In vivo and *ex vivo* Calcium imaging in *Drosophila* larva

C. Eschbach
claire.eschbach@universite-paris-saclay.fr

Technologies des Neurosciences/Methods in Neuroscience
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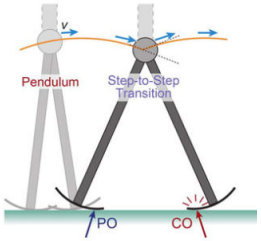
2

Mechanisms allowing locomotion

mechanical aspect: the legs act as a pendulum



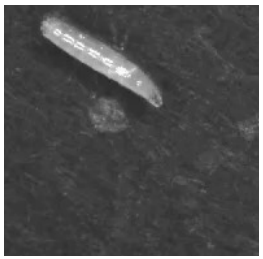
https://www.youtube.com/watch?v=durPmC42MHM
Wooden Walker - Passive Dynamic Walking

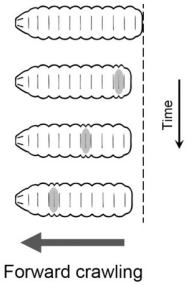
https://www.youtube.com/watch?v=LewVEF2B_pM
STRANDBEEST EVOLUTION 2017

5

Mechanisms allowing locomotion

Larval locomotion as a spring: waves of compression/expansion of the segment (peristalsis) moves the object in a direction





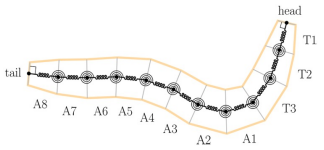
6

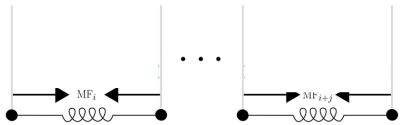
Mechanisms allowing locomotion

A model of larval locomotion by Loveless *et al.*, 2019:

⇒ "exploration-like behavior": forward and backward peristalsis, turning

⇒ "It is necessary to add a **neuromuscular system** to counteract the loss of energy due to friction, and to limit the simultaneous compression of segments."

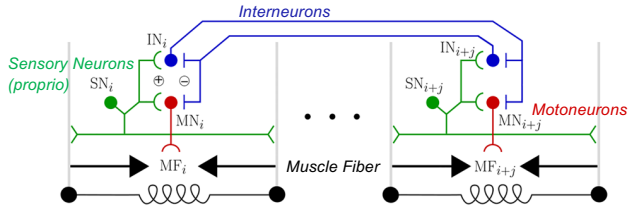




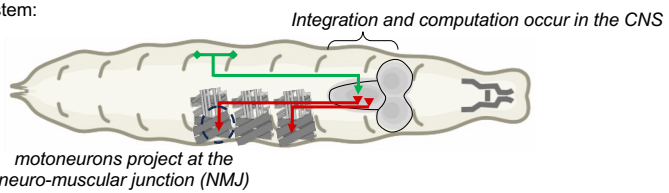
7

Mechanisms allowing locomotion

A model of larval locomotion by Loveless *et al.*, 2019:



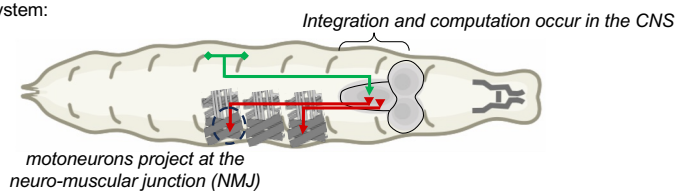
larval nervous system:



8

Mechanisms allowing locomotion

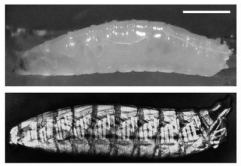
larval nervous system:



In this practical course, we will explore the organisation of the locomotory system in the *Drosophila* larva

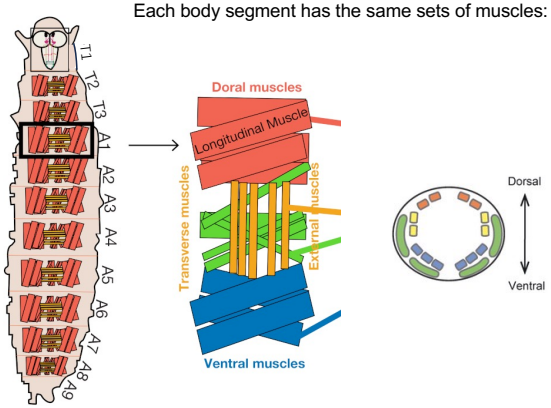
9

Organization of muscles and motoneurons in the larva



GFP expressed in the muscles

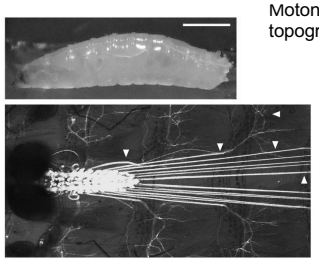
Each body segment has the same sets of muscles:



