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Methods to study *Drosophila* immunity

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ABSTRACT

Innate immune mechanisms are well conserved throughout evolution, and many theoretical concepts, molecular pathways and gene networks are applicable to invertebrate model organisms as much as vertebrate ones. *Drosophila* immunity research benefits from an easily manipulated genome, a fantastic international resource of transgenic tools and over a quarter century of accumulated techniques and approaches to study innate immunity. Here we present a short collection of ways to challenge the fruit fly immune system with various pathogens and parasites, as well as read-outs to assess its functions, including cellular and humoral immune responses. Our review covers techniques for assessing the kinetics and efficiency of immune responses quantitatively and qualitatively, such as survival analysis, bacterial persistence, antimicrobial peptide gene expression, phagocytosis and melanisation assays. Finally, we offer a toolkit of *Drosophila* strains available to the research community for current and future research.

1. Introduction

The *Drosophila* immune system has been the focus of intense studies and is now one of the best-characterized among Metazoans. Studies show that insect immune systems are complex and share many characteristics of the vertebrate innate immune system. The nature of the *Drosophila* immune response is dependent on the mode of infection, the type of pathogen and route of challenge, the tissue(s) affected, developmental stage, genotype and many other physiological parameters including the presence of symbiotic bacteria. Since immune reactions are not as tightly regulated as developmental processes, specific attention to all these physiological parameters is essential before reaching any conclusion. Physiological processes have a multitude of ways of interfering with host survival to infection; as an immune response requires the rapid reallocation of resources it easily enters in competition with other vital processes [1,2]. As a consequence many immune and non-immune factors contribute to survival. Generally, any mutants that affect the general fitness of flies in some way or other tend to exhibit higher susceptibility to infection. For instance, stress and repair programs have recently entered centre stage in host defence as exemplified by accumulating work on the gut [3,4].

This also raises another point to consider when assessing immunity: the immune response is compartmentalized or tissue-specific. We traditionally distinguish the systemic immune response on one hand, which takes place in the body cavity and involves hemolymph, hemocytes and the fat body, and local immune responses on the other hand, that take place in barrier epithelia such as the gut and the tracheae [5]. Humoral and tissue-specific immunity is complemented by powerful intracellular defence mechanisms such as RNAi, which is an essential defence against virus.

In the wild, *Drosophila* is infected at various stage of its life cycle by viruses, parasites (nematodes, parasitoid wasps), protozoans (for example trypanosomes [6]), fungi (yeast, filamentous and Microsporidians) and bacteria. Each of these microbes triggers a rather distinct set of overlapping immune pathways. Flies are also infected by two vertically transmitted endosymbionts, *Wolbachia* and *Spiroplasma*. While there is no evidence that these highly co-evolved symbionts trigger or are affected by the immune system, both classes of endosymbionts can promote symbiont-mediated defence; *Wolbachia* protects flies against viruses while *Spiroplasma* exerts protection against wasps and nematodes [7,8]. The presence of *Wolbachia* which is found in many lab stocks should be taken into consideration.

The immune response can be analysed after challenge or under basal conditions. The basal immune response is affected by environmental microbes associated with the flies such as bacteria found in the gut or ingested from the substratum. In these cases, germ-free (axenic) or gnotobiotic (i.e. with defined microbiota) fly culture allows defining the contribution of these microbes to

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the basal level of infection. The systemic immune response is traditionally divided into a cellular response that involves hemocytes and the humoral response that includes and melanisation and the production of antimicrobial peptides and other effectors. Studies have shown that all *Drosophila* stages can mount effective immune responses, although some of them are stage-specific (for example, the exclusively larval lamellocyte production during encapsulation of parasitoid wasp eggs). Embryos and pupae are interesting models to study wound healing reactions, hemocyte motility and phagocytosis of apoptotic cells (that will not be described here). Humoral and cellular immune responses are usually studied in both larvae and adults. Since the maturation of immune responsiveness is strongly dependent on ecdysone [9,10], immune pathways in the adult stage are less affected by developmental timing. It should be noted that AntiMicrobial Peptides (AMPs) (and many immune genes) show very high levels of inducibility (equivalent to heat-shock genes). Their expression profiles are influenced by multiple parameters and not surprisingly, these genes are picked up in many microarray studies. It is also important to determine whether the observed immune activation has a direct or an indirect cause. For instance, most mutations disrupting the gut compartmentalization lead to higher antimicrobial peptide gene expression in this tissue [11]. This is similar to the observation that many mutations can cause melanotic tumours (cellular like immune response) in a number of indirect ways [12].

Many mutations affect the immune response and in some cases, the differences observed can be down to the genetic background for all the reasons stated above (effect of background on survival for example is illustrated in Fig. 2A). To study subtle immune phenotypes, it is therefore recommended to compare mutants and wild-type in several different background contexts. For instance, use two wild-type stocks such as $Canton^S$ and $Oregon^R$ and different combinations of mutants (for example mutation over a deficiency, mutant and RNAi).

After raising these points of caution, we describe below some of the methods used in our lab and others to study the immune response to microbes and parasites, with an emphasis on antimicrobial defence mechanisms.

2. Commonly used pathogens and culture conditions

Various types of microbes can be used to challenge the *Drosophila* immune system. Rather than using mammalian pathogens, the organisms of choice are entomopathogenic microorganisms, which naturally infect wild *Drosophila* populations, and are particularly informative as both parties have evolved attack and survival mechanisms. Among these, genetically tractable organisms in particular have the added value of allowing parallel screening of pathogen virulence genes and host resistance/tolerance genes. Of note, strains from the same bacterial species group can behave differently as virulence factors differ from strain to strain.

2.1. Bacteria

Among the large variety of commonly used bacteria, not all are naturally infecting insects. Below we describe two commonly used Gram-negative entomopathogenic bacteria, with further infectious lab strains listed in Table 1. Where available, bacteria carrying fluorescent markers or resistance genes can be used for ease of quantification or re-isolation from the host.

2.1.1. Gram-negative bacteria

Pectobacterium (old name *Erwinia*) *carotovora carotovora* is a Gram-negative bacterium that causes soft rot in plants and is naturally transmitted by insects. The strain *Ecc15* is particularly attractive as it is genetically tractable to some extent, stable, can naturally infect *Drosophila*, and triggers a very strong immune response while exhibiting a mild lethality in wild-type flies. The use of this strain has been instrumental in studying the systemic immune response as well as the local immune response in trachea or gut and. *Ecc15* is grown as a shaking culture in LB medium at 29 °C and can be reliably identified by its "rotten potato" smell. To infect flies, an overnight culture of 50–100 ml is pelleted by centrifugation (15 min at 3200*g*) and adjusted to OD_{600} = 200. Running plates of *Ecc15* can be kept for a month at 4 °C to start liquid cultures. The *Ecc15* wild-type strain carries genomic rifampicin resistance, and a GFP-transformed strain (spectinomycin-selectable) is available for fluorescent tracing of bacteria. Note that the GFP fluorescence contributes slightly to absorbance at 600 nm and pellet density needs to be adjusted accordingly [13,14].

Another naturally occurring lethal Gram-negative entomopathogen is *Pseudomonas entomophila*, isolated from a fly in Guadeloupe. *P. entomophila* is cultured like *Ecc15* but is more sensitive to pelleting, because compacted or air-exposed bacteria tend to lyse. This bacterium is genetically unstable and avirulent GacA-like mutants are observed at high frequency. Verifying strains can be done by growing on milk-agar plates to test the proteolytic activity (wildtype but not GacA) [15–17]. A variety of useful *P. entomophila* mutants have been isolated, including the non-virulent GacA strain, or protease and toxin mutants.

Alternative Gram-negative bacteria are *Providencia* [18], *Serratia marcescens* (notably the strain *Sm DB11-40*, [19]), or *Pseudomonas aeruginosa* [20,21], which can be found associated with flies in the wild. Laboratory strains of *Escherichia coli*, although not associated with wild flies, have also been used to probe the immune system, notably the Imd pathway which is activated by DAP-type PGN.

2.1.2. Gram-positive bacteria

Gram-positive bacteria can be divided into two types based on their peptidoglycans. Lysine-type peptidoglycan bacteria are strong inducers of the Toll pathway while DAP-type peptidoglycan Gram-positive bacteria including the *Bacillus* and *Listeria* genus activate both the Toll and Imd pathways.

Most Lysine-type Gram-positive bacteria are pathogenic to flies upon injection, with the notable exception of *Micrococcus luteus* that can be used as a non-lethal inducer of the Toll pathway. *Enterococcus faecalis* and *Staphylococcus aureus* have been used to probe the Toll pathway in survival experiments, and occasionally *Aerococcus viridans* has been used to stimulate a Toll-dependent immune response. *S. aureus* is especially interesting to probe the role of phagocytosis and melanization [22,23,131]. Among DAP-type Gram-positive bacteria, *Listeria monocytogenes*, *Listeria innocua, Bacillus megaterium* and *Bacillus subtilis* are currently being used (for example [24]).

Mycobacterium marinum has been used as a *Drosophila* model for tuberculosis and exhibits the slow killing and wasting characteristics of the disease in humans [25,26].

