Chapter 35
Calcium Imaging in *Drosophila melanogaster*

Nicola Vajente, Rosa Norante, Paola Pizzo, and Diana Pendin

Abstract *Drosophila melanogaster*, colloquially known as the fruit fly, is one of the most commonly used model organisms in scientific research. Although the final architecture of a fly and a human differs greatly, most of the fundamental biological mechanisms and pathways controlling development and survival are conserved through evolution between the two species. For this reason, *Drosophila* has been productively used as a model organism for over a century, to study a diverse range of biological processes, including development, learning, behavior and aging. Ca^{2+} signaling comprises complex pathways that impact on virtually every aspect of cellular physiology. Within such a complex field of study, *Drosophila* offers the advantages of consolidated molecular and genetic techniques, lack of genetic redundancy and a completely annotated genome since 2000. These and other characteristics provided the basis for the identification of many genes encoding Ca^{2+} signaling molecules and the disclosure of conserved Ca^{2+} signaling pathways. In this review, we will analyze the applications of Ca^{2+} imaging in the fruit fly model, highlighting in particular their impact on the study of normal brain function and pathogenesis of neurodegenerative diseases.

Keywords Calcium imaging · *Drosophila* · Calcium indicators · GECI · Calcium signaling · Neurodegenerative diseases

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35.1 A Brief History

The path of *Drosophila* as a research model is a history of groundbreaking achievements, underpinned by 6 Nobel Prizes since 1933. The first went to Thomas Hunt Morgan, who delineated the theory of inheritance by using *Drosophila* to define genes location on chromosomes [1]. Some years later, Hermann Muller defined the effects of X-rays on mutation rate in fruit flies [2], opening the field to modern genetics. These seminal discoveries allowed the generation of genetic tools that still prosper, *e.g.*, balancer chromosomes, special chromosomes that, preventing meiotic crossing-over, are used to maintain complex stocks with multiple mutations on single chromosomes over generations [3]. New genetic tools developed over the years allowed the fruit fly to move with times. As a significant example, CRISPR/Cas9 genome editing strategies allow simple and rapid engineering of the fly genome [4].

What makes *Drosophila* the model organism of choice of many researchers is the observation that relevant genes, cellular processes and basic building blocks in cellular and animal biology are conserved between flies and mammals [5]. Moreover, compared to vertebrate models, *Drosophila* has considerably less genetic redundancy, making the characterization of protein function less complicated. The function of a gene product can be inferred by generating fly lines for its up- or down-regulation and then analyzing the resulting phenotypes. The fruit fly represents also an ideal model organism to study human diseases. Remarkably, over 60% of known human disease-causing genes have a fly orthologue [6]. Most of the cellular processes known to be involved in human disorders pathogenesis, including apoptosis signaling cascades, intracellular calcium (Ca^{2+}) homeostasis, as well as oxidative stress, are conserved in flies. Of note, the high accessibility of the nervous system at different developmental stages, makes also neuroscience experiments feasible in the fly model. Moreover, flies exhibit complex behaviors and, like in humans, many of these behaviors, including learning, memory and motor ability, deteriorate with age [7, 8].

Box 35.1 Advantages of Using *Drosophila* as a Research Animal Model
Beside genetics, the strongest selling point of using *Drosophila* as an animal model are: (i) *Drosophila* are relatively inexpensive and easy to keep, as they are raised in bottles or vials containing cheap jelly-like food. (ii) Generally, there are very few restrictions, minimal ethical and safety issues on their laboratory use. (iii) Flies life cycle is very fast, lasting about 10–12 days at 25 °C. Newly laid eggs take 24 h to undergo embryogenesis before hatching into first instar larvae, which develop into second, and then third instar larvae. The duration of these stages varies with the temperature: at 20 °C, the average length of the egg-larval period is 8 days; at 25 °C it is reduced to 5 days. Larvae transform into immobile pupa, undergo metamorphosis and eclose in the adult form 5–7 days later. (iv) A single fly can produce hundreds

(continued)

Box 35.1 (continued)
of offspring within days, thus it is relatively easy to quickly generate large numbers of embryos, larvae or flies of a given genotype. Individual flies are easily manipulated when anaesthetized with carbon dioxide, allowing identification of selectable phenotypic features under a stereomicroscope [9].

35.2 *Drosophila* Ca^{2+} Toolkit

The Ca^{2+} ion is the major intracellular messenger, mediating a variety of physiological responses to chemical and electrical stimulations. Therefore, cell Ca^{2+} concentration [Ca^{2+}] must be tightly controlled in terms of both space and time, a task that is accomplished by several Ca^{2+} transporting and buffering systems. Easily accessible knock-down and knock-out strategies, applicable to cell lines, as well as to living animals, have helped discovering in flies a number of molecules involved in Ca^{2+} signaling. As in mammals, basal cytosolic Ca^{2+} levels are controlled in flies by the interplay of Ca^{2+} transport systems, localized in the plasma membrane (PM) and the membranes of intracellular organelles that function as internal Ca^{2+} stores (Fig. 35.1). This toolkit, together with a number of Ca^{2+}-binding proteins, concurs in creating and regulating the dynamics and spatial localization of Ca^{2+} signals. The major players in Ca^{2+} signaling in *Drosophila* are briefly described below; the interested reader is referred to a more extensive review on the topic [10].

