

22

Drosophila Immunity

Methods for Monitoring the Activity of Toll and Imd Signaling Pathways

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Summary

Invertebrates lack an adaptive immune system and rely on innate immunity to resist pathogens. The response of *Drosophila melanogaster* to bacterial and fungal infections involves two signaling pathways, Toll and Imd, both of which activate members of the nuclear factor (NF)- κ B family of transcription factors, leading to antimicrobial peptide (AMP) gene expression. In this chapter, we present the current methods used in our laboratory to monitor the activity of both signaling pathways.

Key Words: *Drosophila* immunity; Toll and Imd signaling pathways; antimicrobial peptides; reporter genes; RT-qPCR; survival analyses.

1. Introduction

Innate immunity serves as a first-line defense against microbial invaders and is common to all metazoans. *Drosophila melanogaster*, the fruit fly, shows a potent host defense when challenged by various microorganisms. Studies on the fly immune response have provided evidence for similarities between mammalian and *Drosophila* innate immunity. Hence, this organism appears to be a suitable model system for studying the innate immune defense.

The *Drosophila* immune response consists of multiple cellular and humoral response mechanisms including activation of phagocytosis by specialized blood cells (hemocytes and plasmatocytes), melanization, coagulation, and

synthesis of antimicrobial peptides (AMPs) by the fat body (functional equivalent of the mammalian liver). Seven classes of inducible AMPs with activity spectra directed against bacteria or fungi have been identified in *Drosophila*. Their expression is controlled by members of the nuclear factor (NF)- κ B family transcription factors, which are activated by two evolutionary conserved signaling pathways, Toll and Imd (1,2). The Toll pathway is activated mainly by Gram-positive bacteria and fungi, whereas the Imd pathway mostly responds to Gram-negative bacterial infection (3,4).

In this chapter, we describe the techniques currently used in our laboratory to monitor the Toll and Imd pathway activities by measuring AMP gene expression after infection. We also include methods used to perform survival analyses, because inactivation of either of these pathways can lead to increased susceptibility to microbial infection.

2. Materials

2.1. Fly Strains

1. Wild-type flies: Canton^S and Oregon^R.
2. Toll pathway mutant strain: *spz^{rm7}/TM6C*.
spz^{rm7} is a null mutation generated by ethyl methane sulfonate (EMS) mutagenesis in *spätzle* that encodes the ligand of Toll (5). Several markers of the original stock (M317, Tübingen stock center) including *ebony* were removed by recombination (6). *spz^{rm7}* homozygous flies are viable, but females are sterile and lay dorsalized embryos.
 Other mutations affecting the intracellular components of the Toll pathway (*Tl*, *pll*, *tub*, *dif*, *MyD88*) can be used instead of *spz^{rm7}*.
3. Imd pathway mutant strain: *Rel^{E20}*, *e⁺* (3).
Rel^{E20} is a deletion of *relish* (that also affects a nearby gene), which encodes the NF- κ B targeted by the Imd pathway (7).
 Other mutations affecting the Imd pathway (*imd*, *dredd*...) may be used as positive controls.
4. Strains carrying reporter genes are described in **Table 1**.

2.2. Bacterial Strains

1. Gram-negative bacteria: *Escherichia coli* strain 1106, *Erwinia carotovora carotovora* 15 rif^R (*Ecc15*) and *Pseudomonas entomophila* rif^R. After oral infection, *Ecc15* induces a systemic immune response in larvae but not in adults (8), whereas *P. entomophila* is an entomopathogenic bacterium that can trigger a systemic immune response in both larvae and adults (9).

Table 1
Fly Strains Carrying Reporter Genes

Name	Genotype	Reporter genes	Chromosomal location	References
Dpt-lacZ	<i>P[Dpt - lac, ry⁺]; ry506</i>	Dpt-lacZ	I	(21)
DD1	<i>y, w, P[Dpt - lacZ, ry⁺], P[Drs - GFP, w⁺]</i>	Dpt-lacZ	I	(19)
Drs-lacZ	<i>w, P[Drs - lac, w⁺]</i>	Drs-lacZ	I	(19)
DIG	<i>w; P[Dpt - GFP, w⁺]D3 - 2, P[Dpt - GFP, w⁺]D3 - 4</i>	Dpt-GFP	III	(9)

Drs, Drosomycin; *Dpt*, Diptericin. Flies carrying each of these reporter genes in combination with Toll and Imd mutations are used as positive controls. The expression of the *Drs-lacZ* reporter gene is strong and shows a significant basal activity in larvae (19).