2.2. Fungi

Beauveria bassiana and *Metarhizium anisopliae* area naturally occurring insect pathogens which infect by breaching the cuticle during sporulation, thanks to a battery of proteo- and lipolytic enzymes. *B. bassiana* is cultured on malt agar plates at 18–29 °C, in the dark. Higher temperatures result in faster growth rates but may favour contaminating bacteria; to prevent this, plates can be sealed with parafilm. Usually cultures take 1 month to sporulation, which can be induced by switching to 29 °C. It is important to keep

Table 1
Commonly used pathogens.

Bacterium	Gram	Culture conditions	Dose, route and temperature of infection
Escherichia coli	Negative, DAP-type	LB, 37 °C	OD_{600} = 400 (S), OD_{600} = 200 (O), 29 °C
Erwinia carotovora carotovora 15	Negative, DAP-type	LB, 29 °C	OD_{600} = 200 (S), OD_{600} = 100 (O), 25–29 °C
Pseudomonas entomophila	Negative, DAP-type	LB, 29 °C	OD_{600} = 200 (O), 29 °C
Enterobacter cloacae β12	Negative, DAP-type	LB, 37 °C	OD_{600} = 10 (S), 22–29 °C
Salmonella typhimurium	Negative, DAP-type	LB, 37 °C	OD_{600} = 0.1–10 (S), 22–29 °C
Listeria monocytogenes	Positive, DAP-type	BHI, 37 °C	OD_{600} = 0.01–200 (S), 22–29 °C
Bacillus subtilis	Positive, DAP-type	LB, 37 °C	OD_{600} = 5–20 (S), 22–29 °C
Micrococcus luteus	Positive, Lys-type	LB, 29 °C	OD_{600} = 200 (S), 29 °C
Staphylococcus aureus	Positive, Lys-type	LB, 37 °C	OD_{600} = 0.5–200 (S), 22–29 °C
Staphylococcus saprophyticus	Positive, Lys-type	LB, 37 °C	OD_{600} = 5 (S), 22–29 °C
Enterococcus faecalis	Positive, Lys-type	LB, 37 °C	OD_{600} = 0.01–20 (S), 22–29 °C
Fungus, yeast			
Candida albicans		YPG broth, 29 °C	OD_{600} = 200 (S), 22–29 °C
Beauveria bassiana 802		Malt agar, 25–29 °C	Roll flies in sporulating dish, 29 °C
Metarhizium anisopliae KVL 131		Malt agar, 25–29 °C	Roll flies in sporulating dish, 29 °C
Aspergillus fumigatus			Spore suspension (S), 29 °C

Note that lower doses should be used when microbes are micro-injected. Infectious doses are indicated as a range; lower concentrations are useful for sublethal challenges. Abbreviations: S, systemic infection; O, oral infection; YPG, yeast peptone glucose; LB, Luria Bertani; BHI, Brain–Heart-Infusion.

plates dry – wipe the lid with clean paper if condensation droplets form. To propagate cultures, flip sporulating plates to new mal agar. Running plates can be kept up to 1.5 months at 18 °C or until dark spots appear on the fungal lawn.

Other fungi need to be injected to trigger an immune response. *Candida albicans* is a yeast-like fungus that triggers a strong activation of the Toll pathway. As this fungus exhibits little proteolytic activity, it will elicit a GNPB3-Toll immune response, which has been well characterized [27]. Injection of spores of *Aspergillus fumigatus* likewise triggers a strong systemic antifungal immune response.

Recently, the immune response to microsporidian species has been addressed [28].

2.3. Parasites

In the wild, young *Drosophila* larvae are often infested by a variety of parasitoid wasps [29]. Some are specific to *Drosophila melanogaster* (for example *Leptopilina boulardi*) while others are generalists (for example *Leptopilina heterotoma*, *Asobara tabida*), and parasitoid strains differ in their virulence. The more virulent wasps can be propagated in wild-type strains of *Drosophila*, while less virulent wasps, such as *A. tabida*, are better cultivated in *PPO1*, *PPO2* double mutants or *Drosophila subobscura*. Parasitoid wasps will emerge 7–10 days later than flies. The adult parasitoid wasps can be kept on sucrose/agar or honey.

For more information, consult: http://www.jove.com/video/3347/an-introduction-to-parasitic-wasps-drosophila-antiparasite-immune.

Few papers have analyzed the fly response to protozoans (*Crithidia*, [30]) and nematodes. For nematodes, recently updated methods are available [31–33].

3. Infectious routes

Drosophila are naturally exposed to pathogens while foraging on decaying fruit, so the most common route of access is by oral infection to the digestive system or by contact with the tracheal system. However, wild insects also sustain injuries to the cuticle and subsequent introduction of microorganisms into the hemocoel. Sporulating fungi likewise are able to breach the cuticle and extend hyphae into the sterile body cavity. The latter two modes of infection are most easily reproduced in the lab by pricking with a pathogen-contaminated needle.

3.1. Systemic infection

To introduce pathogens into the body cavity, anaesthetized adult flies are pricked into the thorax with a thin metal needle dipped in a concentrated bacterial pellet or a suspension of fungal spores (for illustration, see [34]). Pricking into the thorax rather than the abdomen reduces the risk of damaging the intestines, which can easily result in secondary bacteraemia from microbiota. Individuals normally recover quickly after pricking, and the wound site usually displays melanisation within a few hours. To avoid scoring lethally injured individuals, dead flies should be counted at 2 h after pricking and removed from further analysis.

While needle pricking is a quick and efficient way to deliver a shot of infectious agent, a more precise dosage may be required for certain experiments. For this kind of experiment, it may be useful to microinject a defined volume (nanoliters) of bacterial pellet using a pulled glass capillary mounted on a Nanoject™ apparatus (for illustrations, see [34]). Capillaries however tend to cause larger wounds, a stronger melanisation reaction and longer recovery times than needles. Alternative methods have been described such as eye injection, genitalia infection [35,132] and placing flies with cut-off legs on contaminated medium. Food vials with freshly injured flies can be kept sideways to prevent weakened individuals from sticking to the medium.

Larval systemic infection is performed with a finer tungsten needle into the posterior lateral side of larvae immobilized on a pre-chilled rubber pad. Larvae can be deposited straight into the bacterial pellet for ease of manipulation. Injured larvae are placed on sealed 5 cm petri dishes containing apple juice agar or normal fly medium.

After infection, flies or larvae are transferred to the temperature most adapted to optimal pathogen growth (see Table 1).

For infection with fungi, spores can be directly injected as described for bacteria, but for entomopathogenic fungi, the method of choice is to place CO_2-anaestethized flies directly on the sporulating fungal lawn and to shake the culture plates until flies are uniformly covered in spores (for illustrations, see [34]). Flies will become gradually infected as spores germinate and breach the cuticle [36].

3.2. Oral infection

For oral infection, 2–4 day old adults are dehydrated/starved for 2–3 h in empty vials at 29 °C to ensure synchronous feeding of all individuals when flipped into infection vials. An infection vial consists of standard medium (but without live yeast) completely covered by a Whatman paper disk (Fig. 1A and [15]). The disk is soaked in control solution (10% sucrose) or bacterial pellet mixed with control solution. 120–150 µl are sufficient to soak a 22 mm diameter disk. Starved flies are flipped into the infection vials and kept at optimal temperature for pathogen growth. Experimental evidence suggests that bacteria on the filter disk do not remain viable beyond a couple of hours, therefore it is possible to flip infected flies onto fresh medium after an initial infection phase (24 h for example). Other methods include placing flies on paper towels soaked with bacteria. In this case, fresh bacterial or sucrose solution should be frequently added to avoid dehydration (resulting in continuous infection) [19]. The method of infection (for example with or without prior starvation, continuous or one-shot delivery of bacteria) can have a strong influence on the outcome of the infection.

For natural infection of larvae, individuals are incubated in concentrated solution of bacteria alone or mixed with crushed banana [13]. To prevent larvae from crawling out of the infectious food, transfer them to Eppendorf tubes and plug with a foam stopper. After 30 min, larvae are removed from the mix and transferred to normal food vials. Larvae continue to be infected in the vials. Washing larvae allows stopping the ingestion of bacteria and monitoring persistence. Tracheal infections of larvae are usually carried out at a lower temperature to allow sufficient time, before pupariation, for the development GFP fluorescence from reporter genes. Monitoring the percentage of infected trachea by expression of Drs-GFP is described in Section 4.1.4.

3.3. Injection of immune elicitors

Injection of microbes leads to complex immune responses because they involve growth of the microbe within the host. Injection of dead bacteria allows circumventing this problem. To activate a specific branch of the immune response pure elicitors can be used such as TCT (ligand for the Imd activating receptors PGRP-LE and PGRP-LCx/a), DAP-type peptidoglycan (to activate PGRP-LC and PGRP-LE and to a lesser extent PGRP-SA), Lysine-type peptidoglycan (PGRP-SA), β-glucan (GNBP3), proteases (to activate the psh-Toll pathway). Note that pure LPS has no effect on antimicrobial peptide gene expression but most commercially available LPS preparations (Sigma for example) do show activation of Imd because of trace contaminations by peptidoglycan [37,38]. Pure products can be purchased from InvivoGen or obtained from laboratories specialising in bacterial cell wall biochemistry.

