27 Methods for Studying Infection and Immunity in *Drosophila*

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List of abbreviations

AMP:	Antimicrobial peptide
Ecc15:	*Erwinia carotovora carotovora* 15

INTRODUCTION

Research on pathogens such as *Listeria, Yersinia, Salmonella, Shigella, Escherichia,* which are particularly suitable to genetic manipulation, have increased our understanding of the molecular interactions between bacterial factors and host cellular components (Finlay and Cossart, 1997). Despite present advances, however, the overall spectrum of interactions between infectious microbes and their hosts remain poorly understood. To date, the model host organism systems used to analyze host/bacteria interaction (cell culture or mouse) have not allowed for a systematic identification by genetic screening of the host factors involved in the infection process and corresponding host immune responses.

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Nevertheless, many of the mechanisms underlying host innate immune responses, as well as invasive strategies used by pathogenic microbes, appear to be conserved across phylogeny, pointing to their ancient origin (Hoffmann *et al.*, 1999; Tan and Ausubel, 2000). These results highlight the potential of non-mammalian model organisms that are amenable to genetic analysis for studying host–pathogen interactions.

During the last century *Drosophila melanogaster*, the fruit fly, has been a widely-used model organism for genetic studies. A facile genetic system, reliable husbandry techniques, and fully sequenced genome all contribute to the usefulness of this organism. In addition, *Drosophila*, like other insects, shows efficient constitutive and inducible host defense responses that display striking parallels with mammalian innate immune responses (phagocytosis by macrophage-like hemocytes, antimicrobial peptides, proteolytic cascades). Consequently, *Drosophila* is especially suitable for the analysis of the interplay between microbes and the innate immune defense. Flies, like mammals, possess respiratory and digestive tract tissues that are also the target for invading pathogens. Although the physiology of these organs are significantly different from their mammalian counterparts they share some basic properties as barriers to microbial infection. Finally, our good understanding of the evolution as well as the ecology of *Drosophila* in relation to natural pathogens in the wild can be relevant for host–pathogen analysis. In keeping with this idea, it should be pointed out that flies function as vectors in the spread of many human and plant pathogens.

In this review, we describe the basic techniques currently used to both infect *Drosophila* and to monitor corresponding immune responses.

◆◆◆◆◆◆ MICROBIAL INFECTION OF *DROSOPHILA* LARVAE AND ADULTS

In our laboratory, we use two methods for infecting *Drosophila*: (1) Microbial injection—a direct introduction of bacteria and fungi into the body cavity of the fly; and (2) Natural infection without injury.

Introduction of microbes into larvae and adults

Microbial injection is used to assay *Drosophila*'s immune responses and survival to different bacteria and fungal species. Basically, microbes are introduced inside the body cavity using either a needle (septic injury) or a micro-injector. In addition to the introduction of microbes inside the body cavity, this stimulus results in an injury and the activation of melanization reactions at the wound site. Clean injury experiments differ from a microbial injection only by the fact that the needle used to puncture the fly has not been previously dipped in a bacterial or fungal solution.

Septic injury

Third instar larvae

Wandering third instar larvae are washed in water and placed in a small drop of water on a black rubber block (Figure 27.1A). From an overnight bacterial culture, a bacterial 'pellet' is generated with an OD close to 200, a thin needle (entomology needle used for dissection) is dipped into it, and larvae are punctured on their posterior lateral side (Figure 27.1B). Injected larvae are transferred to a filter paper moistened with water (or 0.2% glucose solution) in a vial containing fly medium. The site of the puncture heals and remains visible as a pronounced dark spot (due to the melanization reaction). Nearly all third instar larvae that are injured with a needle will die a few hours after injury (before or during the pupal stage). To obtain a better survival rate, we challenge larvae with a more appropriate needle, such as a 0.2 mm diameter tungsten wire which has been sharpened in a 0.1 M NaOH solution by electrolysis. To avoid damaging the needle, a drop (50 to 100 μl) of bacterial solution can be placed directly onto the same rubber block recipient where the larvae will be pricked.

Under these conditions, nearly 50% of injured larvae give rise to adults. Overall, larval injury induces a rather high lethality rate, which is possibly due to the strong internal pressure, or to a critical developmental stage just prior to metamorphosis.

Adult flies

To inject adult flies, a thin metal needle is used, mounted on a small handle (Figure 27.1C). The bacterial and fungal pellet (see above) is deposited inside a cut-off microfuge tube lid. We dip the thin needle into the bacterial pellet and puncture the dorsal or lateral side of the thorax of a CO_2-anesthetized fly (Figure 27.1D), then gently separate the fly from the needle with a paint brush. The use of cold light is recommended to avoid dessication of the challenged animals. They are then transferred to a clean vial of standard corn-meal fly medium where they normally recover within 5 to 20 minutes. Less than 5% of them immediately die when injected with a non-pathogenic strain probably due to gross inner lesions. Flies that recover exhibit normal viability. The site of injury heals and remains visible as a small dark spot. This method is straightforward and allows large samples to be tested in a short period of time, i.e. 300 flies or more can be infected per hour.

Injection

Septic injury with a small needle is probably the simplest way to infect *Drosophila*. This technique, however, does not allow for accurate quantification of injected microbes or microbial components. When necessary, we use the Drummond's Nanoject (automatic) injector to deliver a defined volume of microbial solution (Figure 27.1E). Glass capillary tips can be pulled under high heat, backfilled with mineral oil and then mounted onto