Ca^{2+} enters the PM through Ca^{2+} channels, *e.g.*, voltage- and ligand- gated. As for voltage-gated Ca^{2+} channels, the fly genome encodes three α1 subunits (Ca-α1D, cacophony, Ca-α1T) forming Ca_v1, Ca_v2, and Ca_v3 type channels, respectively, mainly expressed in the nervous system and muscles [10]. Among ligand-gated channels, glutamate-gated ionotropic receptors (iGluRs) are represented in *Drosophila* by 15 genes encoding different subunits. As in other animal species, *Drosophila* uses glutamate as a fast neurotransmitter in neuromuscular junctions (NMJs), and highly Ca^{2+} permeable iGluRs are clustered in active zones in postsynaptic motor neuron terminals [11]. Cations enter sensory neurons through Transient Receptor Potential (TRP) channels. The gene encoding the first member of the trp superfamily was identified in *Drosophila* photoreceptors as a PM Ca^{2+} permeable channel, required for mediating the light response [12]. A vast number of trp homologs were found in vertebrates that have been classified in seven major subfamilies in metazoans.

Cytosolic Ca^{2+} increase can be also triggered by the activation of phospholipase C (PLC), which produces inositol 1,4,5-trisphosphate (IP_3) that interacts with Ca^{2+} channels located in the Endoplasmic Reticulum (ER) and Golgi apparatus (GA), causing their opening. Three IP_3 receptor (IP_3R) isoforms are expressed in mammals, while a single IP_3R is present in *Drosophila* (itpr) [13]. The channel shares the highest level of similarity as well as functional properties (channel conductance, gating properties, IP_3- and Ca^{2+}-dependence) with the mouse IP_3R1. The release of Ca^{2+} from intracellular stores occurs also through Ryanodine

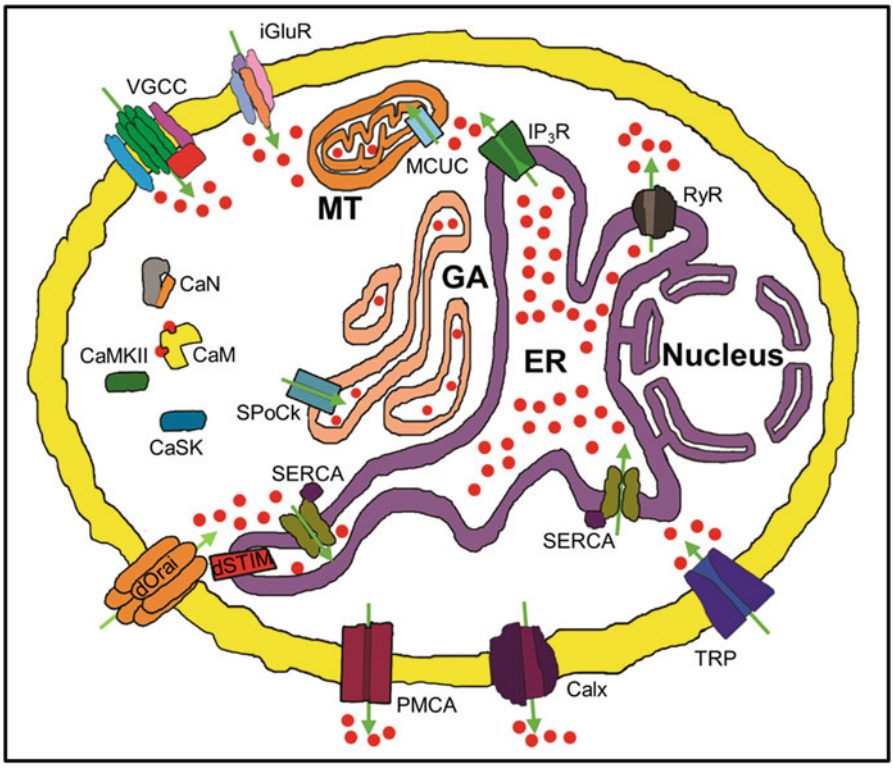

Fig. 35.1 A *Drosophila* cell with its Ca^{2+} toolkit. The movement of Ca^{2+} ions (red spots) are indicated as green arrows. The Ca^{2+} handling proteins inserted in the PM, from the upper left corner, are: Voltage-Gated Ca^{2+} channels (VGCC), glutamate-gated ionotropic receptors (iGluRs), Transient Receptor Potential (TRP) channels, Na^{+}/Ca^{2+} exchanger (Calx), PM Ca^{2+} ATPase (PMCA) and ORAI1 oligomers forming a channel. In the ER membrane from the upper left side are present: inositol 1,4,5-trisphosphate receptor (IP_3R), Ryanodine Receptor (RyR), Sarco-Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) and STIM1. In the GA membrane is present the Secretory Pathway Ca^{2+} ATPase (SPoCk). The inner mitochondrial membrane hosts the mitochondrial calcium uniporter complex (MCUC). Different Ca^{2+} interacting proteins are resident in the cytosol: Calcineurin (CaN), Calmodulin (Cam), Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII), and Ca^{2+}/Calmodulin-dependent serine protein kinase (CaSK)

Receptor (RyR), located in the sarco/endoplasmic reticulum (SER) membrane. In vertebrates, three isoforms are described (RyR 1–3), while the *Drosophila* genome contains a single RyR gene that encodes a protein with approximately 45% identity with the vertebrate family members [14, 15].

Ca^{2+} release from intracellular stores is most often accompanied by Ca^{2+} influx through PM channels in the regulated process of Store-Operated Ca^{2+} Entry (SOCE). The molecular basis of SOCE, whereby Ca^{2+} influx across the PM is activated in response to depletion of ER Ca^{2+} stores, has been under investigation for more than 20 years and was finally revealed thanks to the identification of the two

molecular key players in RNAi screens performed in *Drosophila* S2 cells [16, 17]. The presence of a single fly homologue for stromal interacting molecule 1 (STIM1) and ORAI1, whereas mammals have two and three copies respectively, offered a flying start for the identification of the proteins.