3.4. Wasp parasitisation

For wasp infection, 20–30 second instar wild-type larvae are co-housed with 3–4 *A. tabida* females for 2 h. Parasitized larvae are kept at ⩽25 °C until capsule dissection 4–6 days post-infection. Lamellocyte differentiation can be visualized by phalloidin staining of hemolymph preparations or with the use of lamellocyte markers.

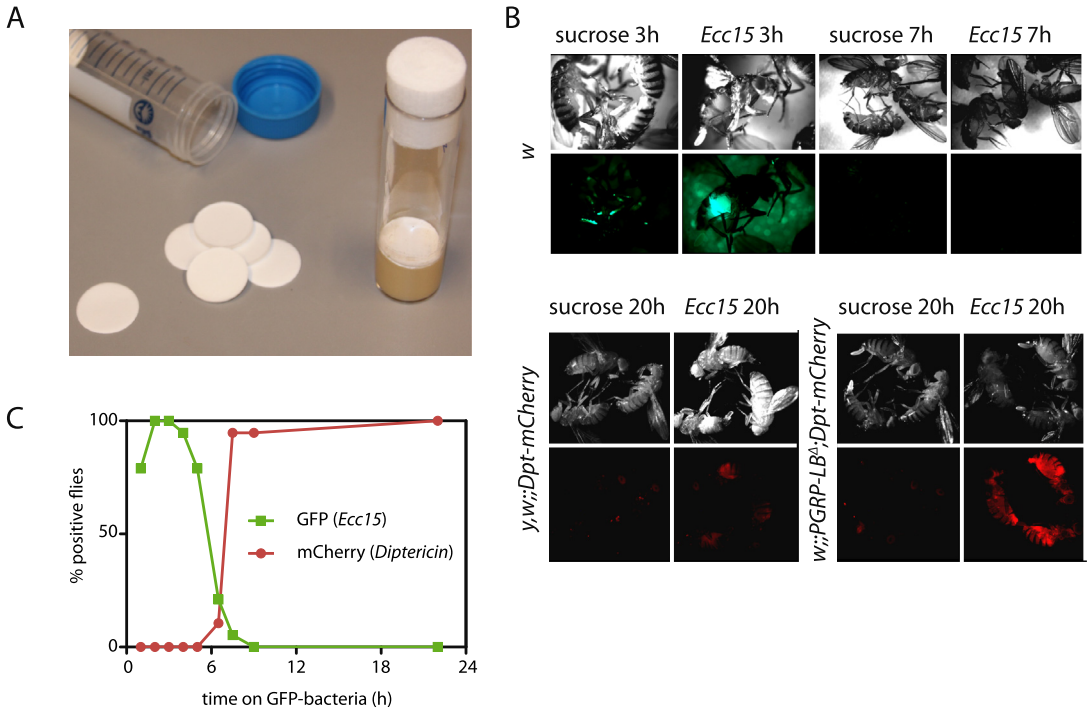

Fig. 1. Oral infection methods and read-outs. (A) Preparation of fly vials for oral infection. Whatman filters (can be sterilized) are layered on top of the medium and soaked with 120–150 µl bacterial solution shortly before infection. (B) Fluorescent monitoring of bacterial presence and systemic immune response to oral infection. Top panels: flies were fed a GFP-tagged non-lethal Gram- bacterium, *Erwinia carotovora carotovora 15* (*Ecc15*), or sucrose as control, for indicated time points. The presence of *GFP-Ecc15* can be tracked under a fluorescence binocular as bright fluorescence within the abdominal area. After 6–8 h, the fluorescence disappears due to gut emptying and food uptake blockage occurring during infection. Bottom panels: reporter flies carrying *Dpt-mCherry*, with or without the *PGRP-LB* mutation, were subjected to the same diet as in a, but monitored at 20 h for systemic induction of *diptericin* (mCherry) under a fluorescence binocular. The fat body of reporter flies, deficient for *PGRP-LB*, shows a strong induction of mCherry [108]. (C) Kinetics for disappearance of bacteria and appearance of immune response signal, derived from observations as in B.

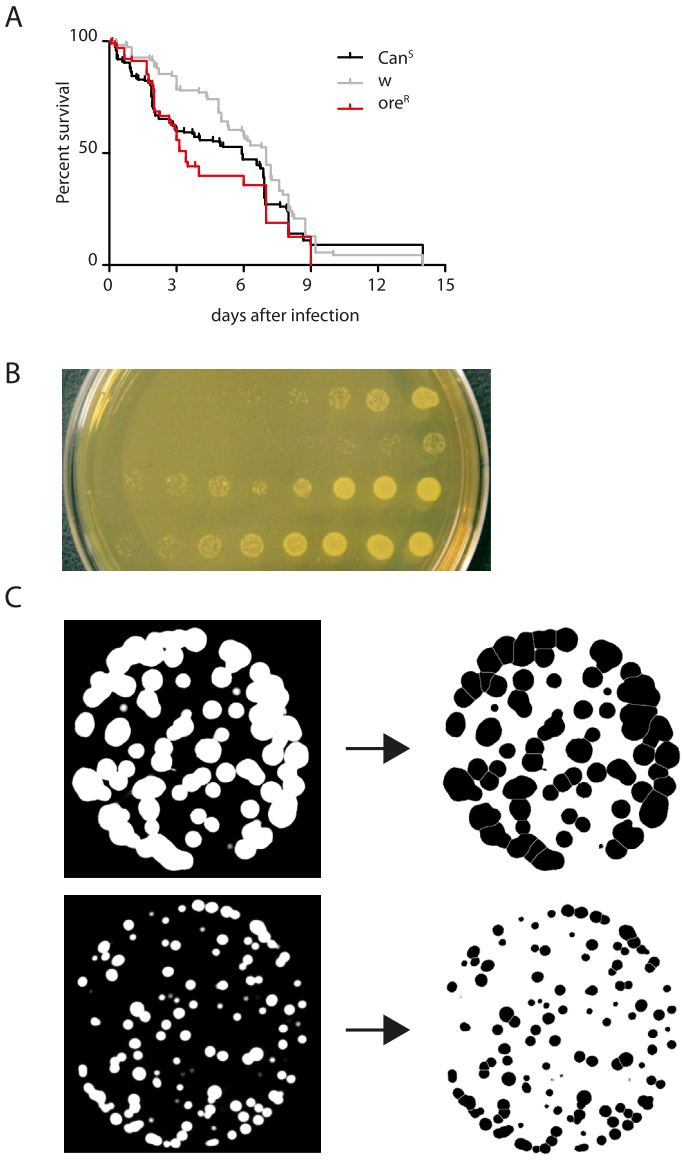

Fig. 2. Survival and colony counting. (A) Survival of different wild-type strains to systemic infection with *Ecc15* (needle pricking, OD600 = 200, at 29 °C). Data represent cumulated counts (*ore*^{R}, 211 males; *Can*^{S}, 553 males; *w*, 266 males) from 2 different experimenters over a span of 4 years. Median survival: *ore*^{R} 82 h, *Can*^{S} 142 h, *w* 168 h. *p* values by log-rank test: *ore*^{R} vs *w* $p < 0.0001$; *Can*^{S} vs *w* $p = 0.0068$. *p* values by Gehan–Breslow–Wilcoxon test: *ore*^{R} vs *w* $p < 0.0001$; *Can*^{S} vs *w* $p < 0.0001$. The increased survival of the w strain is reflected by a stronger Imd response in these flies. (B) Serial dilutions of fly lysates 24 h after infection. Lines 2 and 3 are immuno-compromised and show enhanced bacterial growth. (C) ImageJ processing of colonies for automated counting. Images need to be taken with high gamma distortion to optimize sharpness of colonies. Using FIJI/ImageJ [109], convert to an 8bit image, use the commands "convert to mask" then "watershed" then "analyse particles" to obtain colony counts per image. Note that the watershed process is only able to some extent to separate colonies that have grown into each other (compare top panel to bottom panel). Grow plates at room temperature instead of 29 °C overnight to prevent colony overlap and to optimize the accuracy of automated counting.

3.5. Germ-free animals

The *Drosophila* gut lumen is an environment with relatively low bacterial diversity and numbers (1–30 species, 103–105 CFU/fly). While the number of bacteria is quite consistent at the third larval stage, numbers in adults are extremely variable and the existence of a stable population of dividing bacteria residing in the gut has not yet been formally demonstrated [39–41]. Bacteria found in the *Drosophila* gut are also found in the substratum, suggesting constant contamination through feeding. Germ-free flies are easily generated by dechorionating embryos with chlorox and then surface-sterilizing them in 70% ethanol. Sterile dechorionated embryos are transferred to autoclaved fly medium in sterile vials, taking special care not to transfer larvae along with the embryos as their gut contents would re-infect the entire population. Note that germ-free flies will not spontaneously acquire microbiota and need to be placed in the presence of bacteria (derived from other flies or from a culture) to re-acquire their microbiota. Recent papers have added to our understanding of the nature of *Drosophila* microbiota. Note that germ-free conditions do not only change the gut microbial environment but also the substratum.