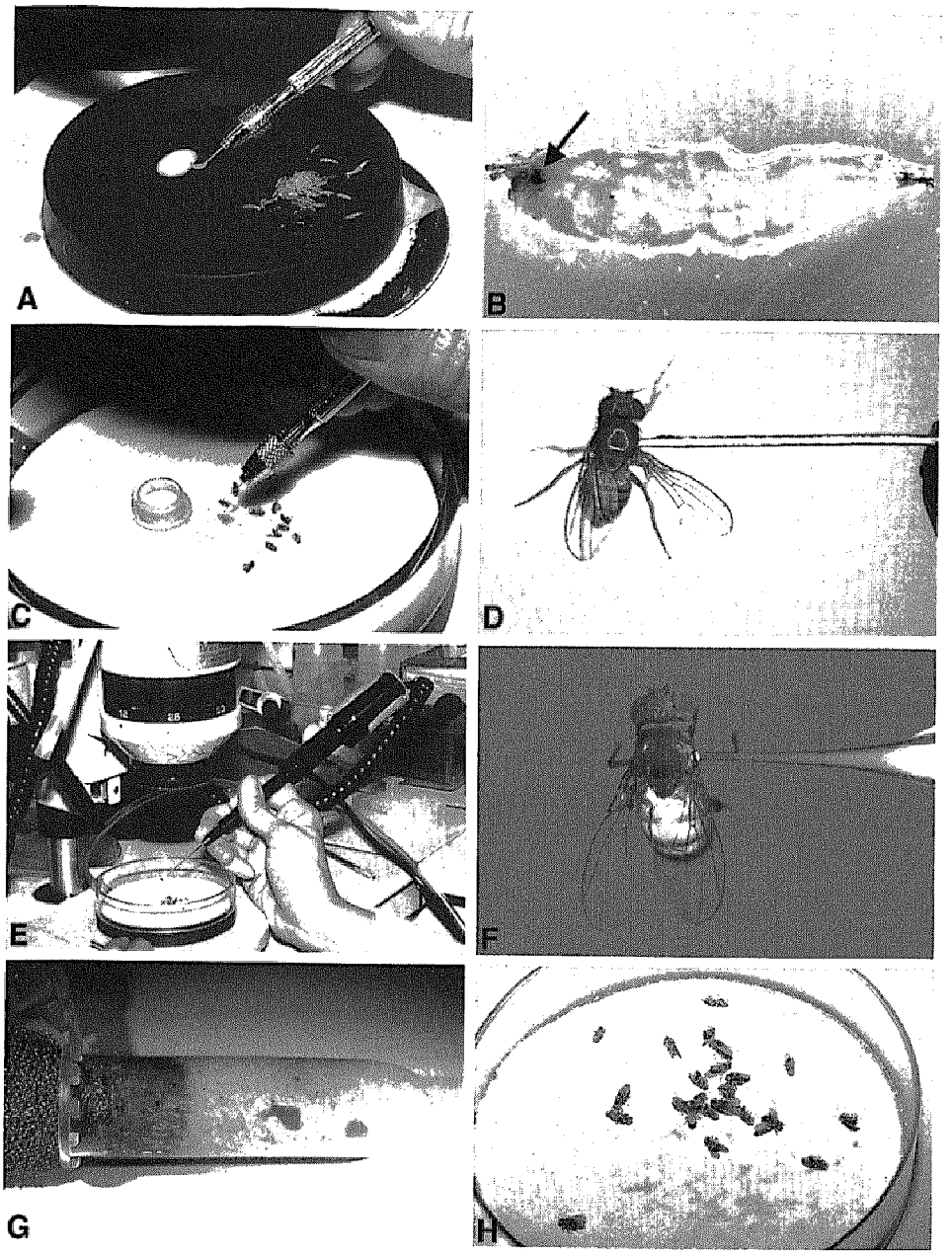

Figure 27.1. Microbial infection of *Drosophila*. (A) Septic injury of larvae is performed with a tungsten needle. A rubber block is employed to protect the needle from damage. A drop of microbial solution is placed on the rubber block, and larvae are placed inside a drop of water. (B) Larva are punctured on their posterior lateral side, triggering a melanization reaction at the injury site (arrow). (C) The needle is mounted on a handle to prick adult flies. The CO_2 pad (Inject + Matic™, Geneva) provides a convenient way to anesthetize flies. The microbial solutions are concentrated and placed into the cap of a microfuge tube. (D) An adult fly is pricked on the dorsal side of the thorax. (E) Drummond Nanoject injector: a fine capillary tip is backfilled with mineral oil before mounting onto the injector handle, and dipping into microbial solution to load. After specifying the quantity, at each pulse, the injector will release the exact amount (varies from 4 to 73 nl) into the body cavity of the fly. (F) An adult fly being injected with 9.2 nl of GFP expressing bacteria (OD = 100). (G) Natural infection of larvae is performed by mixing the following in a centrifuge vial: crushed banana, bacterial pellet, and third instar larva. After 30 min of incubation at room temperature, the larvae and the bacterial mixture are directly transferred to a standard fly vial. (H) Natural fungal infection is done by covering the flies thoroughly with fungal spores. Flies are anesthetized and shaken on a Petri dish containing a sporulating fungal species. (This figure is also reproduced in colour between pages 276 and 277.)

the Nanoject device calibrated for specified injection amount. Injections of 4 to 73 nl into an adult fly are possible (Figure 27.1F). This apparatus can be used for the injection of micro-organisms, chemicals, and purified bacterial compounds, where highly accurate conditions are required. These compounds may be diluted in Ringer solution (Ashburner, 1989). Injection, however, tends to provoke more trauma than septic injury due to the larger needle and the introduction of high amounts of solution.

Natural infection of larvae and adults

Bacterial natural infection

Larvae

Natural infections are performed with bacterial strains such as *Erwinia carotovora carotovora 15 (Ecc15)* that can induce a strong immune response in *Drosophila* in the absence of physical injury (Basset *et al.*, 2000). Approximately 200 third instar larvae are placed into a 2 ml microfuge tube containing a mixture of 200 µl of an overnight bacterial culture concentrated to OD = 200 and 400 µl of crushed banana. For multiple samples, banana may be crushed in a hand-held spice grinder (Krupps). The larvae, bacteria and banana are thoroughly mixed by strongly shaking the capped microfuge tube; afterwards a piece of foam is inserted into the reopened tube to prevent larvae from wandering away from the bacterial mixture (Figure 27.1G). The infection process takes place at room temperature for 30 min. The mixture is then transferred to a standard corn-meal fly medium and incubated at 29°C. When *Ecc15* is used in this infection procedure, 80% of the treated larvae express genes encoding antimicrobial peptides in fat body cells; lower concentrations of *Ecc15* reduce the percentage of larvae that express these genes.

Adults

Adults are dehydrated for a few hours in a dry vial in the absence of food then transferred into a vial containing filter paper hydrated with 5% sucrose/concentrated bacterial solution, and incubated at 25°C. Each day, the paper is rehydrated with the same solution. To date, we have not identified a bacterial species that naturally infects adults and stimulates a systemic immune response. Infection of *Drosophila* adults by *Ecc15* induces the expression of antimicrobial peptide genes in several epithelial tissues (Tzou *et al.*, 2000). The feeding of *Drosophila* with *Serratia marcescens Db11* causes high lethality, likely due to toxin secretion (Flyg *et al.*, 1980). This bacterial strain, however, does not reproducibly induce the expression of antibacterial peptides.