- Gram-positive bacteria: *Enterococcus faecalis* and *Micrococcus luteus*. *E. faecalis*, but not *M. luteus*, kills flies after injection (4).
- Fungi: *Beauveria bassiana* and *Candida albicans*. *B. bassiana*, but not *C. albicans*, kills flies after infection.

2.3. Buffers, Chemicals, and Primers

- Luria Bertani (LB) and YPGA (5 g of yeast extract, 5 g of Bacto Peptone, 10 g of glucose, and 15 g of agar per liter) media.
- Corn-meal fly medium, per liter: 8 g agar, 80 g polenta, 40 g yeast, 40 g sucrose, and 53.6 ml moldex.
- Malt-agar medium, per liter: 1 g peptone, 20 g glucose, 20 g malt extract, and 15 g agar.
- Apple juice medium, per liter: 17.5 g agar, 25 g sucrose, and 250 ml apple juice.
- Phosphate-buffered saline (PBS) 10×: 80 g NaCl, 11.5 g Na₂HPO₄, 2 g KH₂PO₄, 2 g KCl, water qsp 100 ml (adjust pH to 7.3/7.5 with 10 M HCl). Autoclave.
- Glutaraldehyde 25% solution.
- X-gal: 5-bromo-4-chloro-3-indolyl β-D-galactoside, 5% in dimethylformamide.
- Staining buffer: 10 mM NaH₂PO₄/Na₂HPO₄, 150 mM NaCl, 1 mM MgCl₂, 3.5 mM K₃FeCN₆, 3.5 mM K₄FeCN₆ (adjust to pH 7.2). Add 30 μl of X-gal per ml of solution (see Note 1).
- Z buffer: 60 mM Na₂HPO₄, 60 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol (adjust pH to 8 with NaOH).
- ONPG (*o*-nitrophenol-β-D-galactoside).
- TRIZOL[®] Reagent (Invitrogen Cergy Pontoise, France).
- Chloroform, isopropanol, and ethanol.
- Random hexamer primers, dNTP.
- SuperScript[™] II Reverse Transcriptase (SSII-RT), RNaseOUT[™] Recombinant Ribonuclease Inhibitor (RNaseOUT[™] RRI, Invitrogen).

Table 2
Sequence of Oligonucleotide Primers Used for Quantitative PCR (26)

Primer name	Gene	5'–3' Sequence	PCR efficiency
DiptericinF	Dpt	GCTGCGCAATCGCTTCTACT	1.9864
DiptericinR	Dpt	TGGTGGAGTGGGCTTCATG	
DrosomycinF	Drs	CGTGAGAACCTTTTCCAATATGATG	1.9908
DrosomycinR	Drs	TCCCAGGACCACCAGCAT	
Ribosomal protein 49F	rp49	GACGCTTCAAGGGACAGTATCTG	1.891
Ribosomal protein 49R	rp49	AAACGCGGTTCTGCATGAG	

PCR efficiency has been set up for each couple of primers using the LightCycler® 2.0 System.

15. LightCycler® (LC) 2.0 System, LC FastStart DNA Master SYBR Green I, LC Capillaries, LC Carousel Centrifuge 2.0 (Roche Neuilly sur Seine, France).
16. Oligonucleotide primers (see **Table 2**).

3. Methods

The patterns of AMP expression can be classified into three categories (**10**). (1) Systemic response: Injection of microbes into the body cavity or natural infection by specific microbes (*B. bassiana*, *Ecc15*, *P. entomophila*) induces a strong expression of AMP genes in the fat body and a low expression in a fraction of hemocytes. (2) Inducible local response: Studies have shown that many epithelia can express a subset of AMP genes and that the expression can be enhanced upon natural bacterial infection via the Imd pathway (**8, 9, 11**). (3) Constitutive local expression: Several tissues constitutively express AMP genes [e.g., *Drosomycin* (*Drs*) is constitutively expressed in the spermathecae of female flies]. This response is not regulated by the Toll or the Imd pathway (**12**).