The *Drosophila* symbiont Wolbachia can be removed by treating flies with tetracycline as described in [7].

4. Methods for scoring the response to infection

It is becoming increasingly appreciated that immunity to infection does not simply imply getting rid of the causative agent. Homeostasis also requires tissue regeneration and wound healing,

which rely on stem cell mobilization; rebalancing of energy resources which may have implications on fertility and life span; or possible epigenetic changes which poise the individual against subsequent infections. Below we outline the most commonly used methods and tools to assess outcome of infection, in order to inform such post-infection concepts as resistance, tolerance, resilience, hormesis etc.

4.1. Commonly used fly stocks

All flies listed in this publication are publically available through the Bloomington stock centre or will be made available shortly.

4.1.1. Immune deficiency mutants

Table 2 lists commonly used immune deficiency mutants. For the Imd pathway, imd^1 and Rel^{E20} show a complete loss of antimicrobial peptide induction and adults usually die from Gram-negative infection within 24–48 h. For Rel^{E20}, it is advisable to use the cleaned-up background without the *ebony* marker [42,43]. Note that the *E20* deletion affects another gene. The Imd^1 (hypomorph) mutation and null mutations in *dTAK1* and *PGRP-LC* are slightly weaker than null mutations of other members of the pathway (*dredd*, *Ikkß*, *IKKγ*, *Tab*, *dFADD*, *Imd*). The background of the stock influences its overall resistance (see also Fig. 2A) and many mutations are done in the *y,w* background which shows higher immunoreactivity than for example ore^R or Can^S. The best way to activate the Imd pathway is to over-express *Imd*, *PGRP-LC*, *PGRP-LE* or the truncated, transcriptionally active form of Relish (*Rel68*) [44–47].

The Toll pathway has many other non-immune functions and Toll deficient mutants show some zygotic lethality during larval stage with few escapers [48]. Mutations that can be used include spz^{rm7} (perfectly viable); a combination of Toll alleles (*Tlr632/Tl1-RXA*), *pell74* (a strong hypomorph), and *MyD88*. The pathway can be activated by over-expression of many genes (for example *spz*, *Pelle*, *Toll*...) or using a gain-of-function mutation in *Tl* (*Tl10b* or *uas-Tl10b*) or *cactus*-deficient flies. Mutations in serine proteases or pattern-recognition receptors upstream of Toll tend to block one of the extracellular branches upstream of SPE and Spz.

Decent RNAi lines for *in vivo* targeting of the Toll and Imd pathways are also available (for example *FADD-IR*, *Pelle-IR*) [49,50].

To target melanisation, several mutants lacking hemolymph PO have been described. The most historic is *Black cells*, a gain-of function mutation in *PPO1* that affects both PPO1 and PPPO2 proteins ([51], Neyen, unpublished observations). More recently, a double *PPO1, PPO2* mutant was generated which shows complete absence of PO activity in the hemolymph [131].

Mutants that lack all hemocytes have been described (*domino*, *L(3)hem*) but they exhibit many other defects as they lack all diploid cells [52]. Viable flies lacking all or most plasmatocytes are called phagoless and can be generated by crossing a hemocyte gal4 driver (*Hml-Gal4* or *spn-Gal4*) to either *uas-Bax* or both *uas-hid* and *uas-rpr* [53,54].

4.1.2. Methods for identifying novel immune genes based on large scale screens and expression profiling

A powerful large-scale tool to identify genes involved in immune activation or regulation is RNA interference (RNAi), either in cell-based screens or in a slightly more laborious *in vivo* setting [55]. While the patchy efficiency of first generation RNAis relegated *in vivo* screens to fishing expeditions, improved second-generation RNAi libraries promise optimized throughput in RNAi-based screens. S2-based RNAi screens have successfully identified novel Toll, Imd and JNK pathway components [56–59], determinants of intracellular pathogen resistance [60] or phagocytic receptors on hemocytes [61,62], and *in vivo* RNAi screens have contributed to our understanding of *Drosophila* hemocyte development or response to intestinal infection [63,64].

While unbiased reverse genetic screens can be useful to identify novel gene functions, a targeted approach based on contextual gene expression may speed up the process considerably. Microarrays represent a rich source of co-regulated genes in different tissues and infectious conditions, and have been comprehensively reviewed in [65]. The Pathogen Associated Drosophila MicroArray (PADMA) database (www.padmadatabase.org) regroups accessible microarray data sets from a variety of immune challenges [66]. A list of annotated immune genes involved in the *Drosophila* immune response as well as a collection of microarrays giving transcriptional profiles upon systemic infection, intestinal infection and tracheal infection are available here: http://lemaitrelab.epfl.ch/resources.

RNAseq datasets represent another avenue to identify novel immune-regulated genes through their association with known immune genes in co-regulated clusters [67]. Finally, the Drosophila Genetic Reference Panel (DGRP) represents a unique asset for population genomics and genome-wide association studies (GWAS) based on 168 fully sequenced strains that can be subjected to any number of infectious challenges [68]. Importantly, polymorphisms associated with changes in immune function coming from this kind of screen will bring to light regulatory, intergenic regions in addition to protein-encoding genes. A first hit with an immune function identified through this GWAS approach is pastrel, a gene that confers allele-specific resistance to common *Drosophila* viruses [69].

4.1.3. Methods to generate immune deficient cells, tissues or organisms

Many of the classical mutants described in Section 4.1.1 stem from historical EMS (ethyl methanesulfonate) screens. Forward genetic screening based on alkylating agents has the benefit of unbiased targeting of the genome, and can give rise to a variety of mutations from nulls to temperature-sensitive hypomorphs. While mapping EMS mutations can be time-consuming, novel sequencing-based methods have greatly improved efficiency of mapping [70,71]. Transposon mutagenesis, albeit suffering from insertion bias [72], allows for easy retrieval of positional information, and forms the basis for a downstream toolkit of genetic applications including imprecise excision knock-out, Gal4-UAS overexpression of flanking genes, or element replacement by targeting vectors, to name but a few [73–77]. Extensive libraries of P-element-based transposon insertions are available through stock centers, along with deletion and duplication lines [78–81]. Finally, targeted gene knock-out using optimized targeting plasmids in combination with CRISPR will greatly accelerate full KO coverage of the fly genome [82].

To address the cell- or tissue-autonomous function of a gene, or to circumvent lethality issues, clonal analysis is a method of choice [83]. While genetic mosaics have been mostly used to delineate neural development pathways, twin-spot and MARCM techniques can be easily adapted to immune tissues [84–87]. Additionally, RNA interference has been widely used to reduce gene expression in a tissue- and time-controlled manner, preventing developmental effects. For more extensive methodology on genome engineering, RNAi screens or navigating large-scale data sets to retrieve gene expression information, the reader is referred to the relevant contributions to this issue.

4.1.4. Flies carrying reporter constructs

Large-scale screens require easily scorable read-outs that can be quickly visually inspected. For antimicrobial peptide induction, fluorescent and enzymatic reporter genes are available for both Imd and Toll pathways (Table 2 and Section 4.5, and [34]).

Table 2
Useful fly strains.