Kohsaka et al., 2012; Kohsaka et al., 2016; Gowda et al., 2021

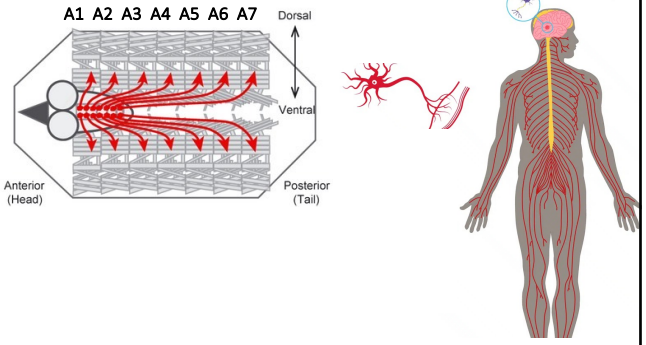
11

Organization of muscles and motoneurons in the larva



GFP expressed in the motoneurons

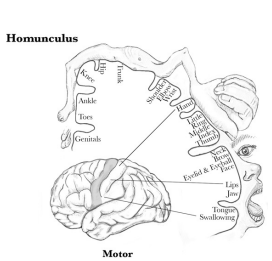
Motoneurons directly connect the CNS to muscles in a topographic way

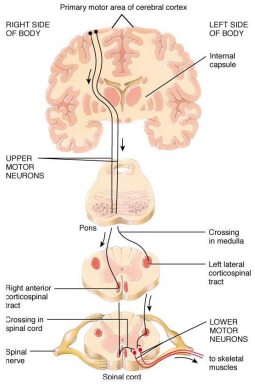


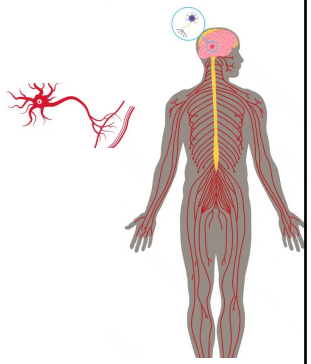
Kohsaka et al., 2016

12

Topographic organization of the motor system



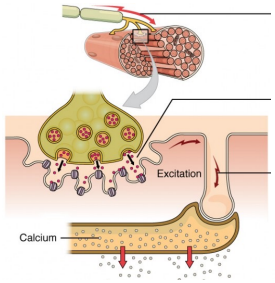




Ragagnin et al., Frontiers in Neuro 2019

13

NEUROMUSCULAR JUNCTION AND CONTRACTION OF MUSCLE FIBER



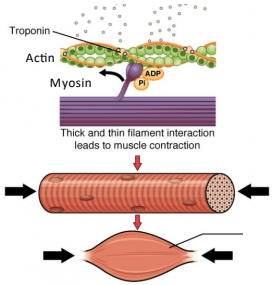
Action Potential from motoneuron

Release of NT (Glu in insects, Ach in mammals)

AP initiated in sarcolemma and travels along T-tubules

Release of Ca^{2+} from storage in the sarcolemma reticulum

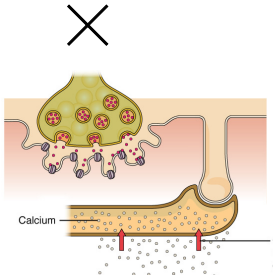
AP → Ca^{2+} → Contraction



Ca^{2+} binds to troponin and drives cross-bridge between actin and myosin > this initiates contraction, sustained by ATP

16

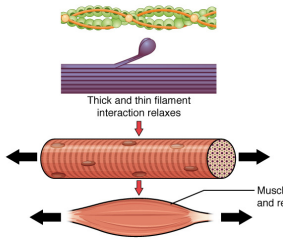
NEUROMUSCULAR JUNCTION AND RELAXATION OF MUSCLE FIBER



End of signalling from the motor neuron

Repolarization of the sarcolemma and T-tubules, voltage-gated calcium channels in the SR close, and Ca^{2+} pumped back

~~AP~~ → ~~Ca^{2+}~~ → Relaxation



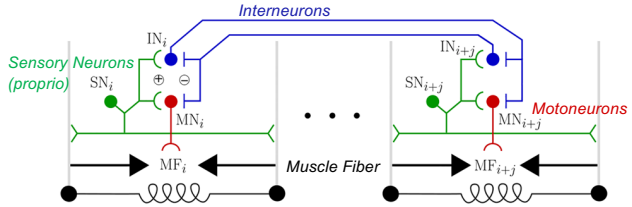
Tropomyosin shields back binding sites and relaxation begins

(also stops contracting if runs out of ATP)

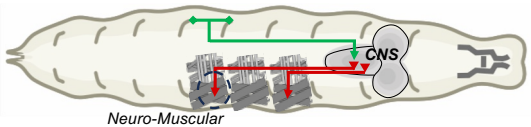
17

Mechanisms allowing locomotion

A model of larval locomotion by Loveless *et al.*, 2019:



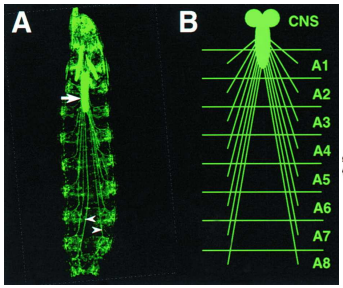
Larval nervous system:

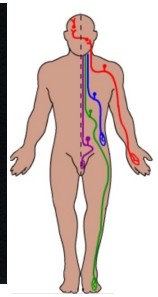


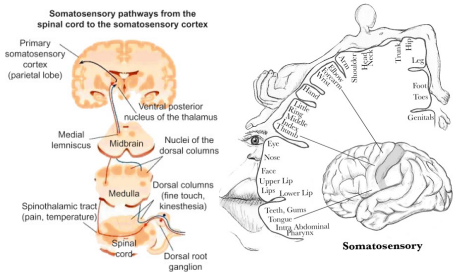
18

Organization of somatosensory neurons in the larva

Somatosensory neurons follow the same topographic arrangement as motoneurons







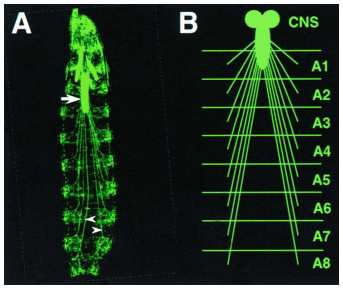
GFP expressed in sensory neurons

Leiserson et al., 2000

20

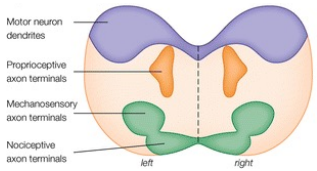
Topographic organization of the motor and somatosensory systems

Somatosensory neurons have the same topographic arrangement as motoneurons



GFP expressed in sensory neurons

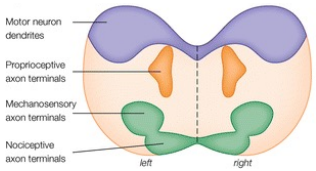
⇒ Communication within segments



Leiserson et al., 2000

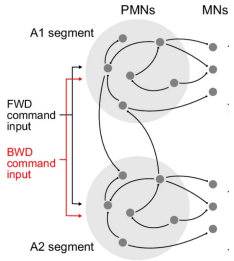
21

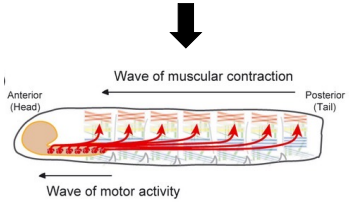
Overall locomotory system in drosophila larva:



Communication within segments

Communication between segments





Zarin *et al*., 2019

22

Mechanisms allowing locomotion

During this practical course, we will explore approaches to study the circuit for locomotion and response to stimuli in *Drosophila* larvae:

⇒Observe peristalsis movements

⇒Observe muscles contractions

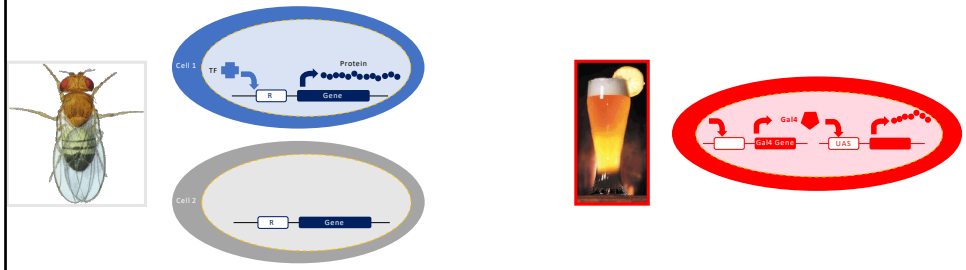
⇒Observe motoneurons activations

We will discuss the following **methods**:

- Genetically encoded fluorescent sensors
- Fluorescent microscopy
- Image processing tools

24

THE GAL4/UAS SYSTEM Brand and Perrimon, 1993

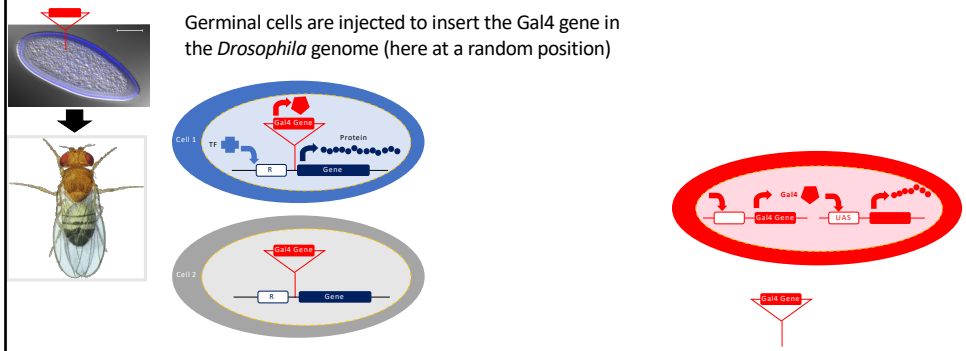


Each cell has its own transcription factors (TF), different from one cell type to the other. Each TF needs specific recognition sites and activates specific genes.

Also, different species have different TF with different recognition sites.