The main route for Ca^{2+} uptake into mitochondria is through the mitochondrial calcium uniporter (MCU) complex, a Ca^{2+}-selective ion channel located at the inner mitochondrial membrane. The channel subunit MCU is regulated through other regulatory components, i.e. MICU1/2/3 and EMRE [18]. A MCU homologue has been identified and characterized in *Drosophila* [19, 20], along with its regulatory subunits MICU1 [19, 20] and EMRE [21].

Ca^{2+} signals are terminated by the combined activities of Ca^{2+} ATPases, located on PM, ER, GA membranes and the Na^{+}/Ca^{2+} exchanger, NCX. PM Ca^{2+} ATPase (PMCA) is a protein present in all animals, characterized by a high Ca^{2+} affinity and a low-transport capacity that extrudes Ca^{2+} from the cytosol to maintain $[Ca^{2+}]$ at the basal value of about 100 nM. In humans and other mammals, four major PMCA isoforms are encoded by separate genes, while the *Drosophila* genome encodes a single, ubiquitously expressed PMCA [22]. The ATPases located on ER and GA membranes acts to re-accumulate the cation in the organelles' lumen. The SER Ca^{2+} ATPase (SERCA), transports inside ER/SR two Ca^{2+} ions per ATP hydrolyzed. In vertebrates, three SERCA protein isoforms are encoded by three distinct genes, while in *Drosophila* a single gene was identified [23]. Fly SERCA has a higher identity with mammalian SERCA1 and SERCA2 (71–73%) and is expressed at a very high level in the central nervous system (CNS) and muscles. A single homolog of the Secretory Pathway Ca^{2+} ATPase (SPCA) is present in *Drosophila*, named SPoCk. The gene results in three isoforms, but only one (SPoCk-A) has been reported to localize in GA membranes, as its mammalian counterpart [24]. The other two variants have been reported to localize in the ER and peroxisomal membranes. The NCX is a non-ATP-dependent antiporter that mediates the efflux of Ca^{2+} ions in exchange for Na^{2+} import. The *Drosophila* NCX, named Calx, is highly expressed in brain and muscle and has 55% identity with the three mammalian isoforms NCX1, NCX2 and NCX3, which are differentially expressed mainly in the heart, brain and skeletal muscles, respectively [25].

35.3 Experimental Set Up for Ca^{2+} Imaging in *Drosophila*

The conserved Ca^{2+} molecular toolkit, together with the advantages of the model depicted above (Box 35.1), set the basis for the fruit fly to be a major model organism for Ca^{2+} signaling research. Thanks to the development of a broad range of Ca^{2+} indicators (Box 35.2), Ca^{2+} imaging procedures have been specifically designed for their application in flies.

Advancements in Ca^{2+} imaging techniques have proceeded through two distinct although interconnected avenues: the improvement of Ca^{2+} indicators and the development of appropriate instrumentations. In the field of Ca^{2+} imaging of live tissues/animals, the application of wide-field microscopy is limited by light scattering across the z-axis of extended pieces of tissue, thus the use of two-photon

(2P) microscopy is usually preferred. 2P microscopy have allowed measurements in the intact brain of an entire transgenic animal, improving spatial resolution by restricting the excitation of chromophores to defined focal planes.

In order to perform optical Ca^{2+} imaging experiments in *Drosophila*, the access of light for excitation to the structures of interest must be assured. The simplest possibility is to excite the genetically encoded Ca^{2+} indicator (GECI, see Box 35.2, Fig. 35.2) directly through the animal's cuticle without any surgical manipulation. When baseline fluorescence of the GECI used is strong enough, the partial transparency of third instar larvae allows for optical access of brain, dorsal sensory neurons and muscles. Since imaging is hurdled by continuous larval movements, a few experimental tips have been developed, *e.g.*, immobilization of the larva on the coverslip with a transparent sticky tape [26] or in microfluidic clamps [27]. Despite the immobilization, slight contractions and movements cannot be completely eliminated, possibly leading to shifts in the focal plane and thus alterations in the fluorescence intensity. The problem can be overcome by using ratiometric GECIs (Box 35.2).

Box 35.2 Ca^{2+} Indicators for Imaging in Flies

Approximately 30 years ago, scientists started to design and engineer organic fluorescent Ca^{2+} indicators, opening the door for cellular Ca^{2+} imaging. Since then, a variety of probes have been developed, which differ in their mode of action, Ca^{2+} affinities, intrinsic baseline fluorescence and kinetic properties. Two major classes of Ca^{2+} indicators have been developed, *i.e.*, chemical probes and genetically encoded Ca^{2+} indicators (GECIs).

Chemical indicators (*e.g.*, fura-2, indo-1, fluo-4) are small fluorescent molecules that are able to chelate Ca^{2+} ions. These molecules are based on BAPTA, an EGTA homologue with high selectivity for Ca^{2+}. The Ca^{2+} chelating carboxyl groups are usually masked as acetoxymethyl esters, making the molecule more lipophilic and allowing an easy entrance into cells. Once the molecule is inside the cell, the Ca^{2+} binding domains are freed by cellular esterases. Binding of a Ca^{2+} ion to the molecule leads to either an increase in quantum yield of fluorescence or an emission/excitation wavelength shift. Chemical indicators are mostly used to measure cytosolic $[Ca^{2+}]$. Early attempts to measure presynaptic $[Ca^{2+}]$ in *Drosophila* employed membrane permeant chemical Ca^{2+} indicators [30]. However, this technique is hardly reliable due to uneven dye loading, high background fluorescence and lack of cell type selectivity. To overcome current limitations, dextran-conjugated fluorescent Ca^{2+} indicators have been loaded in cut axons. The approach allowed to measure resting $[Ca^{2+}]$ and nerve-evoked Ca^{2+} signals during high-frequency activity [31].