Genotype	Stock center	References	Comments
Antimicrobial peptide gene reporters			
P{Dpt-lacZ, ry+};;ry		[110]	Carries *Diptericin-lacZ* insertion on X. Transgene containing 2.2 kb of the *Dpt* promoter; provides an accurate read-out of the Imd pathway in most tissues
y,w, P{Dpt-lacZ, ry+}, P{Drs-GFP, w+}		[5,111]	Carries *Diptericin-lacZ* and *Drs-GFP* on X. Note that the GFP is secreted (fused after position 59 of the *Drosomycin CDS*)
w, P{Drs-lacZ, w+}		[111]	Carries *Drs-lacZ* on X. The *Drs-lacZ* reporter gene is very strong and shows a significant basal activity in larvae
w; P{Dpt-GFP, w+}D3-2,{Dpt-GFP, w+}3-4		[14]	Carries two recombined copies on each third chromosome (four insertions in total). Useful for analysis of *Dpt* expression in barrier epithelia
yw;;P{dpt::cherry-C1, w+}		[53]	Good reporter of *Dpt* expression
w;; P{Dipt-Dpt-HA, w+}		[93]	Carries *Dpt* promoter followed by *Dpt* CDS and tag. Reporter of Dpt expression that allows following the expression of the antibacterial peptide
Mutations affecting the Imd pathway			
Dipt-lacZ I; b, pr, imd^{1}		[112]	Homozygous viable mutation on the second chromosome. *imd^{1}* is a strong hypomorph found in the old BL1046 Bc stock. Susceptible to Gram-negative bacteria
y,w,Dredd^{B118}		[43]	Null mutation in *Dredd*. Homozygous viable.
y^{1},w*,Tak1^{2}	BL26272	[113]	Null mutation in *dTAK1*. Homozygous viable. Susceptible to Gram-negative bacteria
w;; PGRP-LC^{E12}	(see also BL36323)	[45]	Null mutation in the Imd pathway receptor. See also *w∗*; *PGRP-LC$^{\Delta 5}$*
Relish^{E20}, e+		[42,43]	A deletion of relish which also affects a nearby gene. The ebony marker of the original stock (Hultmark lab) was removed by recombination with the *Oregon* stock. Susceptible to Gram-negative bacterial infection
y^{1},w^{67c23},PGRP-LE^{112}	BL33055	[114]	A mutation affecting the pattern-recognition receptor *PGRP-LE* that regulates the Imd pathway in the midgut and in the fat body
y^{1},w^{67c23},PGRP-LE^{112},PGRP-LC^{E12}		[115]	A stock that lacks the two receptors of the Imd pathway
dFADD-IR	NIG 12297R-1	[50]	This RNAi reduces Imd pathway activity
w; UASimd,hspgal4/TM6C		[50]	The *P{uas-imd, w+}* insertion [44] was recombined with *hs-gal4* allowing activation of the Imd pathway
w;; PGRP-LB$^{\Delta 1}$		[108]	A deletion of the negative regulator of the Imd pathway *PGRP-LB*. PGRP-LB scavenges peptidoglycan, thereby determining the level of Imd pathway activation.
y^{1},w^{67c23}; P{EPgy2}pirk^{EY00723}	BL15039	[116–118]	A null mutation the negative regulator *Pirk*
Mutations affecting the Toll pathway			
spz^{rm7}/TM6C (see also spz^{2} ca^{1}/TM1)	(see also BL3115)		*Spz^{rm7}* is a genetically null, homozygous viable mutation in *spz* generated by EMS. Several markers of the original stock (M317 Tübingen stock center) including *ebony* were removed by recombination
Tl$^{1\text{-}RXA}$, e/TM6C (or any other null allele of Toll)			Null mutation in *Toll*. The TM3SerSB balancer of the original stock was replaced by TM6C
Tl^{r632}, ca/TM6C (Tl^{r632} was renamed Tl^{r3})	BL3238		A thermosensitive mutation in *Toll*. Tl deficient mutant larvae and adults can be produced by keeping Tlr632/Tl1-RXA late embryos and young larvae at permissive temperature (18–20 °C) and transferring them to restrictive temperature at the pupal stage
mwh1 e1 Tl^{8}/T(1;3)OR60/TM3, Sb^{1} Ser^{1}	BL30914		Oregon 60 is a dominant male lethal mutation. Tl^{8} (*Tl10b*) is a dominant female sterile mutation (embryos are ventralized). In this stock all the males are *Tl^{8}/TM3SerSb*. To obtain *Tl^{8}* flies, cross these males with wild-type females. *Tl^{8}* constitutively activates the Toll pathway inducing a strong expression of the *Drosomycin* reporter gene and melanotic tumors
P{uas-Tl10b}			Generated by Jean-Marc Reichhart
y,w, DD1,PGRP-SA^{seml}		[119]	Homozygous viable mutation in *PGRP-SA*. Generated by EMS mutagenesis
w;; ModSP^{1}		[24]	This null mutation in *ModSP* blocks the Pattern-recognition branch upstream of the Toll pathway
w; P{w+, uas-MODSP}		[24]	Over-expression of *UAS-ModSP* activates the Toll pathway
Pelle-IR	NIG 5974R-1	[49]	This RNAi stock from the NIG (Mishima) can be used to reduce Toll pathway activity by targeting the kinase Pelle
Rel^{E20}, spz^{rm7}		[120]	A double mutant lacking Toll and Imd pathway activity (no inducible expression of antimicrobial peptide gene expression)
Mutations affecting melanization and clotting pathways			
P{Dpt-lacZ, ry+} I; Bc/Bc	BL1036	[51,112]	Dominant mutation affecting an unknown gene located on the second chromosome. *Bc/+* larvae show 50% proPo activity and melanised crystal cells. *Bc* homozygous larvae show no PO-activity and melanized crystal cells. This *Bc* stock derived from the old BL1046 *Bc imd^{1}* stock. Another *Bc* stock is *Bc^{1} fj^{1} wt^{1}* (BL1036)
w; spn27A^{25A}/Cyo-actin-GFP		[120]	Null mutation by P element excision of *serpin 27A*. *Spn27A* mutants show decreased viability at the pupal stage. *Spn27A* larvae and adults show an excessive melanisation reaction after injury
w; Spn28D^{1}/CyOGFP II		[121]	Deletion of *serpin 28D*. *Spn28D* mutants show lethality at the pupal stage and excessive melanization
w; PPO1[w^{+}]		[131]	Deletion of *PPO1* by homologous recombination (insertion of w+)
w; PPO2[w]		[131]	Deletion of *PPO2* by imprecise excision
w;PPO1,PPO2[w^{+}]		[131]	While mutations in PPO1 reduce melanization, no hemolymphatic melanization is found in PPO1, PPO2 double mutants
Hayan^{1}		[97]	Null mutation by P element excision of *Hayan* which encodes the terminal serine protease of the melanisation cascade. *Hayan* mutants show a systemic wound response defect and a complete absence of hemolymph PO activity
w^{1118}; PBac{WH, #3}Hml^{f0337}4	BL18646	[122]	A null mutation in *hemolectin*. Shows some defect in clotting
w^{1118}; fon$^{\Delta 424A}$/CyO, P{ActGFP}JMR1	BL4344	[123]	A null mutation in *Fondue*. Shows some defect in clotting

Table 2 (*continued*)

Genotype	Stock center	References	Comments
Gal4 Drivers (fat body and hemocytes)			
w; P{yolk-Gal4, w+)/TM3Ser			The *yolk-Gal4* driver strongly expresses Gal4 in the adult fat body of females (and also follicle cells of the ovary). This insertion lowers the viability of flies and as a consequence, *yolk-Gal4* stocks are usually kept at 23 °C. The strength of the driver may be influenced by nutritional state
w; P{GawB}c564	BL6982		The *c564* driver strongly expresses Gal4 in the adult fat body (and in parts of the gut and hemocytes)
P{ppl-GAL4.P}		[124]	The *ppl-Gal4* driver strongly expresses Gal4 in the larval and adult fat body (and also in the gut). It is weaker than *c564* at the adult stage
Hemocyte drivers and markers			
w^{1118}*; P{Hml-GAL4.Δ}2, P{UAS-2xEGFP}AH2*	BL30140	[53,54]	This *GAL4* line expresses in all the plasmatocytes. Note that this line is deleted for NimC1 gene [133]. It can be used in combination with uas-Bax or uas-Hid, uas-Rpr to eliminate plasmatocytes
hemese-Gal4		[125]	This *GAL4* line expresses brightly in most (∼80%) of hemocytes
w; Srp(hemo)Gal4,UASGFP		[126]	This *GAL4* line expresses dimly in all hemocytes and hemocyte progenitor cells
w; pxn-gal4 8.9,uas-GFP		[127]	This *GAL4* line expresses brightly in all plasmatocytes
Lz-Gal4;UAS GFP		[128]	This *GAL4* line expresses brightly in all crystal cells
w, Eater-Gal4, UAS-2xEYFP; BcF6-CFP; MSNF9-mCherry		[129]	This line labels all three hemocytes types; *Eater-Gal4* drives eYFP expression in plasmatocytes, crystal cells express CFP and lamellocytes express mCherry
w; 2x[HmlΔ-dsRed.nuc]/SM6a *w;; 2x[HmlΔ-dsRed.nuc]/TM6c*		[130]	These lines carry two independent insertions on Chr II or ChIII and label all plasmatocyte nuclei dsRed+

Many reporter lines (GFP, RFP, LacZ) are available to measure antimicrobial peptide genes. GFP-reporters tend to be slower and not very quantitative compared to lacZ reporters which are very sensitive but stable. *Diptericin* (*Dpt-lacZ, Dpt-GFP, Dpt-mCherry*) is the best read-out for the Imd pathway. Toll pathway activity can be monitored by the expression of *DIM2* or *Drosomycin*. One should however bear in mind that *Drosomycin* induction receives a significant input from the Imd pathway (especially at early time points) and is regulated by the Imd pathway in tracheae. Lines carrying both the *Dpt-lacZ* and *Drs-GFP* on the X allow assessing both pathways in a same fly.

A new set of reporter genes have been generated that allow identifying each hemocyte population. Some of these reporter genes mark only differentiated cells while other mark all the lineages (see Table 2).

4.2. Survival

Survival to infection is the most holistic approach to assessing defects in immune response. As Drosophilists are not restricted by ethical considerations, sufficiently large numbers of individuals can and should be scored (typically at least 3 biological repeats with cohorts of 30 individuals per genotype and pathogen). Technical issues to consider: if using females, the medium will quickly be worked by hatching larvae and may require more frequent flipping in early stages of an experiment. If using males, the issue of offspring does not arise, but vials containing only males tend to grow sticky with bacteria quicker, and need flipping every 2–3 days throughout the experiment. Usually infections are done on CO_2 anaesthetized flies at room temperature using a cold light. Flies that die within 6 h are removed from the count and are considered dead by injury.