31

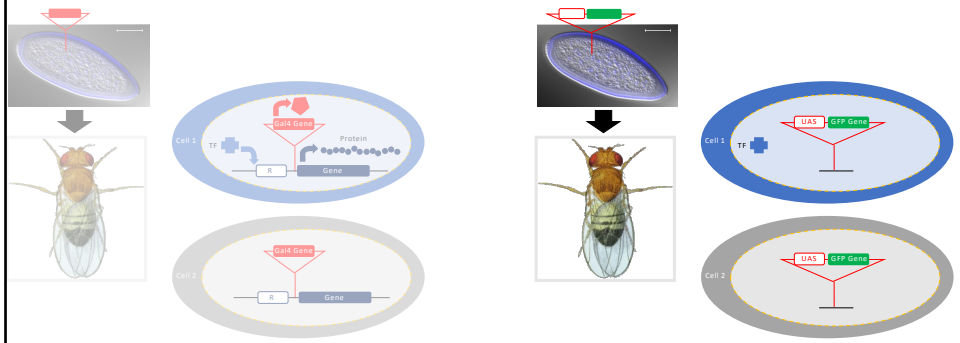
THE GAL4/UAS SYSTEM



Gal4 is only produced in cells where an upstream recognition site – specific to *Drosophila* – is activated.

33

THE GAL4/UAS SYSTEM



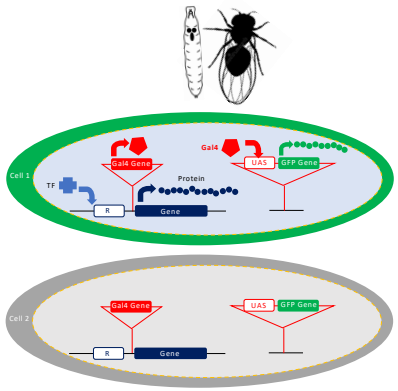
A second Drosophila line is generated : germinal cells are transformed with the effector element in the Drosophila genome.

Without Gal4, the recognition site cannot be activated.

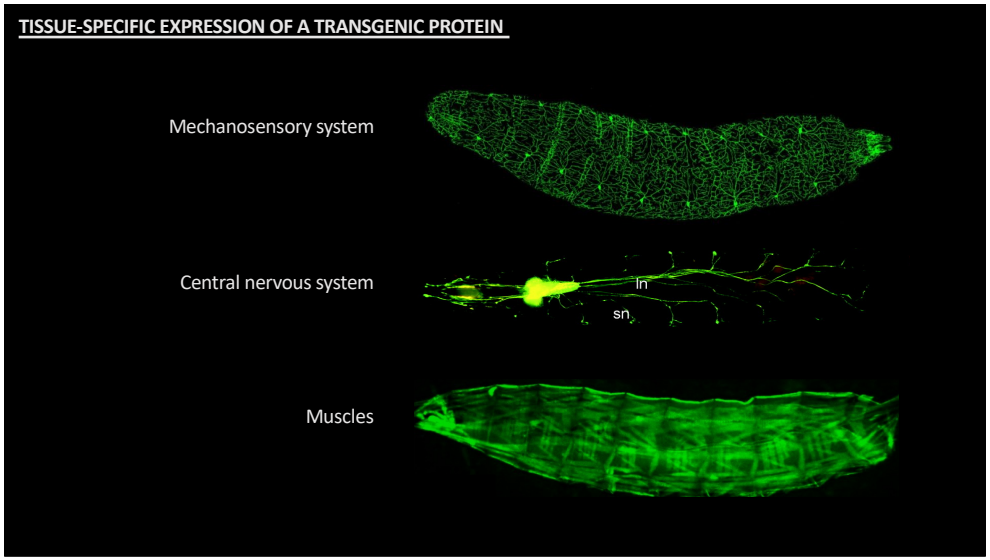
35

THE GAL4/UAS SYSTEM

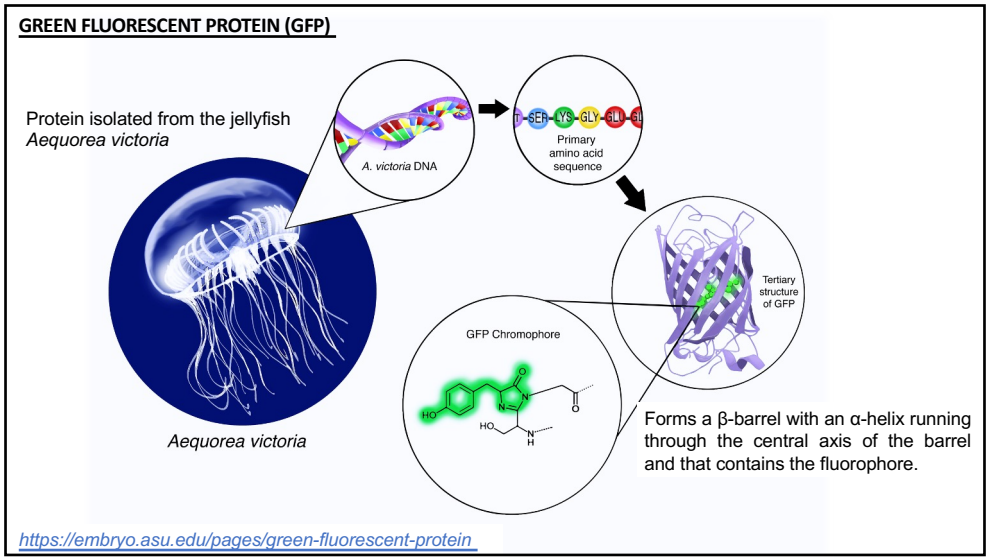
F1 line



37

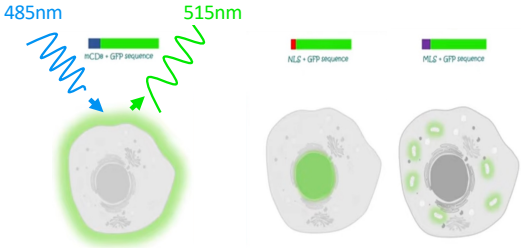

TISSUE-SPECIFIC EXPRESSION OF A TRANSGENIC PROTEIN
Mechanosensory system
Central nervous system
In
sn
Muscles