GECIs include different types of engineered proteins, such as single fluorescent protein-based indicators (*e.g.*, GCaMP), bioluminescent probes (*e.g.*, aequorin) and fluorescence (or Förster) resonance energy transfer (FRET)-based indicators (*e.g.*, cameleons) [32].

(continued)

Box 35.2 (continued)

GCaMP is one of the most used GECIs, based on a circularly-permuted variant of Green Fluorescent Protein (cpGFP). The N-terminus of the cpGFP is connected to the M13 fragment of the myosin light chain kinase, while the C-terminus ends with the Ca^{2+}-binding region of calmodulin (CaM). In the presence of Ca^{2+}, M13 wraps around Ca^{2+}-bound CaM, leading to a conformational change that increases the fluorescence protein (FP) fluorescence intensity [33] (Fig. 35.2, panel a). During recent years, different variants of GCaMP indicators have been developed, with improved characteristics in terms of Ca^{2+} affinity, brightness, dynamic range. Other variants of FPs, *e.g.*, red-coloured, have been used to develop sensors allowing simultaneous measurement in different organelles, making GCaMPs a whole family of great tools to follow Ca^{2+} dynamics.

One limitation in the use of GCaMPs, and in general of single protein-based GECIs, is the sensitivity to movement artifacts as well as focal plane shifts, which can be mistaken for $[Ca^{2+}]$ changes. A method used to correct for this type of artifacts is to co-express a FP together with the GECI [34]. Alternatively, the limit can be overcome by using ratiometric indicators, such as cameleon. This molecule uses the same Ca^{2+} binding domains of the GCaMP (M13 and CaM), that are bound to two different variants of the GFP: a cyan (CFP) and a yellow (YFP) variant. In the absence of Ca^{2+}, the excited CFP emits at 480 nm, while in the presence of Ca^{2+} the interaction between Ca^{2+}, CaM and M13 brings the two FPs at a closer distance (2–6 nm), and the energy released from the CFP is absorbed by the YFP, that emits at a different wavelength (535 nm) (Fig. 35.2, panel b). By calculating the ratio of EYFP/ECFP emissions, one obtains a clear indication of intracellular $[Ca^{2+}]$ variations, excluding changes of fluorescence caused by artefactual movements of the sample.

GECIs allow the monitoring of Ca^{2+} not only in the cytosol, but also in organelles (*e.g.*, ER, mitochondria, GA, etc.) thanks to the addition of specific targeting sequences. GECIs have demonstrated valuable in the measurement of $[Ca^{2+}]$ in cells and within organelles in several in vivo models. Notably, in flies, the ease of transgenesis allowed the generation of several lines for the expression of GECIs, both cytosolic and organelle-targeted. Moreover, the Gal4-UAS expression system [35, 36] allows the targeting of the probes to specific tissues or even cell subtypes. In this two-part approach, one fly strain carries the Ca^{2+} sensor cDNA under the control of an upstream activator sequence (UAS), so that the gene is silent in the absence of the transcription factor Gal4. A second fly strain expressing Gal4 in a cell type-specific manner is mated to the UAS strain, resulting in progeny expressing the probe in a transcriptional pattern that reflects the expression pattern of the Gal4 line promoter.