Natural infection by entomopathogenic fungi

The genes encoding the antifungal peptides *drosomycin* and *metchnikowin* can be selectively induced after massively covering the adults with spores

of the entomopathogenic fungi *Beauvaria bassiana* or *Metharizium anosiplae*. The level of antifungal peptide gene expression increases over several days and is similar to the level obtained after microbial injection challenges (Lemaitre *et al.*, 1997). These two fungi are pathogenic for many insect species and have the ability to cross the cuticle of insects through the secretion of proteases and lipases (Clarkson and Charnley, 1996). Natural infection or injection of these fungi causes a significant mortality in wild-type adult flies.

Production of fungal spores

The seeding of fungal spores should be carried out under sterile conditions. *Beauvaria bassiana* spores are spread onto 5.5 cm *Petri* dishes (or 9 cm and larger for collecting spores) of malt-agar (1 g peptone, 20 g glucose, 20 g malt extract, 15 g agar, in 1 l water, autoclaved). The use of glass beads facilitates homogeneous plating. Incubate the spores at 25 to 29°C. The fungal hyphae will germinate after 3 to 5 days at 25°C. After 10 to 30 days, check for the presence of dust-like spores. Well-sporulated plates are stored at 4°C for infection experiments.

To collect spores, wash the 9 cm dishes with 10 ml of sterile water. Separate spores from hyphal bodies through a funnel lined with glassfibre. Spores are collected into a 50 ml vial and centrifuged at 5000 rpm for 15 min at 4°C. Discard supernatant. Quantify the number of spores per ml using a hemacytometer (generally around 10^9 to 10^{12} sp ml^{-1}) under high magnification. Aliquots of fungal spores in 20% glycerol can be stored for several months at 4°C or several years at −20°C.

Infection of flies

One way to naturally infect adults with fungi is to use a pencil or dropper to place a droplet of concentrated liquid solution of spores onto CO_2-anesthetized adults. This is tedious and requires a high quantity of spores, but it is useful for some fungal species that do not grow easily on Petri dishes. Alternatively, the most efficient and natural way to infect flies with fungi is to place the CO_2-anesthesized flies on a 5.5 cm or 9 cm dish with a well-sporulated carpet of fungi. Hand-shake the Petri dish until the flies are totally covered with spores (Figure 27.1H). Infected flies are transferred into clean vials of normal medium and are incubated at 29°C. Vials should be changed every 2 days.

Larvae can well be rolled on sporulated plates. This treatment induces the formation of melanotic tumors and the induction of the *drosomycin* gene (Braun *et al.*, 1998).

Parameters that influence infection

Several parameters can influence the infection process.

- *The infection procedure.* As mentioned earlier, natural infection provides a cleaner picture of the infection process, without the interference of host

reaction induced by wounding. In the case of fungal infection, for instance, challenge via septic injury with *B. bassiana* triggers an immune response with characteristics and kinetics different from that elicited by a natural infection with the same fungus, suggesting that a different set of recognition signals is switched on by different infection methods. In the case of injection, the size of the needle and the site of injection may influence the infection process. At the larval stage, differences in needle thickness can mean life or death. Adults, however, can better cope with thick needles.

- *The nature of the microbes and their concentration.* The use of various types of bacteria is recommended to compare the pattern of antimicrobial peptide gene expression, since the latter differs according to the microorganisms used (Lemaitre et *al.*, 1997). In our laboratory, we currently use a mixture of Gram-negative (*E. coli*) and Gram-positive (*Microccocus luteus*) bacteria to induce a high level of all AMP gene expression.
- *The temperature of infection.* Flies live well between 16 and 32°C. Temperature can influence both the growth of the microbes and the physiology of the insect. Many fungal species and some *Bacillus*, as well as *Erwinia* species, favor growth at 30°C.
- *The rearing conditions.* Crowded conditions may induce more trauma in flies. Reduced food amount and contaminated medium can also lead to immune-compromised larvae or adult flies. Thus, all lines to be tested should be taken from healthy stocks to minimize pre-existing disadvantages.

Unfortunately, there is no rigorous analysis of these parameters and their influence on the experimental outcome. Differences in infection procedures can lead to divergent conclusions—and may explain a number of contradictory interpretations of results obtained in different laboratories. However, the possibility to infect a high number of flies over a short time course allows the comparison of many samples in one experiment.

Axenic larvae and adult

For different purposes, it can be informative to rear flies under axenic conditions, namely to assess the role of an infection, in the absence of other living microbial contamination. None the less, even if axenic conditions can eliminate other living microorganisms besides the one introduced experimentally, it does not limit the effect of dead fungi as well as tissue contamination that can elicit an immune response. Rearing axenic flies begins by collecting embryos in a small basket (without contamination by larvae), sterilizing them by dechorionation in 50% chlorox bleach for 5 min, rapid washes in sterile water, then in 60% ethanol. They are eventually transferred in a drop of ethanol solution into recently autoclaved glass vials that contain standard fly medium. Note that the development of axenically raised *Drosophila* is severely delayed and asynchronous.

◆◆◆◆◆◆ MEASURING SURVIVAL TO MICROBIAL INFECTION

It is clear that *Drosophila* can be used as a model to study the pathogenicity of microbes, including the mechanisms by which they kill and the mechanisms by which they escape the host immune responses. Alternatively, weakly pathogenic strains can be useful in analyzing the host defense in immuno-deficient mutants. Microbial infection and stress also induce various physiological and behavioral modifications in a fly (e.g. delay or acceleration of metamorphosis, change of behavior) and this is a field that has not yet been investigated.

Survival results are highly dependent on the parameters described above. Previous studies have identified several classes of bacteria that exhibited different interactions with *Drosophila*. Highly pathogenic bacteria kill flies after injection of low doses (such as *Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus*) weakly pathogenic bacteria induce low lethality as in the case of *Erwinia carotovora*; and non-pathogenic strains *such as E. coli, Salmonella typhimurium, Bacillus megaterium* produce little lethality (Boman *et al.*, 1972; Flyg *et al.*, 1980; PT and BL unpublished data).

In our laboratory, survival experiments are carried out in the same conditions for each fly sample tested. Groups of 20 or more 2–4-day-old adults are infected in the same conditions (methods, needle, experimenter, time) and transferred to a fresh vial every 2 to 3 days to ensure fresh growth conditions. Flies that die within 3 h after infection are excluded from the analysis (less than 5% as a norm, see above). Previous observations showed that survival rates may depend on the genetic background. For example, we noted that some homozygous *ebony* (*e*) fly stocks exhibit a low viability after infection as reported by Flyg *et al.* (1980). In order to examine the survival due exclusively to the mutation under analysis, we chose mutated chromosomes carrying a minimal number of markers. The fly strain must also exhibit a good viability in the absence of immune challenge.