Monitoring AMP gene expression is one of the easiest ways to determine Toll and Imd pathway induction. The *Diptericin* (*Dpt*) gene encodes an antibacterial peptide secreted by the fat body in response to Gram-negative bacterial infection. This gene is tightly regulated by the Imd pathway, and its expression profile provides an accurate readout of Imd pathway activity. *Drs* encodes an antifungal peptide. In contrast to *Dpt*, which is exclusively regulated by the Imd pathway, *Drs* is largely regulated by the Toll pathway but is also partially induced by the Imd pathway after Gram-negative bacterial infection (**3**). Both genes are generally used as reporter genes of Toll and Imd pathway activation.

Analysis of *Dpt* and *Drs* transcripts by real-time quantitative PCR (RT-qPCR) together with the use of reporter genes is commonly performed to monitor the pattern of AMP gene expression. The methods described below outline (1) the preparation of microbes, (2) the infection of larvae and adults flies, (3) the use of reporter genes, (4) the β -galactosidase titration, and (5) the RT-qPCR assays to check AMP gene expression.

Besides, mutants deficient in the Toll and/or Imd pathway succumb to microbial infection. Monitoring fly survival after infection is thus another way to estimate the activity of both pathways. Corresponding protocols are described in **Subheading 3.4**.

3.1. Preparation of Microorganisms

3.1.1. Bacterial Pellets

1. Inoculate 50 ml of LB medium or YPGA with the microorganism and let the culture grow overnight at 37°C, except for *P. entomophila*, *Ecc15*, and *C. albicans*, which grow at 30°C.
2. Concentrate microorganisms to $OD_{600nm} = 200$, except for *E. faecalis*, $OD_{600nm} = 30$.
3. Store pellets at 4°C for 1 week.

3.1.2. Fungal Spores

1. Spread *B. bassiana* spores on Petri dishes with malt-agar and incubate at 25–29°C. Use glass beads to obtain well-covered plates. The fungal hyphae will germinate after 3–5 days at 25°C.
2. After 10–30 days, check for the presence of dust-like spores. Store well-sporulated plates at 4°C for infection experiments and keep for 3 months.

To store *B. bassiana* spores, proceed as following:

1. Collect spores into a 50-ml vial by washing the Petri dishes with 10 ml of sterile water and separating spores from hyphal bodies through a funnel lined with glass fibers.
2. Centrifuge spores at $2600 \times g$ for 15 min at 4°C.
3. Quantify the number of spores per ml using a hemacytometer (generally around 10^9 to 10^{12} spores per ml).
4. Store spores in 20% glycerol for several months at 4°C or several years at –70°C.

3.2. Infection of *Drosophila* Larvae and Adults by Microorganisms

Infection of flies is performed either by introducing microbes directly into the body cavity or by natural infection without injury. Additional information on infection procedures can also be found in **ref. 13**.

3.2.1. Septic Injury

3.2.1.1. ADULTS

Infection of adult flies by septic injury requires the use of a thin metal needle (0.5 mm diameter) mounted on a small handle. Sterile injury of flies is used as an internal control to ensure that pricking has no effect on the studied mutants.

1. Dip the needle into a microbe solution.
2. Prick the lateral side of the thorax of a CO₂-anesthetized fly.
3. Separate the fly from the needle with a brush and put them in a clean vial containing corn-meal fly medium.

3.2.1.2. LARVAE

For larval challenge, a tungsten wire, previously sharpened in a 0.1 M NaOH solution by electrolysis, is used.

1. Wash third instar wandering larvae in water and place them in a small drop of water on a black rubber block.
2. Prick larvae on their posterior lateral side.
3. Transfer larvae to a Petri dish containing apple juice medium and seal the plate carefully.

Following pricking, a dark spot appears at the site of injury corresponding to activation of the melanization cascade, which is a good way to ensure that the flies/larvae have been infected. Larvae are more sensitive to septic injury than adults. In some cases, septic injury can be replaced by injection (*see Note 2*).

3.2.2. Natural Infection

Natural infection is a way to infect *Drosophila* in the absence of artificial injury. It relies on oral ingestion of bacteria or on covering flies with spores of invasive fungi.