39

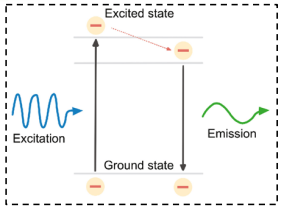

GREEN FLUORESCENT PROTEIN (GFP)
Protein isolated from the jellyfish
Aequorea victoria
A. victoria DNA
Primary
amino acid
sequence
Tertiary
structure
of GFP
GFP Chromophore
Aequorea victoria
Forms a β-barrel with an α-helix running through the central axis of the barrel and that contains the fluorophore.
https://embryo.asu.edu/pages/green-fluorescent-protein

40

GREEN FLUORESCENT PROTEIN (GFP)



GFP does not interfere with biological processes and can therefore be used *in vivo* using a fluorescence microscope.



https://proteopedia.org/wiki/index.php/Green_fluorescent_protein

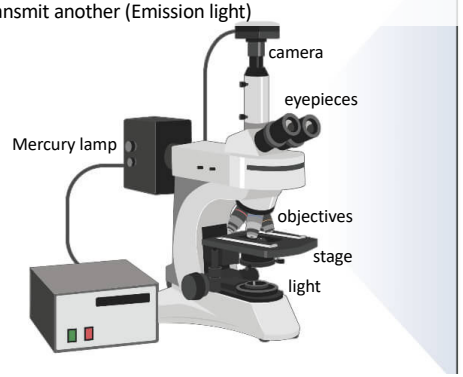
41

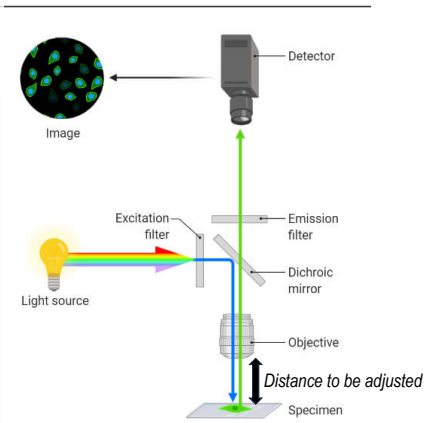
FLUORESCENCE MICROSCOPE

Excitation filter : bandpass filters (for blue)

Emission filter: "barrier", bandpass/longpass filter (for green)

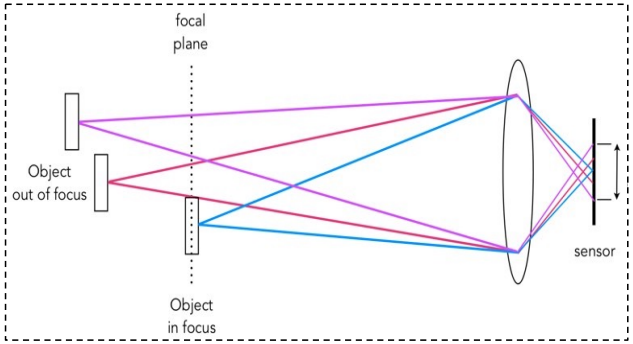
Dichroic mirror: coated glass, reflect 1 color (Excitation light) and transmit another (Emission light)