Depending on the question addressed, infections can be done with lethal or sublethal doses (Table 1). A lethal dose typically should kill all wild-type flies at a constant rate, while a sublethal dose will kill only a fraction of individuals, with the recovered fraction surviving at a plateau before dying from old age or immuno-pathological collateral damage. Statistical considerations: if using a lethal dose, usually the assumption of proportional hazards is true, i.e. death rate for one genotype is constantly proportional to death rate for another genotype, and a direct result of the infection. In this case, the log-rank test is an appropriate and powerful method of analysis, because it gives equal weight to deaths at all the time points. If a sublethal dose was used and death rates taper off after a few days, the assumption of proportional hazards is no longer true, i.e. ratios of deaths per time between groups change with time. In this case, the Gehan–Breslow–Wilcoxon test, which gives more weight to deaths at early time points, is more appropriate. If survival curves cross, neither test is applicable [88].

It should be noted that survival to a given infectious agent is not comparable between different wild-type backgrounds (Fig. 2A) with differences reaching statistical significance. It goes without saying that correctly matching the wild-type background to analysed genotypes and testing different backgrounds is crucial to survival analysis.

4.3. Bacterial persistence

In order to assess the efficiency of an immune response, bacterial clearance can be scored by estimating persistent bacteria within the host. Several more or less quantitative methods are available. First, the presence or absence of fluorescently labelled bacteria can be qualitatively assessed by visual inspection under a fluorescence dissecting microscope. If bacteria are proliferating, systemic infection results in a completely labelled individual. Local retention or proliferation of fluorescently labelled bacteria in the gut can be easily seen through the abdominal cuticle (Fig. 1B). In our hands, GFP-labelled *Ecc15* disappears almost completely from infected guts within 6–12 h post-ingestion (Fig. 1B and C).

A second method consists in counting colony-forming units per fly or per tissue at given times after infection. For systemic infection, we usually encounter considerable variability in CFU per fly when flies are infected by needle pricking, because of slight variation in the infectious dose between individuals. This precludes detection of subtle differences in clearance rates between genotypes. A better approach is therefore to inject an exactly reproducible infectious dose by glass capillary and Nanoject™. To count CFUs, flies are mashed in sterile LB, either manually with a pipette tip or mechanically in a cell lyser and glass-bead filled tubes. The supernatant is then serially diluted in 96-well plates to reach at least 4 sequential countable dilutions when plated. Dilutions are plated on LB-agar with selective antibiotic (if applicable) as 3 µl spots, left to dry for a few minutes and incubated until colonies are visible (Fig. 2B). A quick and reliable way of counting colonies is by automated image analysis (Fig. 2C).

For oral infection, an obstacle to accurate quantification is microbial contamination on the fly's appendages. To eliminate surface bacteria as much as possible, individuals are dipped in 70% ethanol 5 s, then rinsed in sterile water or PBS and dried by touching a tissue paper before collecting. This method however has limitations, because not all flies may be surface-sterilized, and because ethanol can flood the extremities of the digestive tract and kill bacteria in the crop or anal pouch.

A third method is PCR-based quantification of bacterial genes on fly extracts (see [89] for *Spiroplasma* and [26] for *M. marinum*).

4.4. Transcriptional activation, mRNA quantification

A straightforward way of assessing expression of genes independently of reporter engineering is quantitative real-time PCR. Technicalities to consider include numbers of individuals and tissues (as an indication: 10 adults or 15 larvae or 15 fat bodies or 20 intestines), extraction (TriZol and isopropanol precipitation or spin columns), RNA treatment (DNase digestion or not), kinetics of gene expression, target and reference genes. It can also be useful to normalize gene induction to a known entity, for example maximal induction in a given wild-type strain, in order to make sense of "fold changes" upon a test treatment. A selection of reporter genes is listed in Table 3.

Some general considerations on gene expression may be worthwhile pointing out: although immune gene induction is normally robust, many genes display circadian rhythmicity, and measurements might be improved by consistent fly husbandry (12 h light/dark cycle, controlled temperature) [90,91]. NF-κB signalling (Toll/Imd) in particular is oscillatory in nature and kinetics rather than single time points may be more informative for a complete picture of immune homeostasis [92].

While mRNA quantification gives a fairly accurate picture of which genes are switched on, it does not inform on the actual efficiency of the gene products. Indeed, recent publications from different areas of infection biology suggest that translation can be inhibited during infection. Ideally, where antibodies or tagged gene products are available (for example the *Dipt-Dpt-HA* construct described in [93]), protein expression should be assessed in parallel to see whether the kinetics of mRNA induction are matched by the kinetics of effector production (protein or enzymatic product).

4.5. Reporter lines

Reporter constructs which combine immune-inducible promoters with reporter enzymes or fluorescent proteins provide an alternative approach to study gene expression in a spatiotemporal manner. LacZ and GFP enhancer traps are riddling the genome; alternatively, Gal4 traps can be coupled to UAS-reporters of choice to assess gene induction [94]. Below we outline immune-specific reporter strains (listed in Table 2) and how to use them for immune gene quantification.

4.5.1. Non-fluorescent markers of immune activation

A historical way of scoring gene expression is by enzymatic titration of a reporter enzyme (peroxidase, β-galactosidase) driven by the promoter of the gene of interest. P[lac-Z] insertion strains are available from stock centres for a vast number of genes, see also Table 2 for a selection of immune gene-lacZ reporters.

To analyse gene expression by X-gal staining and subsequent microscopic observation, tissues of interest from *P[lac-Z]*-expressing larvae or adults are dissected in PBS at 4 °C and fixed in PBS with 0.5–1% glutaraldehyde at 4 °C. Length of fixation depends on thickness of tissue (seconds for hemocytes, minutes for intestines or fat bodies).

For X-gal staining the following reagents are used: X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) prepared as a 5% solution in dimethylformamide and stored at −20 °C; staining buffer composed of 10 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, 1 mM $MgCl_2$, 3.5 mM K_3FeCN_6, 3.5 mM K_4FeCN_6 and adjusted to pH 7.2 with NaOH. X-gal stock should be added to the staining buffer shortly before use, at 30 µl/ml buffer. After washing the fixed tissues in PBS (1–3 times depending on thickness), incubate in staining buffer with X-gal at 37 °C or at RT. The length of incubation depends on the amount of expressed enzyme (from minutes to overnight). Particular attention should be focused on experimental pH: constitutively expressed β-galactosidase (midgut) is active at pH 6.5, while P[lac-Z]-derived β-galactosidase has an optimum at pH 7.5.

To quantify gene expression by β-galactosidase activity titration, whole animals (3–5 individuals per genotype) or dissected tissues (15–20 intestines, 10–15 carcasses) are collected and lysed in glass-bead filled screw-cap tubes in buffer Z (60 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, adjusted pH to 8 with NaOH). All steps are carried out

Table 3
Primer list.

FlyBase	Name	Symbol	Forward	Reverse
CG7939	Ribosomal protein L32	RpL32	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
CG8175	Metchnikowin	Mtk	AACTTAATCTTGGAGCGA	CGGTCTTGGTTGGTTAG
CG1385	Defensin	Def	GTTCTTCGTTCTCGTGG	CTTTGAACCCCTTGGC
CG10146	Attacin-A	AttA	CCCGGAGTGAAGGATG	GTTGCTGTGCGTCAAG
CG10816	Drosocin	Dro	CCATCGTTTTCCTGCT	CTTGAGTCAGGTGATCC
CG1365	Cecropin A1	CecA1	GAACTTCTACAACATCTTCGT	TCCCAGTCCCTGGATT
CG12763	Diptericin	Dpt	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG
CG14704	Peptidoglycan recognition protein LB	PGRP-LB	CTTGTTTGTTTGTTTATTTTTGTG	CGGTAACCGTCGAGGC
CG11992	Relish	Rel	ACAGGACCGCATATCG	GTGGGGTATTTCCGGC
CG15154	Suppressor of cytokine signaling at 36E	Socs36E	GCACAGAAGGCAGACC	ACGTAGGAGACCCGTAT
CG5963	unpaired 3	upd3	GCGGGGAGGATGTACC	GTCTTCATGGAATGAGCC
CG7850	puckered	puc	TGGCTCTGTTCAAGCG	CCTTATCTCAGTCCCTCG
CG3131	Dual oxidase	Duox	TAGCAAGCCGGTGTCGCAATCAAT	ACGGCCAGAGCACTTGCACATAG
CG31508	Turandot C	TotC	TGGGCTATTCTGACGAGG	TCCGACGTACTTGGTCT
CG14027	Turandot M	TotM	CGGAACATCGACAGCC	CGCTTGACTCCCTCAGA
CG10810	Drosomycin	Drs	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT
CG32283	Drosomycin-like 3	Drsl3	CAGATGATATTCCTGTTTGCT	TGTCCCTCCTCAATGC
CG16844	Immune induced molecule 3	IM3	CGGGAGTCAAGCTCAGA	GTGGCTGTGTGAACTTCAA
CG6134	spätzle	spz	GATCTTCAGCCCACGG	CGAAGTCACAGGGTTG
CG5490	Toll	Tl	AGAGCGACGTATAGGACT	ACCTATAAGAGGGCGACT

on ice. Debris-free supernatants are collected by centrifugation and immediately processed as melanization interferes with the assay. Quantification is done in a 96-well format; samples and control buffer are distributed at 5 μl/well, then 200–250 μl buffer Z with 0.35 mg/ml ortho-Nitrophenyl-β-galactoside (ONPG) is added. Plates are then incubated in a heated microplate reader (37 °C) and measurements taken every 5–10 min at 420 nm. Usually a half-hour time-course is sufficient to obtain accurate quantification; it is advisable to pre-warm the plate for up to 30 min at 37 °C as accumulation of the colour reagent may remain undetectable at first (check for appearance of faint yellow colour in positive samples). In parallel, set up a 96-well format protein quantification (Bradford or BCA) using the same sample volume (5 μl).