In the case of infected larvae, a common practice is to transfer them onto a Petri dish containing apple juice agar or fly medium (Ashburner, 1989) to facilitate the sorting of dead vs live animals. A basic survival count includes the number of pupae and adults that emerge.

◆◆◆◆◆◆ OBSERVING AND COUNTING BACTERIA INSIDE *DROSOPHILA*

We have little information about the fate of bacteria in *Drosophila* larvae and adults. The use of bacteria carrying genomic mutation that confers antibiotic resistance (e.g. rifampicine) is a way to monitor the amount of bacteria introduced into the host. Larvae and adults are collected and washed in water, briefly sterilized in 70% ethanol to eliminate bacteria sticking to the external cuticle, transferred to microfuge tubes containing

LB medium and homogenized. Dilutions are then plated on LB plates with antibiotic selection for bacterial count.

Due to the transparent cuticle of larva, as well as to the ease of dissecting both larvae and adults, GFP reporter gene can be used to monitor bacteria within the host. With a GFP-expressing strain of *Ecc15*, we can observe the localization of live bacteria in larvae of different mutant backgrounds under epifluorescent illumination at high magnification (Basset *et al.*, 2000). We can also study the exact distribution of the bacteria in dissected tissues. Finally, microbes can be observed using classical histology techniques.

◆◆◆◆◆◆ MEASURING *DROSOPHILA* ANTIMICROBIAL PEPTIDE GENE EXPRESSION

In response to infection, *Drosophila* produces a battery of peptides that exhibit distinct activity spectra. This response is regulated at the transcriptional level and AMP gene expression is regulated by distinct signaling cascades that are evolutionary conserved (TOLL, IMD) (reviewed in Khush and Lemaitre, 2000).

The patterns of expression can be classified in three groups (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000): (i) Systemic response injection of microbes into the body cavity induces a strong expression of AMP genes in the fat body and a low expression in a fraction of hemocytes. (ii) Local response; recent studies have shown that many epithelia can express a subset of AMP genes, and that the expression can be enhanced upon natural microbial infection. (iii) Constitutive expression; a number of tissues constitutively express AMP genes (e.g. *drosomycin* is constitutively expressed in the spermathecae of females).

Analysis of transcripts by Northern blots together with the use of reporter genes are the most common ways to monitor the pattern of AMP gene expression. Direct analysis of peptide expression is more troublesome due to the difficulty of obtaining good antisera against these small cationic peptides. Fortunately, HPLC chromatography and Maldi-Tof mass spectrometry have been useful for purification and monitoring of these peptides (for more details on these methods see Hetru and Bulet, 1997; Uttenweiler-Joseph *et al.*, 1998).

Northern blot

This technique has been extensively used to analyze the kinetics of infection-induced AMP gene expression. Total RNA is extracted from samples of 20 flies treated by standard procedures. The possibility to quantify radioactive hybridization signals using a Phosphorimager and to successively re-probe the same nylon or nitrocellulose membrane with the various AMP cDNAs probes makes this approach suitable to compare the expression of AMP genes. cDNA encoding Actin or rp49 are generally used as internal controls since their expression is not modulated by the

immune response. Northern blot of Poly(A^+) RNA, or RT-PCR is used to monitor the expression of genes that are weakly expressed.

Reporter genes

The use of reporter genes is an informative method to analyze the expression pattern of *Drosophila* immune genes. However, it is important to ascertain that the reporter gene faithfully reproduces the pattern of endogenous gene expression.

LacZ reporter genes

Many *Drosophila* lines carrying a *P*-transgene wherein various AMP gene promoter sequences are fused upstream of *lacZ* have been described (*diptericin-lacZ*, *cecropin-lacZ*, *drosocin-lacZ*, *drosomycin-lacZ* (Charlet *et al.*, 1996; Engstrom *et al.*, 1993; Manfruelli *et al.*, 1999; Reichhart *et al.*, 1992)). In addition, *P-lacZ* enhancer trap insertions in *cactus* and *thor* loci that are inducible upon bacterial challenge have also been reported (Bernal and Kimbrell, 2000; Nicolas *et al.*, 1998). These lines allow the analysis of the expression pattern of the corresponding genes by X-gal staining, and the quantification of the expression level by titration of *lacZ* activity.

X-gal staining

This method provides an easy way to study reporter gene expression in larval or adult tissues. Dissect larvae (or adults) in PBS and quickly place the dissected tissues in a small basket immersed in 1 × PBS on ice. Fix 5 to 10 min in 1 × PBS with 0.5 or 1% glutaraldehyde on ice. Wash three times in 1 × PBS on ice. Incubate at 37°C or room temperature in staining buffer from 10 min to overnight. Staining buffer is obtained by adding 30 µl of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside, 5% in dimethylformamide) per ml of staining solution (10 mM NaH_2PO_4/Na_2HPO_4 pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3.5 mM K_3FeCN_6, and 3.5 mM K_4FeCN_6). Pre-incubation at 37°C and centrifugation of this solution can prevent the formation of undesirable crystals. Staining solution can be stored for several months at 4°C. *Drosophila* expresses an endogenous galactosidase in the midgut and few other tissues, the optimal pH of which is 6.5.

β-galactosidase titration

Five larvae, pupae or adults carrying a *lacZ* reporter gene are collected at different time intervals after infection and homogenized in 500 µl of Z Buffer pH 8.0 (stored at −20°C; 60 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-Mercaptaethanol, adjust pH to 8). After a 10 min centrifugation, the supernatant is collected and vortexed. Protein concentration is estimated by classical methods such as the Bradford assay using BSA as a protein standard. *lacZ* activity is determined by measuring the OD at 420 nm in a cuvette incubated at 37°C containing an appropriate

volume of the supernatant mixed with Buffer Z + o-nitrophenol-β-D-galactoside (ONPG) ($[ONPG]_{final} = 0.35\,mg\,ml^{-1}$). Three or more independent measurements for each test are necessary. According to Miller (1972), β-galactosidase activity $= ((\Delta OD)/\Delta T_{min})_V/$(protein concentration in v) $\times 1/0.0045$. The use of microtiter dishes allows the measurement of 100 samples at once.