3.2.2.1. BACTERIAL NATURAL INFECTION

Natural infection is performed with the bacterial strains *Ecc15* or *P. entomophila*. *Ecc15* is not lethal for *Drosophila* and induces a strong systemic immune response only in larvae, whereas in adults it stimulates local expression of AMPs in several epithelial tissues. Conversely, *P. entomophila* is able to induce a local and systemic immune response in both adults and larvae but in addition leads to death of wild-type larvae and flies.

3.2.2.1.1. Adults

1. Dehydrate adults for 2 h in a dry vial in the absence of food.
2. Put a filter paper into a vial with corn-meal fly medium.
3. Hydrate filter paper with a mix of 100 μ l of a bacterial pellet ($OD_{600nm} = 200$) and 100 μ l of a food solution containing a 5% sucrose solution.
4. Transfer flies into the prepared vial and incubate at 25°C.

It is possible to rehydrate the paper each day with additional food solution.

3.2.2.1.2. Larvae

1. Put 200 μ l of a bacterial pellet ($OD_{600nm} = 200$) and 400 μ l of crushed banana into a 2-ml microfuge tube.
2. Place approximately 200 third instar larvae in this tube.
3. Mix thoroughly by shaking the tube.
4. Insert a piece of foam into the tube to prevent larvae from wandering away from the bacterial mixture and to ensure air supply at the same time.
5. Place the tube at room temperature for 30 min.
6. Transfer the larvae together with the bacteria to a vial containing a standard corn-meal fly medium and incubate at 29°C.

3.2.2.2. FUNGAL NATURAL INFECTION

Fungal natural infection is usually performed with the entomopathogenic fungus *B. bassiana*, which has the ability to cross the cuticle of insects, through the secretion of proteases and lipases, and causes a significant mortality in wild-type adult flies (**14,15**).

1. Anesthetize adult flies and place them on a Petri dish containing a sporulating fungus.
2. Hand-shake thoroughly for 30 s to cover the flies with spores.
3. Transfer infected flies to a clean corn-meal fly medium and incubate at 29°C. Vials should be changed every other day.

Larvae can also be rolled on sporulated plates.

Several parameters can influence the infection process (*see Note 3*). In cases where the pathogens are lethal for mutant flies, it is recommended to use non-living compounds instead of the entire microorganisms, such as peptidoglycans purified from bacteria (**16,17**), which are good elicitors of the immune response (*see Note 4*).

3.3. Measuring *Drosophila* AMP Gene Expression

AMP gene expression is measured on samples collected at different time points, according to the expression peak of the monitored gene and the type

of infection. Moreover, previously characterized mutants affecting different components of *Drosophila* signaling pathways, like *spz^{rm7}* and *Rel^{E20}*, are included in each experiment as internal controls. **Table 3** gives the time points at which flies or larvae should be collected for a time course study. Because the range of AMP gene expression can vary from almost undetectable expression to high levels of transcription, it is essential to include unchallenged and bacterial-challenged wild-type flies in each experiment to determine the expression range of the monitored AMP. It is also important to perform experiments applying the same conditions (sex, age of flies, and time of day; see **Note 5**). Combining two independent methods (e.g., reporter genes and RT-qPCR) is the best way to obtain a significant result.

3.3.1. Reporter Genes

The use of reporter genes is an informative method to analyze the expression pattern of *Drosophila* immune genes. Hence, they are the best tools to monitor local expression of AMP genes, which is largely controlled by the Toll and Imd pathways. It is important to ascertain, however, that the reporter gene precisely reproduces the pattern of endogenous gene expression.

3.3.1.1. LACZ REPORTER GENES

Drosophila lines carrying a *P*-transgene wherein AMP gene promoter sequences are fused upstream of *lacZ* (see **Table 1**) allow the analysis of the expression patterns of the corresponding genes by X-gal staining and the quantification of the expression levels by titration of LacZ activity.

Table 3
Time Points to Collect Flies and Larvae During a Microbial Infection

	Monitored peptide gene	<i>Septic injury</i>		<i>Natural infection</i>	
		Bacteria	Fungi	Bacteria	Fungi
Adults	<i>Drs</i>	6–12– 24 –48 h	24–48 h	–	24–48–72 h
	<i>Dpt</i>	1.5–3– 6–12 –24 h	–	6–12–24 h	–
Larvae	<i>Drs</i>	6– 12–24 h	6–12–24 h	–	24 h
	<i>Dpt</i>	1.5–3– 6–12 –24 h	–	24 h	–

Time points in bold letters correspond to the highest expression of each AMP gene when flies are infected by non-persisting microbes (3,27).