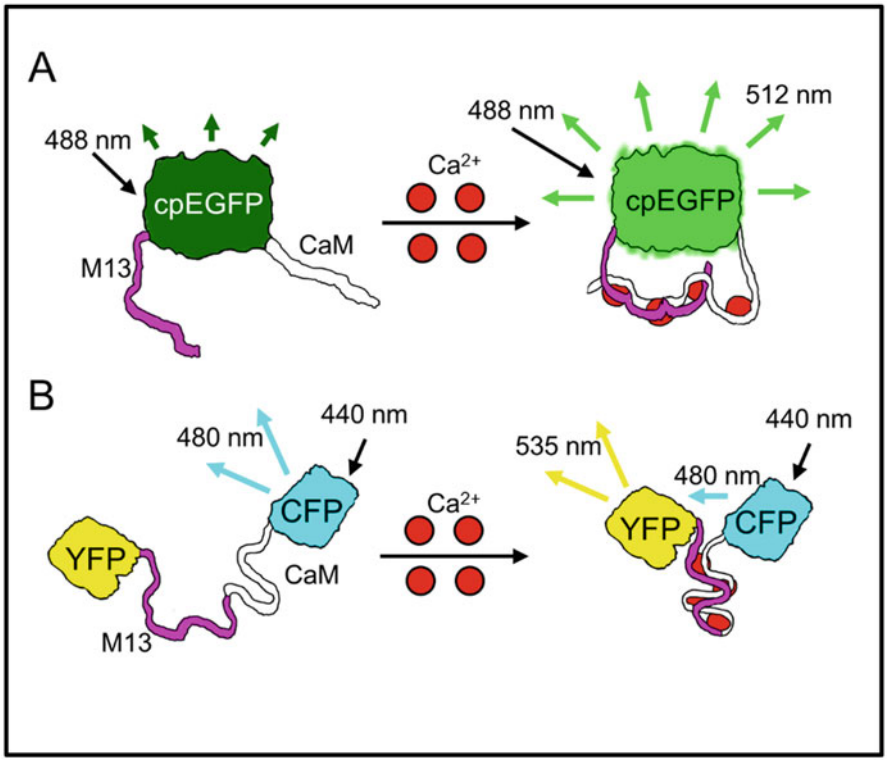

Fig. 35.2 Most used indicators for in vivo Ca^{2+} imaging in *Drosophila*. (**a**) The single-wavelength indicator GCaMP is composed by: the M13 fragment of the myosin light chain kinase domain (M13, pink), the circularly-permutated enhanced Green Fluorescent Protein (cpEGFP, green) and Ca^{2+}-binding region of calmodulin (CaM, white). The FP is excited at 488 nm and the emission is detected at 512 nm. Upon Ca^{2+} binding, a conformational change increases the emitted fluorescence intensity. (**b**) The FRET-based Cameleon probe, composed by: Yellow Fluorescent Protein (YFP, yellow), the M13 domain (pink), the CaM domain (white) and the Cyan Fluorescent Protein (CFP, cyan). The protein is excited at 440 nm and in absence of Ca^{2+} the emission is detected at 480 nm; upon Ca^{2+} binding, conformational changes provide the optimal distance to get Forster Resonance Energy Transfer (FRET), and the YFP emission is detected at 535 nm

Imaging Ca^{2+} activities in the CNS of adult flies usually requires a surgical intervention to achieve optical access to the brain. However, trans-cuticular imaging have also been applied to intact adult brains using 3P microscopy [28]. We report, as an example, a protocol applied to monitor odor-evoked Ca^{2+} dynamics. Anesthetized flies are restrained between a sticky tape and a fine-meshed metal grid which enables air exchange around the abdomen. A small hole cut through the sticky tape into the head capsule allows the exposure of the brain and the antennal lobes expressing the GECI. The odors are then applied to the fly's antennae and the temporal dynamics and spatial distribution of Ca^{2+} activities are monitored using an imaging microscope [26] (Fig. 35.3). Since movements are reduced in these

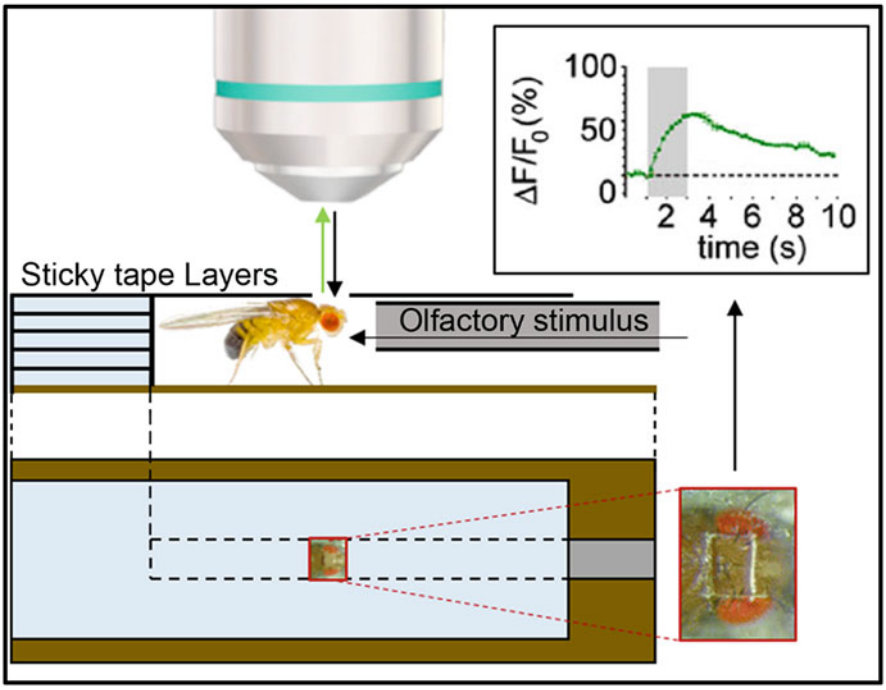

Fig. 35.3 Schematic illustration of a set up for in vivo odor-stimulated Ca^{2+}imaging in Drosophila. A grid is placed on the microscope slide and fixed with several layers of sticky tape. A small passage is cut into the layers of tape, fitting a single fly and a small tube for the delivery of the odor. The chamber is sealed with another layer of adhesive tape where a small window is cut, providing access to the fly's head. The dynamics of intracellular Ca^{2+} in the olfactory sensory neurons of the antennal lobe has been detected upon application of 3-octanol using the sensor G-CaMP 3.0. The duration of the odor stimulus is indicated as a grey bar. (Adapted from: Ref. [29])

preparations, single-wavelength GECIs, such as GCaMPs, are usually preferred, due to their higher dynamic range and because they permit the use of simpler imaging systems.

Thanks to the parallel development of indicators and imaging systems, Ca^{2+} imaging has matured over the years into a powerful tool for imaging of cellular activity in living animals. We propose now an overview of Ca^{2+} imaging experiments that can be performed in *Drosophila*, aware that by far this is not all encompassing, and the list of interesting investigations could certainly be extended.

35.4 Ca^{2+} Imaging: Sensory Neuroscience and Beyond

Drosophila melanogaster contributed to many aspects of neuroscience. In the past, the analysis of fly brain function has been challenging due to the small size of

neurons that initially restricted electrophysiological recordings to specific highly accessible regions, *e.g.*, larval preparations of NMJs [37]. Recently, whole-cell patch-clamp recordings have been performed on fly central neurons [38], providing insights into neuronal activity with an excellent temporal precision. However, as in the brains of vertebrates, *Drosophila* sensory stimuli, motor outputs as well as central processing events, are encoded as spatio-temporal activity patterns that require the concerted activity of many neurons. As a consequence, besides recordings from individual neurons, monitoring the activity across many cells is mandatory to explore complex circuits. In neurons, membrane depolarization is accompanied by fast Ca^{2+} influx via voltage-gated Ca^{2+} channels, as well as slower Ca^{2+} signals deriving from the ER and mitochondrial Ca^{2+} pools [39]. The easiest way to indirectly measure membrane depolarization is measuring the variations in $[Ca^{2+}]$ inside the cells. The development of GECIs allowed for these measurements in vivo in multi-cellular animals, by targeting the probes to specific cells and sub-cellular compartments.