GFP-reporter genes

Lines carrying AMP gene promoters fused to GFP have recently been described (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000). Thus the expression of AMP genes can be monitored in living larvae and adults. The use of GFP reporter genes has revealed the complex expression patterns of the AMP genes. This is due to the ability to analyze the expression of many AMP genes in large samples of individuals and in tissues that are less accessible to classical staining methods, such as the trachea. GFP-expressing *Drosophila* are analyzed directly under a stereomicroscope (e.g. Leica MZFLIII) equipped with epifluorescent illumination (excitation filter 480/40 nm; dichroic filter 505 nm; emission filter 510 nm). Analysis with GFP reporter gene has two major drawbacks. In order to fluoresce, the protein requires cyclization, which results in a lag time and thus GFP detection occurs long after that of *lacZ* β-galactosidase. Second, GFP activity is difficult to quantify although quantification of a *drosomycin-GFP* reporter expression has been used to screen for regulators of the immune response using a spectrophotometer (Jung *et al.*, 2001).

It is thus clear that GFP and *lacZ* reporter genes are complementary tools. Lines carrying both a *drosomycin-GFP* and a *diptericin-lacZ* reporter gene on the X chromosome are currently used to monitor the pattern of expression of both AMP genes in the same animal (Manfruelli *et al.*, 1999).

◆◆◆◆◆◆ PROTEIN ANALYSIS

One advantage of *Drosophila* is the possibility to collect sufficient material from larvae or adults to perform basic biochemical experiments. Larval fat body can easily be isolated (for large-scale collection, see Ashburner, 1989), although the fat body of adults is a loose tissue difficult to excise. However, careful preparation of the adult abdominal dorsal carcass provides predominantly fat body. RNA from such preparations contains minor contamination of epidermal and muscle RNA. The collection of either adult carcass or larval fat body has allowed the analysis of the degradation of Cactus and of the processing of Relish upon infection (Nicolas *et al.*, 1998; Stöven *et al.*, 2000).

Several protocols have been described for immunolocalization of proteins in the fat body (Ip *et al.*, 1993; Lemaitre *et al.*, 1995b; Rutschmann *et al.*, 2000b; Stöven *et al.*, 2000). In our lab, we proceed as follows. Fat bodies are dissected on ice in 1 × PBS and transferred to a small basket immersed in 1 × PBS in 24-well titer plates used in cell

culture. Do not let the tissue dry. All subsequent steps are performed under moderate agitation. Fat bodies are fixed in 4% paraformaldehyde, 2 mM $MgSO_4$, 1 mM EGTA and 0.1 M PIPES buffer for 15 min at room temperature and rinsed in 1 × PBS at 4°C. A brief subsequent fixation at 4°C in 0.5% glutaraldehyde/1 × PBS for 20 s or less can prevent the fat bodies from degradation by Triton. Three 5 min washes in 1 × PBS are followed by permeabilization for 2 h in PBT-A (1% BSA , 0.1% Triton X-100 in PBS). The primary antibody is applied at an appropriate dilution in PBT-A and incubated overnight at 4°C. The preparation is then washed three times for 30 min in PBT-B (0.1% BSA , 0.1% Triton X-100 in PBS). The secondary antibody, usually linked to alkaline phosphatase, is first pre-adsorbed on fixed fat body, then diluted and applied for 4 h to the sample in PBT-B at room temperature. The preparation is fixed for 10 min in 0.5% glutaraldehyde/1 × PBS, washed three times in AP-Sol (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Triton X-100) and incubated for 2 h in the staining solution (0.34 mg ml^{-1} NBT, 0.17 mg ml^{-1} X-Phosphate in AP-Sol). The fat bodies are subsequently mounted in glycerol/ethanol (1/1) on glass slides.

◆◆◆◆◆◆ ANALYSIS OF BLOOD CELL FUNCTIONS

In *Drosophila*, hematopoiesis and blood cell types exhibit specific features according to the developmental stage. During embryogenesis, a macrophage population differentiates in the anterior mesoderm and rapidly migrates to colonize the whole embryo (Tepass *et al.*, 1994). A second blood cell population appears simultaneously in the foregut region that corresponds to crystal cells (Lebestky *et al.*, 2000). At the end of embryogenesis, the larval hematopoietic organ differentiates anteriorly on the dorsal vessel.

During the three larval stages, most of the circulating blood cells are produced by the hematopoietic organ, the lymph glands, that are composed of a variable number of paired lobes along the dorsal vessel (Lanot *et al.*, 2001; Rizki and Rizki, 1984; Shresta and Gateff, 1982). The vast majority of larval circulating hemocytes consist of small round cells, called plasmatocytes, that are characterized by strong phagocytic capacity. Less than 5% of the hemocytes are crystal cells, with typical crystalline inclusion, that are proposed to contain the enzymes and substrate necessary for melanization reactions. A third cell type that only differentiates under given immune conditions is the lamellocyte, a large flattened cell devoted to encapsulation. Such an immune reaction occurs when an invader is too large to be phagocytosed, as is the case for wasp parasitization. At the onset of metamorphosis, the circulating plasmatocytes become highly active macrophages that ingest histolyzing larval tissues. In the lymph glands, large numbers of such phagocytes differentiate in all lobes and are released from the glands. The latter are empty 15 h after pupariation, and subsequently, a typical hematopoietic organ cannot be identified in pupae or adults.

In adults, the only circulating blood cell type is the plasmatocyte: crystal cells and lamellocytes do not differentiate at this stage.

Observation of *Drosophila* hemocytes

In embryos

Embryonic hemocytes can be identified with specific enhancer trap lines or antibodies. Two *lacZ* enhancer trap lines are commonly used: line 197 (Abrams *et al.*, 1992) and line E8-2-18 (Hartenstein and Jan, 1982): the expression of lacZ is detected by the classical reaction using X-gal substrate (Ashburner, 1989), or with anti-β-galactosidase antibodies. Three antibodies directed against hemocyte-specific antigens are available to date. These antigens are peroxidasin, a protein combining both peroxidase and extracellular matrix motifs, which was proposed to participate in extracellular matrix consolidation and in defense mechanisms (Nelson *et al.*, 1994), croquemort, a CD36 homolog that is required for phagocytosis of apoptotic cells by embryonic macrophages (Franc *et al.*, 1996; Figure 27.2A), and lozenge, a Runt-domain transcription factor that in embryos is specifically expressed in crystal cells (Lebestky *et al.*, 2000).