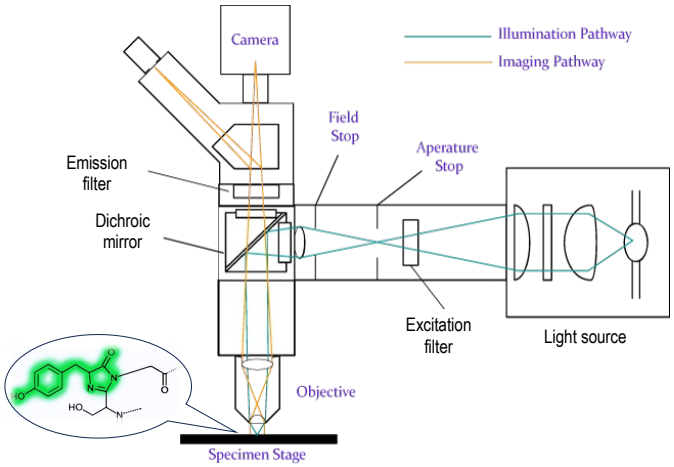
42

BE IN FOCUS



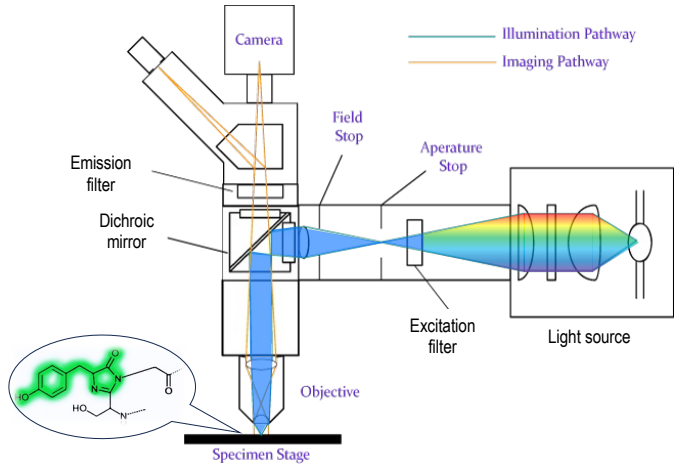
43

OPTIC ELEMENTS IN A FLUORESCENCE MICROSCOPE



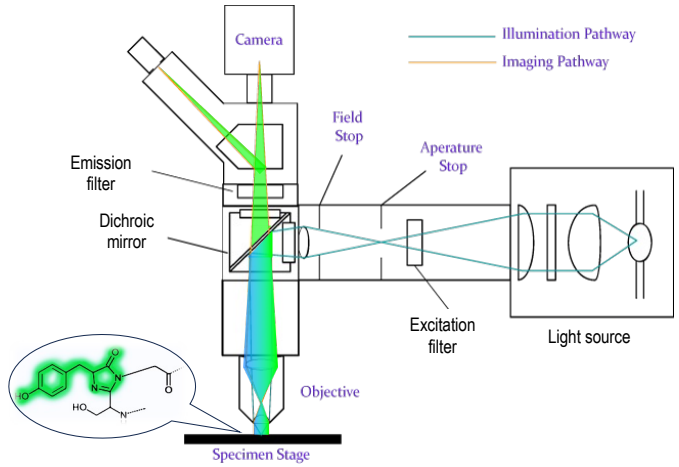
44

OPTIC ELEMENTS IN A FLUORESCENCE MICROSCOPE



45

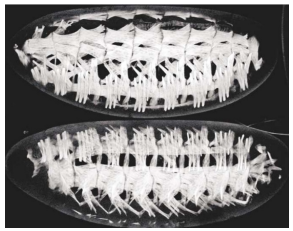
OPTIC ELEMENTS IN A FLUORESCENCE MICROSCOPE



46

With these tools in hands, we can express GFP specifically in the muscles and observe their contraction during movement!

Example study: Crisp *et al*., The Development of Motor Coordination in *Drosophila* Embryos, Development 2008; 135(22):3707-17. *doi: 10.1242/dev.026773.*

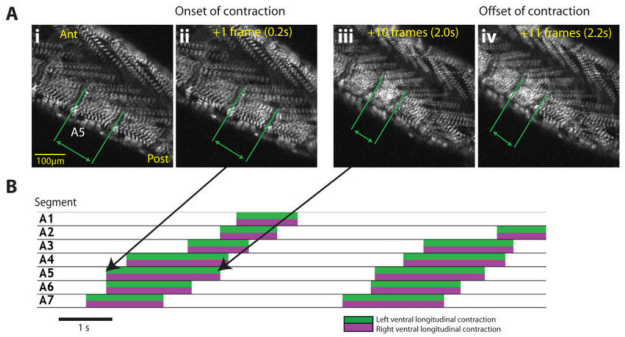


"fly strains carrying GFP traps in proteins expressed at the Z-lines of somatic muscles."

← *Drosophila* embryos

47

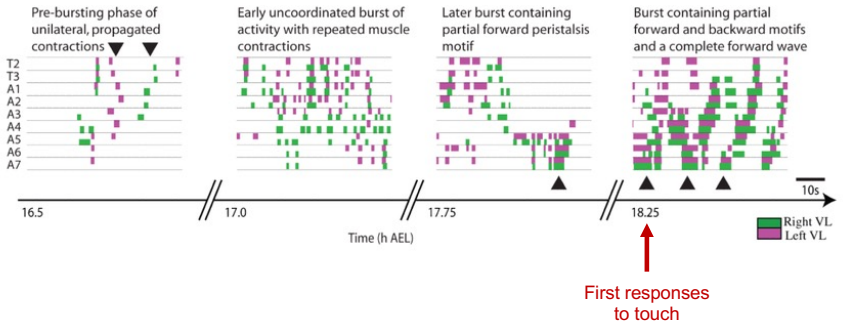
OBSERVE & QUANTIFY MUSCLE CONTRACTION



Crisp *et al*., Development 2008

48

OBSERVE & QUANTIFY MUSCLE CONTRACTION



Crisp *et al.*, Development 2008

49

With these tools in hands, we can express GFP specifically in the muscles and observe their contraction during movement!

Example study: Crisp *et al.*, The Development of Motor Coordination in *Drosophila* Embryos, Development 2008; 135(22):3707-17. *doi: 10.1242/dev.026773.*

With this method, it is not possible to know whether the muscle is **passively** compressed or **actively** contracted.