3.3.1.1.1. *X-Gal Staining*. This method provides an easy way to study reporter gene expression in larval or adult tissues (see **Note 6**).

1. Dissect larvae (or adults) in PBS and quickly place the dissected tissues in 1 × PBS on ice.
2. Fix 10 min in 1 × PBS with 0.5% glutaraldehyde on ice.
3. Wash three times in 1 × PBS on ice.
4. Incubate at 37°C in staining buffer until color develops (from 10 min to overnight).

3.3.1.1.2. *β-Galactosidase Titration*.

1. Collect three sets of five larvae or adults.
2. Incubate tubes at −20°C to freeze the flies (samples can be stored at this step for assaying later).
3. Thaw samples on ice.
4. Add 250 μl of buffer Z to each tube and homogenize for 30 s using an electric pestle or the PrecellysTM24 automated lyser (Berlin Technologies, Saint-Quentin en Yvelines, France).
5. Add 250 μl of buffer Z to each tube and quickly vortex the samples.
6. Centrifuge at 6000 × g for 5 min.
7. Collect the supernatant and estimate the protein concentration with classical methods such as the Bradford assay using bovine serum albumin (BSA) as a protein standard (samples can be stored for few hours on ice).
8. Aliquot 30 μl (*Dpt*) or 10 μl (*Drs*) of the samples into 96-well plates for the readout.
9. Add 250 μl of Buffer Z + ONPG ([ONPG]_{final} = 0.35 mg/ml) to each well rapidly and place plate at 37°C.
10. Monitor β-galactosidase at regular time intervals (2–30 min) by measuring the OD at 420 nm using a microtiter.

According to Miller (1972), the β-galactosidase activity equals $((\Delta OD) / \Delta T_{\min})_v / (\text{protein concentration in } v) / 0.0045$ (**18**).

3.3.1.2. GFP-REPORTER GENES

Lines carrying AMP gene promoters fused to GFP have been described (**10**, **11**). Thus, AMP gene expression can be monitored in living larvae and adults. GFP reporter genes can be useful to analyze gene expression in tissues that are less accessible with classical staining methods, such as the tracheae (see **Note 7**). GFP-expressing *Drosophila* are analyzed directly under a stereomicroscope (e.g., Leica MZFLIII) equipped with epifluorescent illumination (see **Note 8**). *GFP* and *lacZ* reporter genes are complementary tools. Lines carrying both a *Drs-GFP* and a *Dpt-lacZ* reporter gene on the X chromosome are currently used to monitor the pattern of expression of both AMP genes in the same animal (**19**).

3.3.2. RT-qPCR

The northern blot technique has been extensively used to analyze the infection-induced AMP gene expression. A better alternative to study this process is the RT-qPCR method. It consists of a kinetic quantification of a PCR product by measuring a fluorescence signal, which is directly proportional to the amount of accumulated double-stranded DNA. Absolute quantification of the DNA concentration of a sample allows the determination of the corresponding gene expression level. Besides, this technique is safer because it does not require radioactivity.

3.3.2.1. TOTAL RNA EXTRACTION

All steps are performed wearing gloves and using RNase-free products in order to prevent contamination by RNases.

1. Collect 20 adults (or larvae) in a 1.5-ml Eppendorf tube (*see Note 9*).
2. Place tubes at -80°C to freeze flies (samples can be stored at this point for assaying later).
3. Thaw samples on ice.
4. Add 250 μl of TRIZOL[®] Reagent to each tube and homogenize for 30 s using an electric pestle/mortar.
5. Add 250 μl of TRIZOL[®] Reagent and quickly vortex the samples.
6. Incubate at room temperature for 5 min.
7. Add 100 μl of chloroform and vortex for 2×30 s.
8. Centrifuge at $12,000 \times g$ for 15 min at 4°C .
9. Collect 250 μl of the supernatant (aqueous phase) in a clean tube.
10. Precipitate RNA by adding 250 μl of isopropanol stored at room temperature and mix gently but thoroughly by inverting the tube three times.
11. Centrifuge immediately at $12,000 \times g$ for 15 min at 4°C .
12. Remove supernatant by inverting tube.
13. Wash RNA pellet with 400 μl of room-temperature 70% ethanol.
14. Centrifuge at $12,000 \times g$ for 10 min at 4°C .
15. Carefully decant the supernatant without disturbing the pellet.
16. Air-dry the pellet for 10 min and redissolve the RNA in 50 μl of water.
17. Determine the RNA concentration using UV spectrophotometry.