In larvae

It is easy to obtain hemocytes from larvae, especially at the third instar. Individuals are washed in distilled water and dried on a filter paper, then punctured posteriorly and gently squeezed to deposit a droplet of hemolymph (<1 µl) on a poly-lysine coated glass-slide. After 5 min drying, the preparations are fixed for 5 min in a 1% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) solution and mounted in glycerol gelatin then observed by interference phase contrast microscopy (Figure 27.1B). They can also be stained for 1 min in an aqueous 1% toluidin blue/0.1% eosin solution, washed in 95% ethanol, successively transferred to 100% ethanol and to xylene, and finally mounted in Eukitt.

To better visualize hemocytes, or to detect specific cell types, it is possible to use *lacZ* transgenic fly lines. A transgenic strain in which all hemocytes express *lacZ* was produced by Govind (1995). Lineage specific enhancer trap lines were reported by Braun *et al.* (1997), at least for plasmatocytes and lamellocytes (Figure 27.1 C,D).

Method: larvae are washed and dried, then punctured to deposit hemolymph on a glass coverslip. After 5 min drying, the preparations are fixed for 30 s in a 0.5% glutaraldehyde/PBS solution. Staining is in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 3.5 mM $K_4Fe(CN)_6$, 3.5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7.2, overnight at 37°C. Preparations are mounted in glycerol gelatin for observation.

Crystal cells can be visualized in whole animals by heating them at 70°C for 10 min in a water bath (Rizki *et al.*, 1980). This induces specific

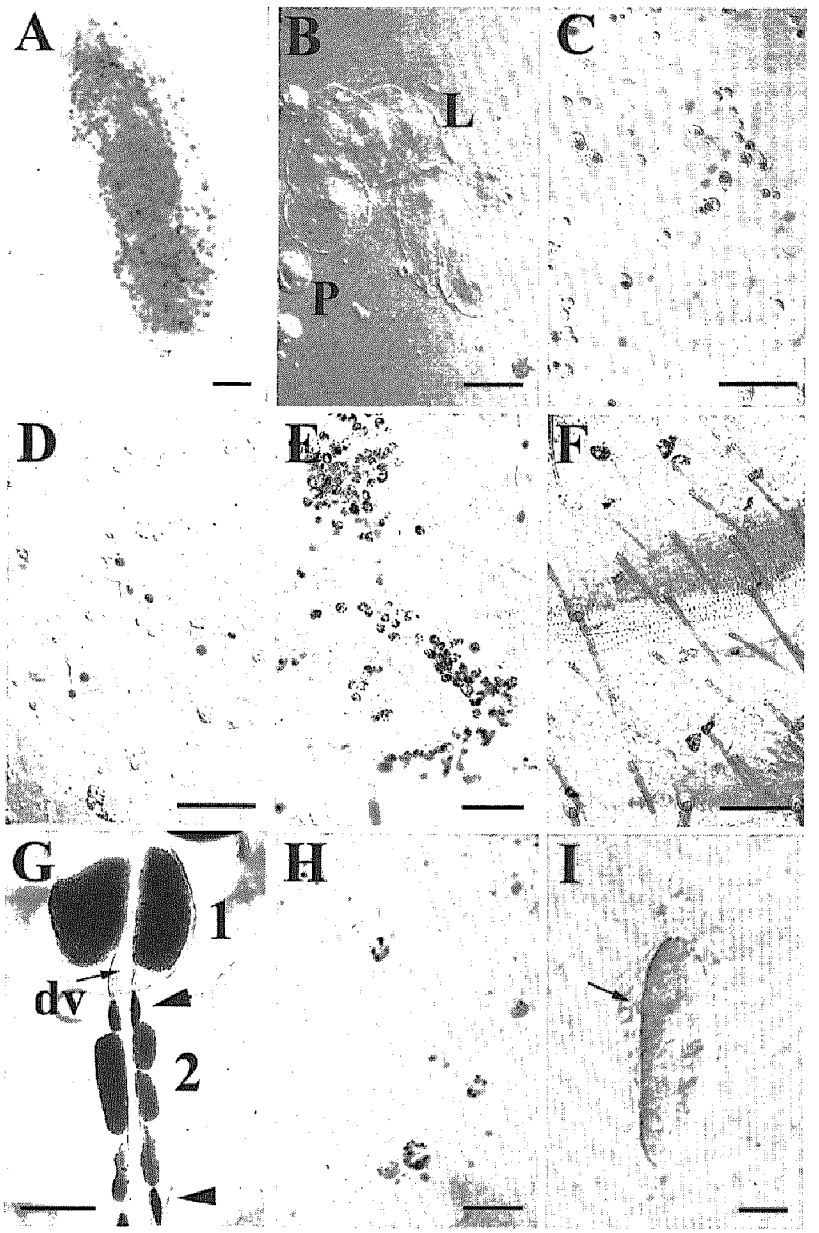

Figure 27.2. Blood cells in *Drosophila*. (A) Distribution of hemocytes in a late embryo, as evidenced by anti-croquemort antibody. Bar: 50 μm. (B) Larval blood cells observed by interference phase contrast microscopy. P: plasmatocyte; L: lamellocyte. Bar: 20 μm. (C, D) *lacZ* expression in larval hemocytes: staining in plasmatocytes in line *l(3)05309* (C) and in lamellocytes in line *l(3)06946* (D) (Braun *et al.*, 1997). Bar: 50 μm. (E, F) Observation of sessile hemocytes through the cuticle after Indian ink injection into a third instar larva (E) or into a *yellow* adult (F). Bar: 50 μm. (G) Dissection of a larval lymph gland attached to the dorsal vessel and stained with osmium tetroxide. Arrow heads: pericardial cells; 1 and 2 designate the first and second lobes of the lymph glands: the second lobes are well developed when larvae are raised at 18°C; dv: dorsal vessel. Bar: 100 μm. (H) Indian ink phagocytosis by larval plasmatocytes observed 2 h after injection. Bar: 20 μm. (I) Encapsulated *L. boulardi* egg in larval hemocoel 24 h after parasitization. The wasp egg is surrounded by lamellocytes (arrow); blackening has not yet occurred. Bar: 50 μm. (This figure is also reproduced in colour between pages 276 and 277.)

blackening of crystal cells that become easily visible in circulation and within the hematopoietic organ, although they disappear during the early hours of metamorphosis.

Immunohistochemistry can be performed on an air-dried droplet of hemolymph following the protocol below.