β-Galactosidase activity is expressed as $\frac{\Delta OD420/\Delta t(min)}{\text{protein concentration} \times 0.0045}$ (in enzymatic units per amount of protein).

4.5.2. Fluorescent markers of immune activation

Similarly to lac-Z reporter genes described for enzymatic quantification, gene expression can also be assessed using fluorescent reporter strains, listed in Table 2.

If a simple off/on answer is sufficient, flies can be inspected simply by eye on a fluorescent stereomicroscope. Systemic and localized immune activation is easily scorable from 4 h onwards using GFP or mCherry fluorescence in whole animals, as illustrated in Fig. 1B and C. Microscopic analysis of live or fixed and stained immune responsive tissues (for example, hemocytes, tracheae, intestines, fat body) can give a more detailed insight into whether the response is restricted to specific cells within a tissue. This is particularly informative when analysing mosaics as it allows comparing immune activation of wild-type versus KO cells in a same tissue exposed to identical levels of infection.

4.6. Melanisation read-outs

Melanisation takes place in the hemolymph compartment and results in the synthesis of an insoluble black pigment called melanin as well as oxidative by-products, which are bactericidal [95,96]. During the melanisation reaction, sequential enzymatic cleavage of secreted serine proteases results in the cleavage of the pro-phenoloxidase PPO into its enzymatically active form PO. Once activated PO catalyses the oxidation of phenols into quinones which subsequently polymerize into melanin. The section below describes the most common assays used to monitor PO.

4.6.1. Collection of hemolymph

Collection of cell-free hemolymph is the first step to any experiments attempting to quantify PO activity. Since pro-phenoloxidases are secreted by crystal cells (see Section 4.6.5), a cell type highly sensitive to mechanical disruption, great care must be taken to avoid collecting cells along with the hemolymph.

The easiest and most efficient way of collection is by gentle centrifugation of intact larvae. 15–20 individuals are placed on a 10 μM filter spin column, covered with 4 mm glass beads and centrifuged for 20 min at 4 °C, 10 K rpm in a microcentrifuge. The weight of the beads is generally sufficient to extract hemolymph during the run, but animals can be pricked with a needle prior to centrifugation in order to optimize recovery. The filter column ensures that most cell debris is retained during centrifugation.

A more accurate but also more time-consuming method is hemolymph collection with a pulled glass capillary mounted on a Nanoject™ apparatus.

Independently of collection technique, the resulting hemolymph samples need to be kept on ice throughout the collection process and supplied with a concentrated protease inhibitor solution (for example Roche cOmplete™ Protease Inhibitor Cocktail Tablets) to avoid any spontaneous proteolytic activation of PO. Hemolymph protein concentration can be determined by any conventional protein quantification method.

Ideal collection times after wounding are 30 min for larvae and 4 h for adults.

4.6.2. DOPA assay

The DOPA test is a quantitative colorimetric assay frequently used to monitor PO activity of hemolymph samples. The assay is based on the conversion by PO of the substrate L-DOPA (L-3,4-dihydroxyphenylalanine) into a red pigment called dopachrome and the measurement of its absorbance at 492 nm. Coloration intensity is dependent on the initial amount of activated PO in the reaction.

In detail, spectrophotometer cuvettes are filled with 5–25 μg of hemolymph protein in a total volume of 200 μl 5 mM $CaCl_2$ solution (containing protease inhibitors, see Section 4.6.1). After addition of 800 μl L-DOPA solution (20 mM in phosphate buffer adjusted to pH 6.6, freshly prepared and protected from light) samples are incubated at 29 °C in the dark. After 30 min the optical density at 492 nm is measured for each sample (a microplate reader can also be used in case of numerous samples) against an L-DOPA control containing no hemolymph.

Note that because proteases inhibitors were added immediately after hemolymph collection, the values reflect the actual *in vivo* PO activity at the time of collection. To measure total PO and PPO activities, protease inhibitors should be replaced by chymotrypsin which facilitates the cleavage of PPO into PO. *Black cells* (*Bc*) and *PPO1*$^{\Delta}$, *PPO2*$^{\Delta}$ mutant flies that lack hemolymph PO activity can be used a negative control for the reaction (see Table 2 for PO-deficient mutants).

4.6.3. PPO cleavage assay

As a complement to the PO assay, a Western Blot using antibodies against *Drosophila* PO1 and PO2 can be performed on larval or adult hemolymph samples [97,131]. By this method, both the naïve PPO (75 kDa) and the mature form of PO (70 kDa), resulting from PO activation upon proteolytic cleavage by the upstream serine protease Hayan, can be detected. To enhance the efficiency of PO cleavage, flies can be infected with a mixture of Gram-positive and Gram-negative bacteria.

4.6.4. Optical read-outs

A very easy but non-quantitative way to monitor the melanisation reaction in adults consists in pricking flies in the thorax with a needle as for systemic infections. The blackening of the wounded area due to melanin deposition can be observed already 30 min post injury (Fig. 3). To image the size and the intensity of the melanisation on the wounded thorax, pictures can be taken using a camera attached to a microscope 4 hours post wounding. Note that the wounding reaction differs according to the bacterial strains; *L. monocytogenesis* and *S. aureus* tend to induce larger melanization spots [98].

In larvae, the melanisation reaction can be observed in larvae after pricking with a thin needle usually on posterior side [34]. 5 min after injury, synthesis of melanin on the surface of the cuticle is already visible. Pictures of the melanisation can be taken 30 min post injury. It is important to keep in mind that the melanisation reaction can vary considerably between animals. In order to have a clear representation of the melanisation reaction in wounded specimen it is essential to repeat the experiment several times and to take a large number of pictures that illustrates the melanised area. *Spn27A*^{1} flies lacking the serpin Spn27A negative regulator show a precocious and uncontrolled melanisation reaction even in absence of injury. *PPO1*$^{\Delta}$, *PPO2*$^{\Delta}$ or *Bc* mutant flies that lack hemolymph PO activity can be used as a negative control of the reaction (see Table 2).

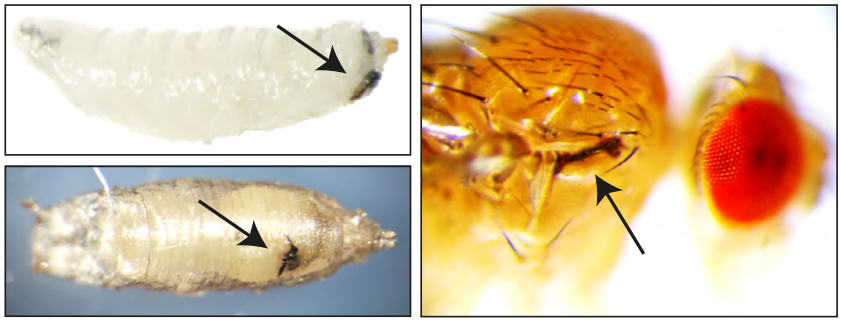

Fig. 3. Melanisation spot on larvae, pupae and adults. *Oregon*^{R} individuals were pricked with a sterile needle and pictures of melanised wound sites were taken at 30 min (larvae) or 3 h (pupae and adults) after injury.

4.6.5. Crystal cell visualisation

Crystal cells are specialised larval hemocytes responsible for the synthesis and the release of PPOs. In larvae, crystal cells can be visualized using a transgenic strain carrying *lz-Gal4, UAS-GFP* which expresses GFP under the control of the crystal cell-specific promoter lozenge (see Table 2). Alternatively, spontaneous activation of PPO in crystal cells can be achieved by incubating larvae in PBS at 65 °C for 10 min [99].

4.7. Oxidative burst

Several ROS quantification methods have been developed over recent years. Here we limit ourselves to ROS detection in the gut. Here we describe one of the more commonly used probes, i.e. 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) that is used to sense specifically H_2O_2 [93,100]. We measured ROS levels in the adult gut of female flies by the addition of 100 μM DCFH-DA fluorescent dye (Sigma) to freshly dissected gut tissue. The dissections are done in the presence of 20 mM NEM (N-ethyl maleimide, Sigma) and the tissue is preserved in NEM until addition of DCFH-DA dye. The tissue is then incubated in the dye for 30 min and then mounted in 70% Glycerol. Sections of anterior midguts should be imaged. The DCFH-DA fluorescent signal is analysed using excitation at 488 nm and emission at 529 nm. An important consideration is growing flies without live yeast as this may influence basal levels of ROS in the intestine. For the quantification we use pixel values of signal intensity and take the average signal measured on representative fields of at least 6 guts.