50

OBSERVE & QUANTIFY MUSCLE CONTRACTION

Similar to GFP, we can express a **calcium sensor** specifically in the muscles and measure the **change** in fluorescence during movement

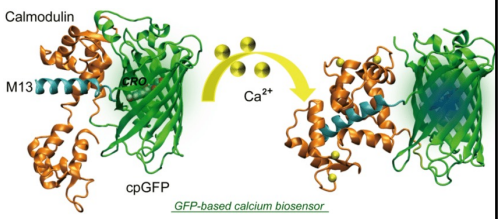
51

GCaMP

A **GECI** (Genetically Encoded Calcium Indicator)

A synthetic fusion of three domains:

- **Calmodulin**: calcium-binding messenger protein
- **GFP**
- **M13**: sequence from myosin light-chain kinase



Principle :

Absence of Ca^{2+}: GFP is exposed to water and is in **a protonated state** => minimal fluorescence intensity.

Ca^{2+} binding: **conformational change of** the CaM domain, which tightly binds to the M13 domain, preventing water molecules from accessing the chromophore => the chromophore rapidly deprotonates and converts into an **anionic form** that fluoresces brightly, similar to native GFP.

53

GCaMP

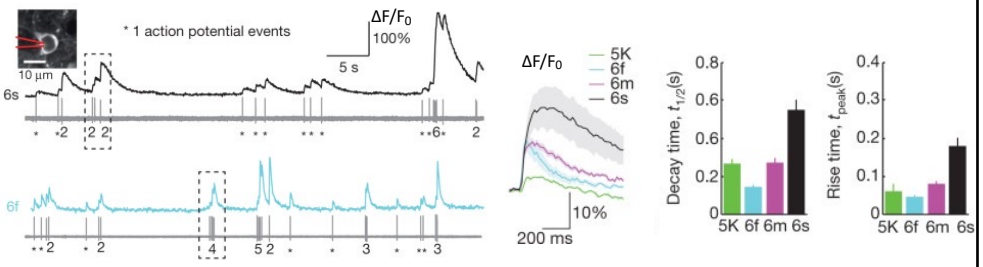
- Nakai *et al.*, 2001: GCaMP1
- Tallini *et al.*, 2006: GCaMP2 > Improvements: brighter, more stable at physiological conditions
- GCaMP3 to GCaMP8 > Brigthness, signal-to-noise ratio, decay time, rise time, spectral sensitivity

54

GCaMP

Chen *et al.*, 2013: GCaMP6:

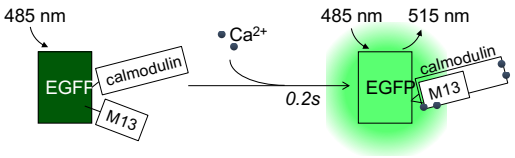
From the raw F, we always compute a **change** in fluorescence **normalized** to a baseline fluorescence (F_0): $\Delta F/F_0$



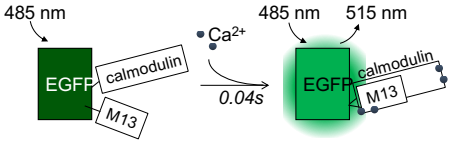
56

GCaMP

*GCAMP6**s**: **s**low rise, high fluorescence*



*GCAMP6**f**: **f**ast rise, but low fluorescence*



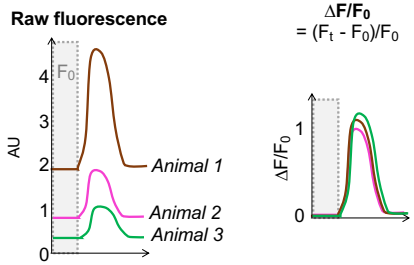
57

GCaMP

To estimate calcium level, the raw fluorescent signal F is showed as $\Delta F/F_0$ corresponding to the raw fluorescence F_t measured at each t time and normalised by the fluorescence F_0 measured at time 0 (in fact typically F_0 is the averaged fluorescence during the first seconds at the beginning of the recording).

Why this normalisation?

because the baseline level of GCaMP fluorescence can vary from one animal to the next.



58

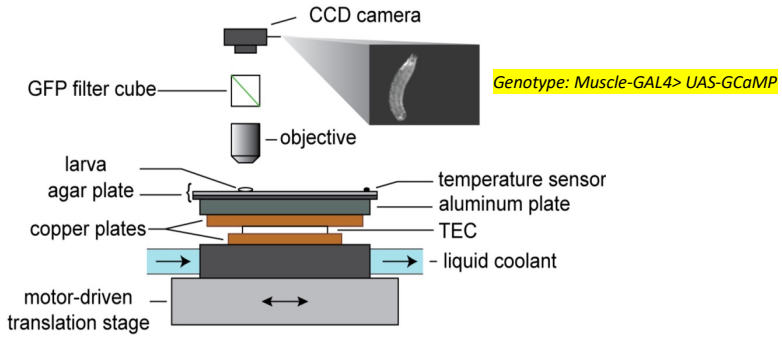
OBSERVE & QUANTIFY MUSCLE CONTRACTION

Similar to GFP, we can express a **calcium sensor** specifically in the muscles and measure the **change** in fluorescence during movement

Example study: Zarin *et al*., A multilayer circuit architecture for the generation of distinct locomotor behaviors in *Drosophila*, eLife 2021 https://doi.org/10.7554/eLife.51781