Different types of scientific questions concerning the function of the *Drosophila* brain can be addressed using optical Ca^{2+} imaging. One of the most extensively explored fields regards the mechanisms of sensory processing, *i.e.*, how neural activity encodes sensory input in behavioral output. Sensory cells and directly coupled downstream neurons encode the sensory stimuli by membrane depolarization-induced action potential frequencies. High-intensity stimuli result in high-firing frequencies, leading to strong intracellular Ca^{2+} transients, allowing to fully exploit the potential of GECIs. A number of studies in this field have helped identify and measure the response of specific brain regions to various sensory stimuli including olfaction, taste, and thermosensation [26]. We present here some significant examples.

Optical Ca^{2+} imaging has been successfully applied to study neuronal activity in the olfactory system of the fly's brain. Flies display robust odor-evoked behaviors in response to cues from plants or other flies. More than 1000 olfactory sensory neurons located in the olfactory sensory organs of the head (*i.e.*, the third segment of the antenna and the maxillary palps) project with their axons to the antennal lobe, the primary olfactory center of the fly's brain. The neuronal terminal arborizations are organized into spherical structures called glomeruli that contact projection neurons and local interneurons. Projection neurons signal to higher brain centers, such as the mushroom body and the lateral horn. Since each sensory neuron expresses a limited number of olfactory receptors with a specific ligand-binding profile, each odor information is represented as a specific spatiotemporal code before it is sent to higher brain centers. Optical Ca^{2+} imaging has been performed in each order of olfactory neurons, including the antennal lobe (example in: [40]; protocol in [41]) and the neurons of the mushroom bodies (Kenyon cells) [42]. The mushroom body has been shown over many years to be a brain region necessary and sufficient for the association of odor stimuli through learning with rewarding or punishing cues [43, 44]. Electrophysiological studies on individual cells allowed to propose a model for odors encoding in the mushroom bodies. The model proposed that only very few out of a large array of Kenyon cells are selectively responding to any given odor

stimulus, due to the convergence of several projection neurons onto a given Kenyon cell, combined with the high firing thresholds of Kenyon cells. The use of GECIs recently allowed to confirm the proposed model: the activity of >100 mushroom body neurons was simultaneously monitored in vivo by two-photon imaging of the Ca^{2+} indicator GCaMP3, allowing the visualization of the distinct patterns of sparse mushroom body neurons activated upon different odors stimulation [45].

Ca^{2+} imaging has also proven to be of enormous value for the analysis of how auditory stimuli are encoded in flies. The structure that has been primarily associated with hearing is the Johnston's organ, located on the second antennal segment. Interestingly, Ca^{2+} imaging experiments performed in intact animals demonstrated that Johnston's organ contains also wind-sensitive neurons. GCaMP-1.3 was expressed under the control of different Gal4 enhancer trap lines in distinct groups of neurons in Johnston's organ. Live flies were mounted in an inverted orientation under a two-photon microscope, and an air flow or a near-field sound, were delivered while recording Ca^{2+} dynamics. Optical Ca^{2+} imaging represented here a powerful tool to dissect this novel circuit, providing evidence that a common sensory organ is used to encode sound-evoked stimuli and air movements [46].

Other relevant aspects for which optical Ca^{2+} imaging using GECIs has been successfully applied to sensory neuroscience comprise: propagation of fly taste perception [47], neuronal plasticity underlying associative learning and memory formation [42], visual circuits dissection [48], mapping of mechanosensory circuits [49].

An interesting recent work, exploiting whole-brain Ca^{2+} imaging in adult flies, aimed at assessing intrinsic functional connectivity. Ca^{2+} signals were acquired from the central brain and functional data were assigned to atlas regions. This allowed to correlate activity between distinct brain regions, providing a framework for using *Drosophila* to study functional large-scale brain networks [50]. Whole-brain imaging has also been attempted during open field behavior in adult flies [51], allowing functional imaging of brain activity of untethered, freely walking flies during sensorial and socially evoked behaviors. Of note, despite imaging over extended periods in live animals is critical to dissect the mechanisms of plasticity, neural development, degeneration and aging, chronic preparations for long-term (>24 h) microscopy have been difficult in *Drosophila*, due to the fly's fragility and opaque exoskeleton. Only recently, laser microsurgery has been employed to create a chronic fly preparation for repeated imaging of neural dynamics for up to 50 days. Ca^{2+} and voltage imaging was performed in fly mushroom body neurons, in particular odor-evoked Ca^{2+} transients were recorded over 7 weeks [52]. This chronic preparation is compatible with a broad range of optical techniques to address in live flies biological questions previously unanswerable.

An interesting approach developed to evaluate functional connections is the combination of Ca^{2+} imaging with genetically encoded optogenetic tools. Optogenetic activation in presynaptic neurons and Ca^{2+} imaging in postsynaptic neurons have been used to map circuits governing different aspects of fly behavior, *e.g.*, antennal grooming behavior [53], courtship [54] and aggression [55]. Some critical aspects need to be taken into account when setting up this kind of experiments, *i.e.*,

minimize spectral overlaps and use independent gene expression systems for the two transgenes [56].

