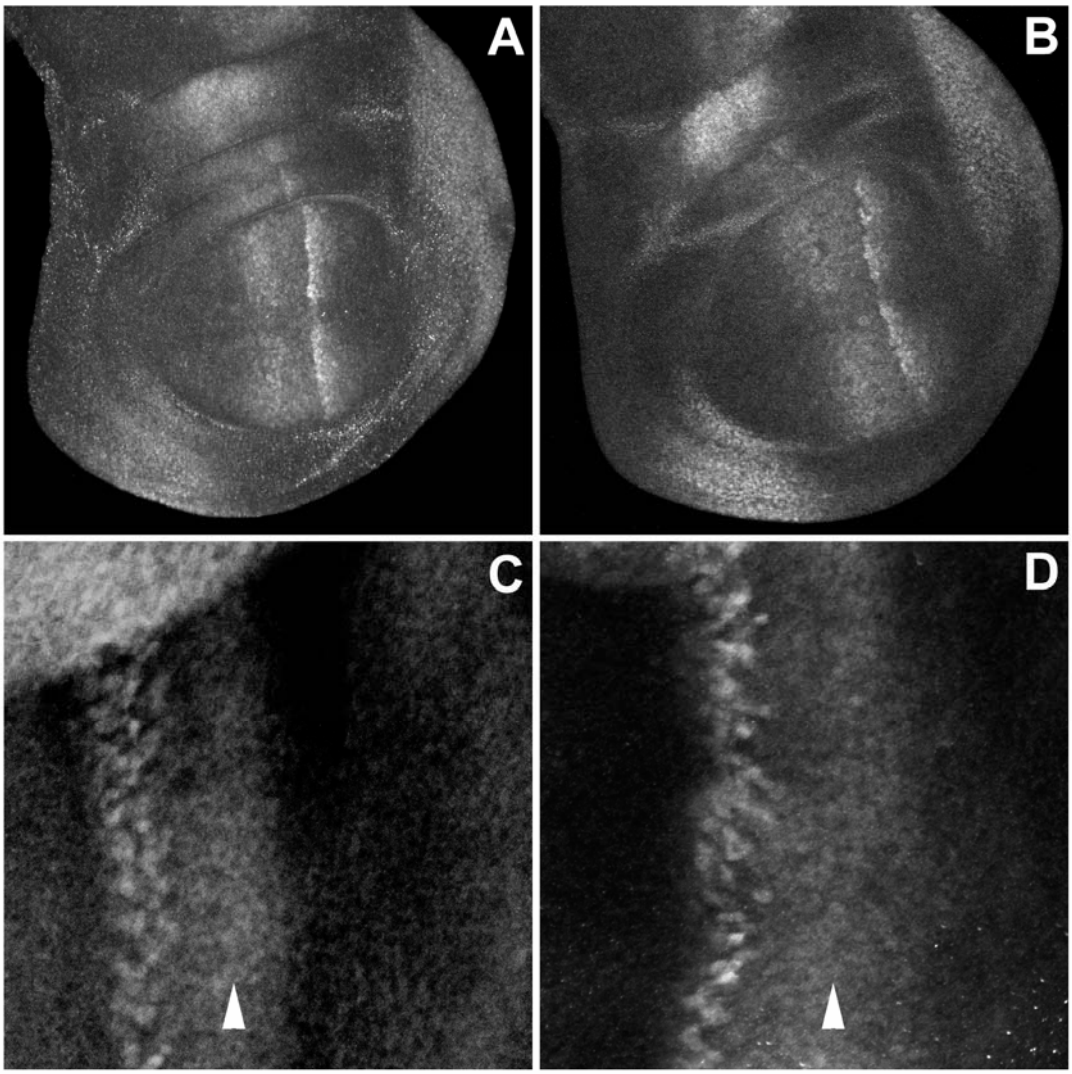

Figure 2. pSmad antibody staining in wild type third instar imaginal discs. (A and B) Confocal projections of wild type wing imaginal discs stained with different anti-pSmad reagents. Similar staining patterns were obtained using the polyclonal PS1 antibody (A) and the monoclonal pSmad1/5 antibody (B). (C and D) Single confocal sections of wild-type eye imaginal discs stained with different anti-p-Smad reagents. Similar staining patterns were obtained using polyclonal PS1 antibody (C) and monoclonal pSmad1/5 antibody (D). The approximate position of the morphogenetic furrow in panels C and D is indicated by the arrowheads. The top left corner of panel (C) is a folded eye disc. For wing discs, anterior is left, and dorsal is up. For eye discs, anterior is right.