Method: fixation is carried out for 5 min in 4% paraformaldehyde, or 4% glutaraldehyde, or in a mix (4% paraformaldehyde; 0.5% glutaraldehyde for instance) in 0.1 M phosphate buffer or in PBS on ice. The fixation conditions depend on the antigen. After three 10 min rinses in PBS-0.1% Tween-20 (PBT), saturation is achieved with 3% pre-immune serum in PBT, for 1h at room temperature. Primary antibody at the appropriate dilution is applied in 3% serum/PBT overnight at 4°C, then after three 10 min rinses in PBT, the preparation is treated with secondary antibody (commercial, usually diluted 1/100 to 1/500) in 3% serum/PBT, 1 h at room temperature. Depending on the secondary antibody (fluorescent, alkaline phosphatase or peroxidase), the samples are processed according to the selected system.

In situ hybridization technique for blood cells is adapted from Tautz and Pfeiffle (1989), with modifications from J.M. Reichhart and G. Grossnibach.

Method: Glass slides are boiled for 5 min in distilled water with soap, rinsed successively under tap water, distilled water and in 95% ethanol before sterilization at 180°C. Hemocytes are deposited on the glass slide, then fixed in Carnoy's medium for 10 min. The preparation is rinsed for 2 min in 95% ethanol and stored (up to several months) at −20°C in absolute ethanol. Before further treatment, the slides must be rehydrated for 2 min in 70% ethanol and 2 min in PBS. For proteinase K treatment, 50 μl of freshly thawed proteinase K are added to 50 ml prewarmed PBS (25 μg ml^{-1} final) and digestion on the slides is allowed to proceed at 37°C for 5 min. Proteinase K digestion is crucial for the procedure and every new batch should be tested first for incubation time. Digestion is arrested by 2 min in 2 mg ml^{-1} glycine in PBT followed by 5 min in PBT. A post-fixation step of 20 min in PBT/5% formaldehyde is followed by a 5 min rinse in PBT, and acetylation. Acetylation solution is obtained by mixing 20 ml of 1 M triethanolamine with 180 ml water, to which 500 μl acetic anhydride are rapidly added. The slides are immediately incubated in the solution for 10 min, then dehydrated in graded ethanol and air dried. For hybridization, the probe is denatured by 4 min boiling then chilling on ice-water: 20 μl of hybridization solution (hybridization solution: 50% deionized formamide; 5 × SSC; 100 μg ml^{-1} salmon sperm; 100 μg ml^{-1} tRNA *E. coli*; 50 μg ml^{-1} heparine; 0.1% Tween-20, in water) containing 2 μl of probe (10 ng of dig-labeled DNA) are deposited on the glass slide. The reaction is covered with an 18 × 18 coverslip and sealed with rubber cement. Hybridization is allowed to proceed at 48°C overnight in a humid chamber. Next day, the coverslip is removed in hybridization solution, washes are successively for 20 min in hybridization solution at 48°C, for

20 min in 50% hybridization solution/50% PBT at 48°C, for 20 min in PBT at 48°C and three times for 5 min in PBT at room temperature. 300 μl of pre-adsorbed anti-dig antibody at appropriate dilution in PBT are deposited on the preparation, covered with a large coverslip and incubation is performed at room temperature for 2 h in a humid chamber. After four 5 min rinses in PBT, it is possible to introduce an amplification step (Vectastain kits) if a low signal is expected. Detection of the signal follows two 2 min rinses in levamisol-AP-9.5 buffer (100 mM NaCl; 50 mM $MgCl_2$; 100 mM Tris pH 9.5; 1 mM levamisol ; 0.1% Tween-20). The staining mixture contains 1 ml levamisol-AP-9.5 buffer, 4.5 μl NBT (Nitro-blue tetrazolium 75 mg ml^{-1} in 70 DMF/water) and 3.5 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate 50 mg ml^{-1} in DMF). For staining, 300 μl of this solution are added to the slide and incubated in a humid chamber at room temperature. Incubation time varies between 15 min and 16 h depending on the probe, with replacement of the staining solution every 2 h, which should greatly reduce the background. The reaction is stopped by 2 min in PBS and 10 min in water, the preparation counter-stained with 0.5% acridine orange in water and mounted in glycerol gelatin.

In adults

Adult hemocytes are more difficult to analyze as it is difficult to obtain hemolymph at this stage; moreover the hemocytes are mostly attached to internal tissues. An efficient way to visualize them is to inject Indian ink into the hemocoel (see below): the nested plasmatocytes take up the Indian ink particles and become visible through the epidermis, in adults as well as in larvae (Figure 27.2 E, F).

Observation of *Drosophila* larval hematopoietic tissue

Lymph glands are very often lost in crude dissections of larvae, as they are tiny and loose. To preserve them for observation, we dissect larvae in PBS, ventral part facing up, after pinning them anteriorly and posteriorly with thin needles. The abdominal integument is cut medio-longitudinally in order to open the larva like a book, exposing the different organs in place. The brain complex is toppled over anteriorly. The trachaea, fat body and gut are successively removed, without pulling, but by delicately detaching them from the nervous and tracheal networks. Thus the dorsalmost organs are displayed: the two dorsal tracheal trunks framing the dorsal vessel, and, anteriorly, the paired lymph gland lobes (see Figure 27.1 G).

Immunohistochemistry can be performed on such preparations, which are fixed for 5 min in 4% formaldehyde/0.5% glutaraldehyde in PBS, washed several times in PBS, then blocked for 2 h in 3% serum/PBT, and incubated overnight in primary antibody. The following steps are as previously described above.

Measure of phagocytosis

Plasmatocytes are the most abundant hemocytes, and they are very efficient phagocytes. Several techniques have been developed to visualize phagocytosis.

Method: The injected material is either Indian ink (Pébéo, Gemenos, France) diluted 1/30 to 1/60 in PBS ((Lanot *et al.*, 2000), Figure 27.2 H), GFP-labeled bacteria (Basset *et al.*, 2000), FITC-labeled bacteria (Elrod-Erickson *et al.*, 2000) or fluorescent polystyrene beads (Elrod-Erickson *et al.*, 2000) at the appropriate dilution. Injection is carried out with a Nanoject injector (Drummond); the volume is usually 40–50 nl per individual. Whereas injection into adults can be done under the routine fly room dissecting microscope into the thorax of anesthesized flies, injection in larvae requests more precaution. The injector is fixed under the dissecting microscope. The larva is impaled onto the needle while keeping it under tension with a pair of tweezers. Ideally, the needle should penetrate the larva in the last intersegmental space at the rear. Before removing the needle after the injection, it is recommended to wait a couple of seconds to allow the injected volume to diffuse throughout the larva. It is often necessary to humidify the contact zone between the needle and the cuticle to prevent damage to the cuticle. Once the larva has been removed from the needle with a paint brush, it is deposited on a humid filter paper. Injected adults or larvae can be examined as soon as 2 h later for phagocytosis, directly under the dissecting microscope. In the case of adults, ink-labeled plasmatocytes are best observed in a *yellow* background.