Another example of the power of combined functional imaging in the fly takes advantage of both GECIs and Genetically Encoded Voltage Indicators (GEVIs) (reviewed in [57]). Yang and colleagues compared voltage and Ca^{2+} responses within compartments of the same neuron, using the ultrafast GCaMP6f sensor together with a newly developed GFP-based voltage sensor, named Asap2f. *In vivo* two-photon imaging of the two indicators was performed in the *Drosophila* visual system. Remarkably, intracellular [Ca^{2+}] do not simply follow the decay of voltage signals. Instead, Ca^{2+} responses appear compartmentalized, *i.e.*, they are different in their amplitude and kinetics among distinct regions of the same cell [58]. Voltage and Ca^{2+} signals appear distinct and neurons may convey varying information to their postsynaptic partners in different synaptic layers. The unprecedented resolution afforded by both indicators allowed to shed light on local neural computations during visual information processing.

In addition to adult flies, also other developmental stages can be subjected to Ca^{2+} imaging. In a recent paper, insight in the molecular pathway underlying network refinement was obtained by performing Ca^{2+} imaging at the embryonic NMJs. The authors demonstrated that oscillatory Ca^{2+} signals via voltage-gated Ca^{2+} channels orchestrate the activity of several kinases and phosphatases, key components in pruning aberrant synapses during embryonic synaptic refinement [59].

Another developmental stage much studied and appreciated for its accessibility and well-established organization is the larval stage. Ca^{2+} imaging has been performed in intact larvae, as well as in isolated larval CNS. An interesting recent example that underpins the power of the fly model is represented by a screen of unknown compounds for their potential to function as anticonvulsants [60]. In this work, GCaMP was expressed in motoneurons and the isolated CNS of third instar larvae was imaged to evaluate the effectiveness of novel anticonvulsive compounds to reduce seizure-like CNS activity.

Whole-brain imaging has also been performed in larvae (*e.g.*, imaging of ventral nerve cord during motor programs execution [61]; imaging of isolated CNS during coordinated motor pattern generation [62]).

Photoactivatable GECIs have been exploited for targeted neuronal imaging in cultured neurons and in fruit fly larvae [63]. Light-induced photoactivation allows single cells to be selected out of dense populations, for visualization of morphology and high signal-to-noise measurements of activity, synaptic transmission and connectivity. This tool combines the reporting Ca^{2+} activity with the selective highlighting of individual cells *in situ* in live tissues, facilitating tracking fine neuronal processes with a clarity that cannot be achieved with dense expression of standard FPs.

Besides the obvious importance in neurotransmission, Ca^{2+} signaling is fundamental for the survival and welfare of all cell types. Indeed, Ca^{2+} imaging experiments have been performed in other tissues, most relevantly in fly muscles. As an interesting recent example, an attempt to image all flight muscles together has

been tried in intact flying animals. In flies, wing motion is adjusted for both quick voluntary maneuvers and slow compensatory reflexes using only a dozen pairs of muscles. By applying visual motion stimuli while recording the pattern of activity across the complete set of steering muscles using GCaMP6f, the authors propose a model whereby the motor array regulates aerodynamically functional features of wing motion [64].

35.4.1 Ca^{2+} Imaging Inside Organelles

Organelle Ca^{2+} handling plays a fundamental role in cell Ca^{2+} homeostasis. At the cellular level, techniques to measure intra-organelle Ca^{2+} are nowadays available and routinely used. However, this type of measurements is still poorly exploited in living animals and few examples are available in *Drosophila*. Among them, the most commonly measured is mitochondrial Ca^{2+} [20, 65] although some attempts have also been done in other organelles.

As a recent example, Drago and Davis revealed a developmental role for the MCUC in memory formation in adult flies. The authors generated a transgenic line for a mitochondria-targeted GCaMP (named 4mtGCaMP3) that was expressed in MB neurons to measure mitochondria Ca^{2+} uptake upon downregulation of the MCUC components MCU or MICU1. Ca^{2+} imaging experiments have been associated to behavioral studies, demonstrating that the inhibition of mitochondrial Ca^{2+} entry in the developing fly MB neurons causes memory impairment [20].

A sensor of the GFP-aequorin protein (GAP) family, optimized for measurements in high-[Ca^{2+}] environments have been also developed and used in drosophila [66]. Among other applications, the authors propose the imaging of SR Ca^{2+} dynamics in the muscle of transgenic flies in vivo, providing evidence for a valuable tool to explore subcellular complex Ca^{2+} signaling in flies.

35.5 *Drosophila* Models of Neurodegenerative Diseases

Changes in intracellular [Ca^{2+}] mediate a wide range of cellular processes that are relevant to neurodegenerative disorder etiology, including learning and memory, as well as cell death. Indeed, a close link between the pathogenesis of different neurodegenerative disorders and Ca^{2+} regulating systems, the so called "Ca^{2+} hypothesis of neurodegenerative diseases", has been convincingly corroborated by several experimental findings [67, 68]. The potential of the approaches described above makes flies a powerful model system to elucidate pathogenic processes in neurobiology. Indeed, a wide collection of fly models of neurodegenerative disorders have been developed. Often, the model consists of targeted expression of human disease-associated protein. In the case of loss of function pathologic mutations, also knock out/knock down approaches have proved successful in

mimicking the pathology. In many cases, robust neurodegeneration is observed in these models.