Results and Discussion

pSmad detection in embryos

To determine whether the monoclonal pSmad1/5 antibody is an acceptable alternative for the PS1 antibody, we first examined the pSmad1/5 staining pattern in wild type embryos. The earliest detectable pSmad1/5 staining was in blastoderm embryos (stage 5) and consisted of a stripe of approximately 5-7 dorsal cells (Figure 1A and B). This stripe of pSmad1/5 staining at the dorsal midline persisted through stages 7 and 8 (Figure 1C). At stage 9, pSmad1/5 staining was retained in a narrow dorsal stripe in the anterior cephalic region. In the segmented region, staining was evident in the presumptive dorsal ectoderm (Figure 1D). By stage 11, the staining was lost in most of the dorsal ectoderm, remaining only in two stripes of variable intensity, one at the dorsal edge of the dorsal ectoderm, and the other near the ventral edge of this tissue (Figure 1E). Weak staining was evident in the amnioserosa. At stage 13, strong staining was detected in the visceral mesoderm of parasegments 3 and 7. At stage 16, staining was detected in the developing midgut, gastric caecae and the central nervous system (Figure 1G). For stages 5 through 11 and in the nervous system, the pSmad1/5 staining patterns are very similar to the previously reported patterns of PS1 staining (Dorfman and Shilo, 2001; Marquéz *et al*., 2002; Sutherland *et al*., 2003). For stages 13, 16, and others not shown, the patterns of pSmad1/5 staining were appropriate for previously reported patterns of *dpp* RNA accumulation (Hursh *et al*., 1993; Jackson and Hoffmann, 1993).

To confirm that the antigen detected by pSmad1/5 is a form of the Mad protein, we examined staining in embryos that lack Mad protein. We chose to examine late-staged mutant embryos that bear a deletion of the entire *Mad* gene, *Df(2L)C28* (Sekelsky *et al*., 1995). We reasoned that these embryos should have low levels of Mad gene products during late stages, because *Mad* mutant embryos fail to make the second midgut constriction during stage 16 (Newfeld *et al*., 1996). No pSmad1/5 staining was observed in *Mad Df(2L)C28* homozygous embryos between stages 13 and 17; these embryos were identified by the absence of green fluorescent protein expressed from *CyO, Kr-GFP* (example in Figure 1H). These studies suggest that phospho-Mad is the predominant antigen detected by pSmad1/5.

pSmad detection in imaginal discs

To test whether the monoclonal pSmad1/5 antibody accurately reports BMP signaling activity in imaginal discs, we compared it to PS1 in side-by-side immunohistochemical stainings of both wing and eye imaginal discs from third instar larvae.

In the third instar wing imaginal disc, Dpp is produced along the anterior-posterior (A/P) boundary (reviewed in Tabata, 2001). BMP signaling occurs in cells surrounding the A/P boundary, and signal strength decreases with increasing distance from the boundary, leading to graded levels of pMad accumulation. pMad is present along the posterior margin of the wing disc as well as in a spot at the anterior margin of the disc near the distal pouch (Figure 2A and Tanimoto *et al*., 2000). A nearly identical staining pattern was obtained using the monoclonal anti-phospho-Smad1/5 antibody (Figure 2B).

In the third instar larval eye imaginal disc, Dpp is expressed in the cells of the morphogenetic furrow (Blackman *et al*., 1991), a transient furrow in the eye field that moves from posterior to anterior during fine patterning of the retinal cells. A stripe of strong pMad accumulation was reported at the posterior edge of the furrow (Vrailas and Moses, 2006). We detected a similar stripe of strong staining at the posterior edge of the furrow using either the PS1 antibody (Figure 2C) or the monoclonal pSmad1/5 antibody (Figure 2D). In our preparations, both antibodies detected a graded pattern of weaker staining in the anterior portions of the furrow. The difference between our observations and the previous report may result from the higher concentration of PS1 antibodies used

in our protocol, or it may reflect variability between aliquots of the PS1 polyclonal antiserum. Both PS1 and PSmad1/5 antibodies revealed stronger, wider patches of pMad in the morphogenetic furrow at both the dorsal and ventral margins of the eye disc (data not shown). Finally, the commercial phospho-Smad1/5 antibody stains a ventral wedge of the antennal region in the eye-antennal disc tissues (data not shown), consistent with the site of Dpp expression in this tissue (Blackman *et al*., 1991).

Conclusion

Based on these data, the monoclonal pSmad1/5 antibody accurately reproduces the staining patterns previously reported for PS1 antiserum during embryogenesis as well as in the third instar wing, eye and antennal imaginal discs. A monoclonal antibody is advantageous both because of the absence of non-specific antibodies and because of the potentially unlimited supply. Thus, we conclude that pSmad1/5 monoclonal antibody is a useful reagent for detection of endogenous BMP signaling in multiple *Drosophila* tissues.

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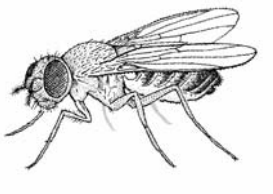

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