Measure of encapsulation

Differentiation of lamellocytes is induced when parasitoid wasps lay eggs in *Drosophila* larvae. Some 50 drosophilid parasitoids have been reported (Carton *et al.*, 1986), among which *Leptopilina boulardi* has been well investigated. Wasp eggs are deposited in second instar larvae and, in resistant flies, the parasitoid egg is rapidly neutralized by encapsulation/melanization (Figure 27.2 I).

Method: We use 2–10-day-old *L. boulardi* adults which have not been in contact with *Drosophila* over the last 48 h. *Drosophila* 24 h egg-laying from ca. 50 females are allowed to develop until the second larval instar. 20 *L. boulardi* females and five males are added to the vial for 4 h, then withdrawn. This treatment results in an efficient parasitization rate of the larvae, and black capsules can be seen on live individuals 48 h later. Differentiation of lamellocytes is already detected 10 h following wasp parasitization. It is better ascertained with the use of a lamellocyte-specific enhancer trap line such as *l(3)06946* (Braun *et al.*, 1997).

Measure of melanization

Humoral melanization is a function of blood cells in *Drosophila*. Melanin formation involves the activity of phenol and polyphenol oxidases that

catalyze the conversion of tyrosine to DOPA, dopamine, N-acetyldopamine, quinone and subsequently melanin. Tests for phenoloxidase activity in cell-free hemolymph and blood cells have been described in detail (Shresta and Gateff, 1982). Two very simple tests are given below.

Assay of phenoloxidase activity

Filter paper is soaked with 10 mM phosphate buffer (pH 6.5) containing 2 mg ml^{-1} L-DOPA (L-3,4-dihydroxyphenylalanine, Sigma) and phenoloxidase activity is detected in blood samples by dropping the hemolymph of a single larva on the paper. For quantification of phenoloxidase activity, 3 μl hemolymph samples are added to 50 μl of 10 mM phosphate buffer (pH 5.9) containing 10 mM L-DOPA, and the OD is recorded for 30 min at 470 nm.

◆◆◆◆◆◆ MUTATIONS AFFECTING *DROSOPHILA* IMMUNE RESPONSE

Several mutations affecting different components of *Drosophila* immune response have been described (listed in Table 27.1). More information on these genes can be found on Flybase (http://flybase.bio.indiana.edu:82/). A more extensive list of genes involved in *Drosophila* immunity is available on the web (http://www.cnrs-gif.fr/cgm/immunity/Drosophila_immunity_genes.html).

◆◆◆◆◆◆ *DROSOPHILA* BLOOD CELL LINES

Several cell lines are available from *Drosophila* that either exhibit hemocyte features (capability of phagocytosis, inducibility of defense genes, expression of hemocyte markers), or are derived from tumorous hemocytes. For the analysis of immune gene regulation, the most commonly used cell lines are SL2 (Schneider, 1972) and mbn-2 cells (Gateff *et al.*, 1980), in which endogenous antimicrobial genes can be induced by bacteria or LPS treatment (Kappler *et al.*, 1993; Samakovlis *et al.*, 1992). The well-known Kc cell line was tested independently in several laboratories and, as no immune-inducible gene expression was evidenced, it has not been exploited so far as a model system for immunity. Kc cells, however, produce many proteins constitutively expressed by blood cells (Fessler *et al.*, 1994).

Transfected SL2 and mbn-2 cells have been successfully used in a number of studies to dissect antimicrobial gene promoters, to analyze interactions between immune transactivators and promoters, or to investigate protein/protein interactions between various immune effectors (Engström *et al.*, 1993; Han and Ip, 1999; Kappler *et al.*, 1993; Kim *et al.*, 2000; Silverman *et al.*, 2000; Stöven *et al.*, 2000; Tauszig *et al.*, 2000).

Table 27.1 List of mutations known to affect *Drosophila* immune response

L(3)*hem*, *dom*	Severe reduction of the hemocyte number and melanization	(Braun *et al.*, 1998; Gateff, 1994)
nec, *spz*, Tl, *tub*, *pll*, *cact*, *dif*, *dl*	These mutations affect the Toll pathway that regulates a subset of AMP genes and many other effector genes. This pathway is required for survival after fungal infection. Some of these mutations also alter cellular response (hemocyte count, lamellocyte differentiation). Mutations that constitutively activate or block this pathway have been described.	(Lemaitre *et al.*, 1996; Levashina *et al.*, 1999; Manfruelli *et al.*, 1999; Meng *et al.*, 1999; Qiu *et al.*, 1998; Rutschmann *et al.*, 2000a)
imd, dredd, rel, IKKγ	These mutations affect the Imd pathway which regulates a subset of AMP genes and is required for survival after Gram-negative infection. These mutations are homozygous viable and seem to have no apparent effect on the cellular response.	(Hedengren *et al.*, 1999; Lemaitre *et al.*, 1995a; Leulier *et al.*, 2000; Rutschmann *et al.*, 2000b)
Bc, *lz*	These mutations affect crystal cell function and block proPhenolOxidase activation.	(Lebestky *et al.*, 2000; Rizki and Rizki, 1984)
hop, stat92	These mutations affect components of the JAK-STAT pathway that controls the expression of complement-like protein as well as hemocyte differentiation and division. Mutations that constitutively activate or block this pathway have been described.	(Hanratty and Dearolf, 1993; Harrison *et al.*, 1995; Yan *et al.*, 1996)

◆◆◆◆◆◆ CONCLUSIONS

Most of the techniques described here are simple and do not require extensive skills or complex devices. Therefore, it requires very little to test the effect of each experimenter's favorite pathogen on *Drosophila*; if the pathogen shows an effect, powerful genetic and molecular tools can be applied to identify host factors that are the target of these micro-organisms. In this context, we should keep in mind that some of the medically important bacteria are also pathogenic for fly. In this case, *Drosophila* also provides an easy assay to screen mutated microbes for the loss of virulence factors.

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