3.3.2.2. cDNA SYNTHESIS

Filter tips are used to prevent contamination of the samples by foreign amplicons. The reverse transcription is performed in a 20 μl final volume. For each experiment, include two control samples: one without RNA and the other without SSII-RT.

1. In a clean 0.2-ml PCR tube add:
 - a. RNA (500 ng/ μ l) 2 μ l
 - b. Random hexamers (50 ng/ μ l) 2 μ l
 - c. dNTPs mix (10 mM) 1 μ l
 - d. H₂O 7.5 μ l
2. Denature samples at 65°C for 10 min.
3. Quick-chill on ice and centrifuge briefly.
4. In each tube add:
 - a. 5 \times SSII-RT buffer 4 μ l
 - b. Dithiothreitol (DTT) (0.1 M) 2 μ l
 - c. RNaseOUT™ RRI 0.5 μ l
 - d. SSII-RT 0.5 μ l
5. Place the tubes in a thermal cycler (heated lid at 70°C) and run the following program:
 - a. Incubate at 42°C for 50 min.
 - b. Denature at 60°C for 15 min.
6. Dilute RT products to 1/50 in water.
7. Store diluted RT products at -20°C.

3.3.2.3. RT-QPCR

Expression levels of the *Drs* and *Dpt* genes are used as readouts for the Toll and Imd pathways, respectively, after being normalized to *rp49* (constitutively expressed in *Drosophila*) expression level (see **Note 10**). Experiments are performed in 20- μ l glass capillaries using the LightCycler® 2.0 System (other systems exist; see **Note 11**). Include a control sample containing water instead of RT product.

1. Prepare the following PCR master mix including for each sample:
 - a. DNA Master SYBR Green I mix 1 μ l
 - b. MgCl₂ 25 mM 1.2 μ l
 - c. Forward primer 10 μ M 0.5 μ l
 - d. Reverse primer 10 μ M 0.5 μ l
 - e. PCR-grade H₂O 1.8 μ l
2. Add in each glass capillary:
 - a. PCR master mix 5 μ l
 - b. Diluted RT product 5 μ l

3. Run PCR using the LightCycler® 2.0 System with the following program:
 - a. Denaturation: 95°C for 10 min.
 - b. Cycling: 95°C for 5 s, 60°C for 5 s, 72°C for 15 s, repeated 40 times.
 - c. Melting curve: 95°C for 5 s, 60°C for 5 s, 72°C for 15 s, 70°C→95°C with 0.1°C/s ramping.
4. Monitor the absolute quantification using the LightCycler® software and calculate the gene expression level (R) using the formula: $R_x = [E_{rp49} \exp(CP_{rp49})] / [E_x \exp(CP_x)]$, where E = PCR efficiency, CP = crossing point, x = *Drs* or *Dpt*. The PCR efficiency for each couple of corresponding primers is indicated in **Table 2**.

3.4. Survival Analysis

Survival analyses are performed using several classes of bacteria that exhibit different interactions with *Drosophila*. Highly pathogenic bacteria (such as *E. faecalis*) kill flies after injection of low doses, weakly pathogenic bacteria induce low lethality as in the case of *Ecc15*, and non-pathogenic strains such as *E. coli* cause low lethality. Survival experiments must be carried out in the same conditions (methods, needle, experimenter, time) for each sample of flies. As a prerequisite, the fly strain must also exhibit a good viability in the absence of an immune challenge. A classical survival analysis in adults can be carried out by using *Ecc15*, *E. faecalis*, *C. albicans*, and *B. bassiana* as pathogens and *Rel^{E20}* and *spz^{rm7}* as positive controls.

1. Collect twenty 2–4 day-old adults.
2. Infect flies using a needle previously dipped into a bacterial or fungal solution. For *B. bassiana*, proceed as described in **Subheading 3.2.2.2**.
3. Incubate flies at 29°C and transfer them to a fresh vial every 2–3 days to ensure healthy medium conditions.
4. Count dead flies twice a day during 1 week.