Ca^{2+} imaging experiments performed in fly models of neurodegenerative disorders have helped unravel the pathogenesis of diseases, including Alzheimer's disease (AD), Parkinson's Disease (PD), Huntington Disease (HD). AD is a neurodegenerative disorder characterized by deposition of amyloid β (Aβ) in extracellular neuritic plaques, formation of intracellular neurofibrillary tangles and neuronal cell death. Among familial (FAD) cases, approximately 50% have been attributed to mutations in three genes: amyloid β precursor protein (*APP*) [69], presenilin 1 (*PSEN1*) [70] and presenilin 2 (*PSEN2*) [71]. The fly genome encodes a single Presenilin gene (*Psn*) [72] and a single APP orthologue (*Appl*) which encodes for β-amyloid precursor-like protein. *Drosophila* models of AD have been developed, mostly expressing wild type and FAD-mutant forms of human APP and presenilins, reproducing AD phenotypes, such as Aβ deposition, progressive learning defects, extensive neurodegeneration and ultimately a shortened lifespan [73]. It is now accepted that FAD-linked presenilins mutants are responsible for a dysregulation of cellular Ca^{2+} homeostasis. An imbalance of Ca^{2+} homeostasis is supposed to represent an early event in the pathogenesis of FAD, but the mechanisms through which FAD-linked mutants affect Ca^{2+} homeostasis are controversial [74]. Using a fly model of FAD, Michno et al. [75], showed that expression of wild type or FAD-mutant Psn in *Drosophila* cholinergic neurons results in cell-autonomous deficits in Ca^{2+} stores, highlighted using the chemical Ca^{2+} probe Fura-2. Importantly, these deficits occur independently of Aβ generation. They also describe a novel genetic, physiological and physical interaction between Psn and Calmodulin, a key regulator of intracellular Ca^{2+} homeostasis. More recently, a study conducted by Li et al. [76] exploited Ca^{2+} probes and confocal imaging to demonstrate that Imidazole, by decreasing the level of intracellular Ca^{2+}, can rescue the mental defect in Aβ42-expressing flies.

PD is the most common movement disorder, typically affecting people between 50 and 60 years of age. The disease is mostly sporadic, only a small fraction of PD cases have been linked to mutations in specific genes, including a-synuclein [77], parkin [78], PINK1 [79]. Cytoplasmic aggregates mainly formed by α-synuclein protein, called Lewy bodies [80], are usually found in the *substantia nigra* of brain tissue. Dopaminergic neurons are the most susceptible to degeneration in PD. Fruit flies are largely used as model for PD. Expression of human α-synuclein in flies leads to selective loss of dopaminergic neurons in the adult brain over time and accumulation of protein in cytoplasmic inclusions [81]. Pan-neural expression of α-synuclein either wild type or carrying PD-linked mutations results in premature loss of climbing ability. Noteworthy, the first animal models revealing an interaction between the two PD genes homologues Pink1 and parkin have been developed in *Drosophila* [82, 83]. Recently, Ca^{2+}-induced neurotoxicity have been explored in a *Drosophila* model of retinal degeneration. The authors found that increasing the autophagic flux prevented cell death in mutant flies, and this depended on the Pink1/parkin pathway [84]. The results indicated that maintaining mitochondrial homeostasis via Pink1/parkin-dependent mitochondrial quality control could potentially alleviate cell death in a wide range of neurodegenerative diseases.

HD is a progressive brain disorder characterized by uncontrolled movements, emotional problems, and loss of cognition. The disease is caused by autosomal dominant mutations in the gene encoding for huntingtin (*HTT*), resulting in an abnormal expansion of the number of CAG triplets, encoding for Glutamine. A *Drosophila* model of HD have helped investigating the mechanisms by which expanded full-length huntingtin (htt) impairs synaptic transmission [85]. The authors showed that expression of expanded full-length htt led to increased neurotransmitter release and increased resting intracellular Ca^{2+} levels, compared to controls. Moreover, mutations in voltage-gated Ca^{2+} channels restored the elevated [Ca^{2+}] and improved neurotransmitter release efficiency, as well as neurodegenerative phenotypes. This suggests that a defect in Ca^{2+} homeostasis contributes to the pathogenesis of the disease, which is in agreement with observations in mammalian systems [86–89].

Drosophila models have been created recapitulating many other diseases affecting the neuronal system, e.g. Hereditary Spastic Paraplegias (HSPs), are a group of inherited neurodegenerative disorders characterized by retrograde degeneration of corticospinal neurons, leading to muscle weakness and spasticity of the lower limbs. HSPs are highly genetically heterogeneous, with over 70 spastic paraplegia gene (SPG) loci associated [90]. Despite this diversity, it is now clear that one of the most common causes of HSPs are mutations in genes encoding proteins that, directly or indirectly, regulate ER morphology and/or distribution. Proper shape is necessary for the ER diverse functions, that are crucial for neuronal welfare [91]. Among these functions, Ca^{2+} sequestration and release play a fundamental role in shaping cytosolic signals [92]. *Drosophila* models have been generated for many HSP-related genes, among them the homologues of Atlastin-1 (SPG3A) [93], Spastin (SPG4) [94], Reticulon-2 (SPG12), ARL6IP1 (SPG61) [95]. Available tools for Ca^{2+} imaging applied to these models would provide valuable insights in the role of Ca^{2+} in the pathogenesis of HSPs.

35.6 Conclusions and Future Perspectives

Ca^{2+} signaling plays a critical role in cellular physiology and, in particular, in fundamental neuronal functions, such as synaptic transmission, synaptogenesis, neuronal plasticity, memory and cell survival. Understanding how the concerted action of neurons, synapses and circuits underlie brain function is a core challenge for neuroscience. The examples we described in this review underscore the contribution of *Drosophila* as a model system to explore cellular and circuits neurophysiology, highlighting potential future directions in the field. Given its genetic accessibility, complex behavioral repertoire and functional similarities with mammalian brain, the fruit fly represents an attractive model organism to approach relevant physiological and pathological questions. The combination of Ca^{2+} imaging with other tools, such as voltage indicators or optogenetics, provides a valuable developing strategy for investigating neural function and dysfunction. New variants of both green and red Ca^{2+} indicators are continually developed, offering improved sensitivity, brightness, photostability and kinetics and can be fruitfully applied to the *Drosophila* model.

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