Recently, the group of Won-Jae Lee has employed a novel probe for measuring ROS in *Drosophila* intestine [101]. This probe (R19S) is a rhodamine-based sensor which specifically reacts with HOCl and not with other ROS [102]. In the same publication, the group utilized 100 mM of Amplex UltraRed reagent (Invitrogen) and 0.2 U/ml of horseradish peroxidase (Sigma) reaction buffer to measure H_2O_2 in *Caenorhabditis elegans*. The probe DCFH-DA and Amplex UltraRed reagent have been used previously for fluorescence-based quantitative measurements in human cell culture. For *Drosophila* a similar quantitative measurement has yet to be reported.

For protocols on how to measure oxidative burst by fluorimetric indicators coupled to immunohistochemistry, the reader is referred to the excellent methods developed by the Banerjee lab (http://www.nature.com/protocolexchange/protocols/414).

4.8. Hemocyte observation

Basic protocols to observe hemocytes were described in [34].

4.9. Phagocytosis assays

The phagocytosis assay described below has been optimised in our lab from a combination of similar methods [23,103,104]. In this protocol, larval hemocytes are collected and mixed with fluorescent heat-killed bacteria, incubated and then run on a flow cytometer to measure the fraction of cells phagocytosing and the intensity of phagocytic uptake. Phagocytosis can be reduced by injecting beads in the body cavities [105]. Recently, assays to monitor phagosome maturation have been developed [106].

To assay phagocytosis, 2–5 day old 30 female and 15 male flies are grown in large vials ready-sprinkled with yeast and containing 10 mL 0.05% (w/v) bromophenol blue fly food at 25 °C. Cultures are flipped every morning (24 h) to yield a rearing density of 100–150 eggs per vial. Collect an excess of wandering L3 larvae with light blue to white guts. Before dissection, larvae are washed in H_2O to remove food, and re-suspended in PBS but not totally submerged such that they remain oxygenated. Prior to bleeding, larvae are dried on precision tissues (Kimtech, Kimberly-Clark).

The number of larvae to bleed depends on genotype (see below). Bleeds are collected in 120 μl cold Schneider's medium (Gibco) containing 1 nM phenylthiourea (PTU, Sigma, freshly prepared) bounded by a hydrophobic ring, drawn using an ImmuEdge pen (Vector Laboratories Inc., US) on a glass slide. The glass slide is atop a 9 cm petri plate filled with ice, used to chill the larvae.

To bleed, chilled larvae are dissected by holding the posterior with 45°-angled tweezers (Inox, No. 5) and with second pair, pinching posterior cuticle and pulling anteriorly to release hemocytes into the medium. A 100 μl volume of cells is transferred to an ultra-low attachment 96-well plate (Corning, Costar no. 3474) and incubated at RT for 10 min. The bacteria are then added: 2×10^6, 2×10^7 or 2×10^8 Alexa-Fluor 488 heat-killed *E. coli* or *S. aureus* (Molecular Probes, for preparation see Section 5.8.3 below) in 10 μl Schneider's/PTU to each well. The bacteria and hemocytes are mixed by gentle pipetting 7 times. The cells are incubated at RT for 20 min to enable phagocytosis. Immediately afterwards, 50 μl 0.4% Trypan blue (Sigma, store at RT) is added to quench the fluorescence of extracellular bioparticles. Trypan blue should be pre-spun, 10 min, 13,000 rpm to pellet insoluble particles and kept at RT to avoid precipitation. Immediately run the quenched sample on a flow cytometer (BD Accuri, US) and measure the mean fluorescence intensity of the single cell population relative to a non-fluorescent control (no bacteria added).

4.9.1. Calculation of phagocytic index

% of Cells phagocytosing = 100 × [number of singlets in fluorescence positive gate]/[number of singlets in fluorescence negative gate].

Phagocytic index, PI = [mean fluorescence intensity of singlet population in bacteria-added sample]/[mean fluorescence intensity of non-fluorescent, no bacteria added, control].

Typical flow cytometry measurements of hemocyte phagocytosis of AF488-labelled heat killed *S. aureus* and a calculation of the phagocytic index are shown in Fig. 4.

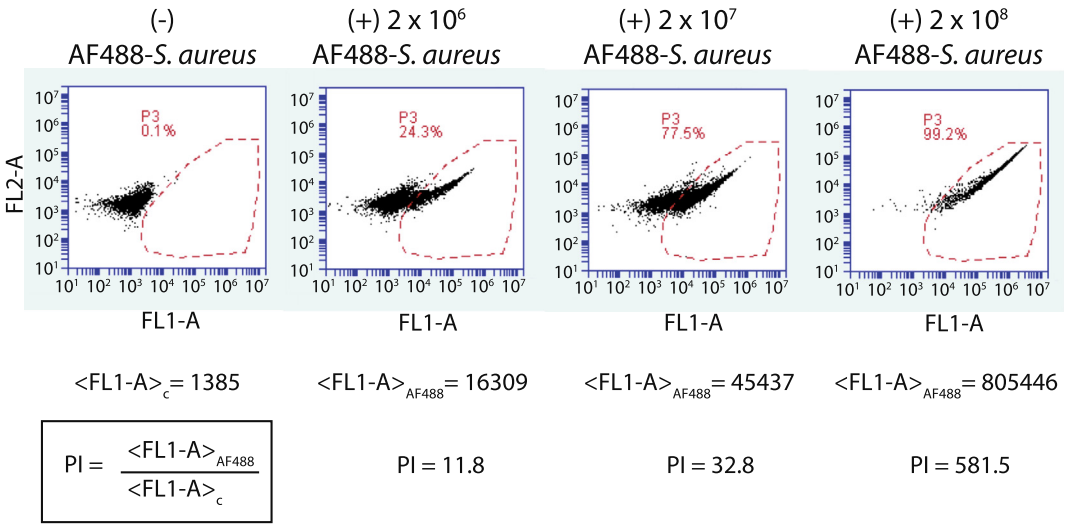

Fig. 4. Measurement of the phagocytic index of *ex vivo* larval hemocytes incubated with AF488-labelled heat killed *S. aureus*. Shown are the fluorescence intensities of *ex vivo* singlet hemocytes from 20 $Oregon^R$ L3 larvae after incubation with 0, 2×10^6, 2×10^7 or 2×10^8 AF488-labelled heat-killed *S. aureus*. Each plot represents a single experiment. Each dot represents a singlet hemocyte. The fluorescence intensity 2 (FL2-A, red) is plotted against the fluorescence intensity 1 (FL1-A, green, AF488 emission). When no bacteria are added, autofluorescence is measured. Addition of 2×10^6 AF488 heat-killed *S. aureus* caused 23.4% of singlet hemocytes to appear in fluorescence gate P3. Calculations of the phagocytic index, PI, from $\langle FL1 \rangle_c$, the mean FL1 intensity of the no bacteria added control, and $\langle FL1 \rangle_{AF488}$, the mean FL1 intensity after addition of bacteria are shown directly below each plot. After addition of 2×10^8 AF488-*S. aureus*, phagocytosis was around 50 times higher than after addition of 2×10^6 AF488-*S. aureus*.

4.9.2. Hemocyte numbers

Circulating hemocyte number in third instar larvae varies between genotypes, therefore, to achieve cell-matched assays across genotypes, it is necessary to adjust the number of larval bleeds between genotypes. On average, 20 larval bleeds from $Oregon^R$ yields ~5000 singlet hemocytes and w^{1118} (BL5905) yields ~7500 singlet hemocytes using the above protocol. Therefore, before doing phagocytosis assays, use the flow cytometer to measure the hemocyte yield per 20 larval bleeds for each genotype and compare to wild type $Oregon^R$. Then use hemocyte yield-adjusted numbers of larval bleeds to achieve 20 $Oregon^R$ larval bleed equivalents or ~5000 singlet hemocytes for all assays across all genotypes.

4.9.3. Preparation of bioparticles

To prepare fluorescent heat-killed Alexa-Fluor conjugated *E. coli* or *S. aureus* (Molecular Probes), 2 mg lyophilized bacteria are dissolved in 2 mL sterile PBS and vortexed for 1 min at top speed. The bacterial particles suspension is further homogenised by passaging 40 times through a 25G × 1.5″ hypodermic needle (Sterican, Braun) mounted on a 2 mL syringe. To determine particle number, dilute a sample serially 10-fold to 1/1000 in PBS, 0.05% Tween-20 and count using a hemocytometer, ×40 objective. Typically $1.0–6.0 \times 10^{10}$ particles are present per mL. Keep on ice and make 10 µl aliquots, freeze overnight at −80 °C and store at −20 °C. While aliquoting, it is extremely important to vortex the bacterial suspension for 5 s between aliquots.

Before use in a phagocytosis assay, aliquots are diluted to the desired concentration and vortexed for 30 s after preparation and for 15 s before each use.

4.10. Clotting assay

Several protocols have been developed to monitor clotting in third instar larvae. This includes various histological methods to visualize the clot, bead aggregation assays, draw out techniques to monitor the viscosity of hemolymph. For details see [107] as well as a movie of a drawout of a hemolymph sample from fondue mutant larvae http://booksite.elsevier.com/9780123739766/.

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