Flies that die within 2 h after infection are excluded from the analysis (<5% as a norm; see **Note 12**). Infected larvae may be transferred onto a Petri dish containing apple juice agar or fly medium (**20**) to facilitate the sorting of dead versus live animals. A basic survival count for larvae includes the number of pupae and adults that emerge.

4. Notes

1. Preincubation at 37°C and centrifugation of the staining solution can prevent the formation of undesirable crystals. It can be stored for several months at 4°C.
2. Septic injury does not allow for an accurate quantification of injected microbes or microbial components. When necessary, a Drummond's Nanoject™ (automatic) injector can be used to deliver a defined volume of microbial solution. The glass

capillary tips are pulled under high heat, backfilled with mineral oil, and then mounted onto the Nanoject device calibrated for the specified injection volume. Injections of 4–73 nl into an adult fly are possible. This apparatus can be used for the injection of microorganisms, chemicals, and purified bacterial compounds, where highly accurate conditions are required.

3. Parameters that can influence the infection process are:

The infection procedure. For instance, challenge via septic injury with *B. bassiana* triggers an immune response with characteristics and kinetics different from those elicited by a natural infection with the same fungus, suggesting that a different set of recognition signals is switched on in response to various infection methods. In the case of injection, the needle size and the site of injection may influence the infection process.

The nature of the microbes and their concentration. The use of various types of bacteria of the same microbial class is recommended to compare the pattern of AMP gene expression, as the latter differs according to the microorganisms used (15).

Temperature. 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 preexisting disadvantages.

4. Bacterial compounds may be diluted in Ringer solution (20) or water and injected into flies.
5. Parameters influencing AMP gene expression:

Fly. Males usually express AMP at a higher level than females after systemic response. Unchallenged females constitutively express *Drs* at a low level (10). This basal level of expression can influence the induction rate after microbial infection.

Age of flies. We usually use 2–4 day-old flies, because younger flies still possess remainders of the larval fat body. *Dpt* expression is strongly dependent on the larval stage (21,22).

Time of day. Microarray studies suggest that AMP gene expression can fluctuate with the circadian rhythm (23).

6. *Drosophila* expresses an endogenous galactosidase in the midgut and few other tissues, the optimal pH of which is 6.5. It is possible to use fly lines deficient for the *Drosophila gal* gene to prevent monitoring of this background.

7. Strong induction of *Drs*-GFP in trachea is obtained by naturally infecting larvae with *Ecc15* and keeping them at 18°C for 2 days.
8. The use of GFP reporters has two major drawbacks. First, in order to fluoresce, the protein requires cyclization, which results in a lag time, and thus GFP detection occurs long after that of β -galactosidase with the same promoter. Second, GFP activity is difficult to quantify although quantification of a *Drs*-GFP reporter expression has been used to screen for regulators of the immune response using a spectrophotometer (24).
9. RNA extraction can be also performed from fly/larval tissues like gut or fat body. In this case, collect about 50 samples of the tissue.
10. RT-qPCR analysis allows the course of a PCR to be visualized as a curve similar to a population growth curve. The initial lag phase lasts until the fluorescence signal from the PCR product is greater than the background fluorescence of the probe system. The exponential log phase starts when sufficient product has been generated to be detected above background and ends when the reaction enters the plateau phase and the reaction efficiency falls. Determination of the PCR cycle (called “crossing point” or CP) for which the fluorescence of a sample rises above the background fluorescence allows to calculate the expression level of the studied gene.
11. Alternatively, 96- or 384-well plates can be used. Besides, other suppliers have developed RT-qPCR systems, which can be used. However, the assays must be set up each time a parameter (primer, system, kit assay, etc.) is changed. In this case, PCR efficiency (which depends on the hybridization temperature of the primers), dilution of RT products, and melting curve (which gives the number of amplified products) have to be determined to ensure that only the designated PCR product is amplified.
12. Previous observations showed that survival rates may depend on the genetic background. For example, we noted that homozygous *ebony* fly stocks exhibit a low viability after infection as reported by Flyg et al. (25). In order to examine the survival due exclusively to the mutation under analysis, we choose mutated chromosomes carrying a minimal number of markers (3).

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