60

OBSERVE & QUANTIFY MUSCLE CONTRACTION WITH GCAMP

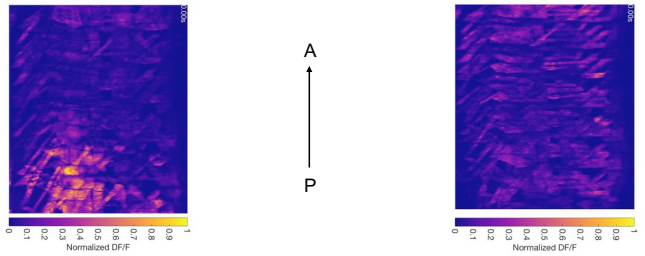


Genotype: Muscle-GAL4> UAS-GCaMP

Zarin *et al*., eLife 2021

61

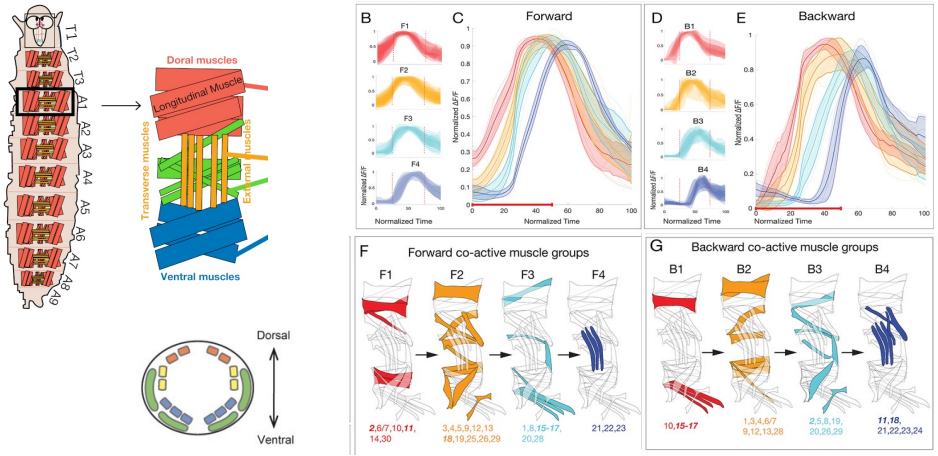
OBSERVE & QUANTIFY MUSCLE CONTRACTION WITH GCAMP



Zarin *et al.*, eLife 2021

62

OBSERVE & QUANTIFY MUSCLE CONTRACTION WITH GCAMP



Zarin *et al.*, eLife 2021

63

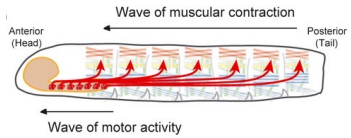
VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS

Instead of the muscles, we can express the **calcium sensor** specifically in the **motoneurons** and measure spontaneous changes in fluorescence

65

ACTIVITY OF MOTOR NEURONS

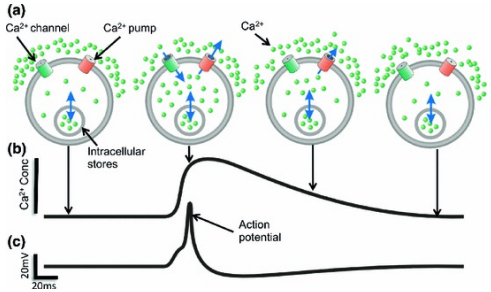
If the motor neurons drive the muscles, we should observe a wave of their activity, similar to the wave observed in muscle contraction



> How can we observe a wave of activity in the motor neurons?

66

VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS



Ca2+ ↗ during neuronal activity:
- voltage-gated calcium channels (VGCC),
- transient receptor potential type C channels (TRPC),
- Glutamate or nACh receptors
- release from internal stores.

Image: Castanares et al., Advanced Optical Methods for Brain Imaging 2018

67

VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS

Instead of the muscles, we can express the **calcium sensor** specifically in the **motoneurons** and measure spontaneous changes in fluorescence

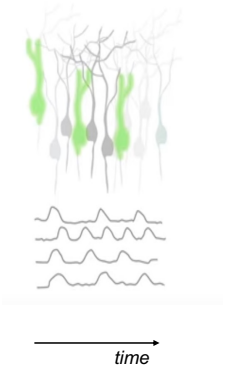
Example study: Pullver *et al.*, Imaging fictive locomotor patterns in larval *Drosophila*, Journal of Neurophysiology 2015
https://doi.org/10.1152/jn.00731.2015

68

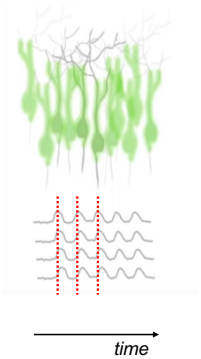
VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS

When visualizing the activity of a population of neurons, one can observe different types of activity:

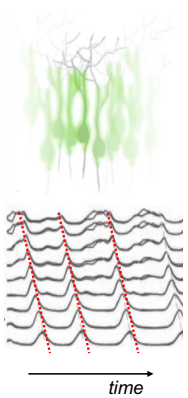
Asynchronous activity



Synchronous oscillatory activity

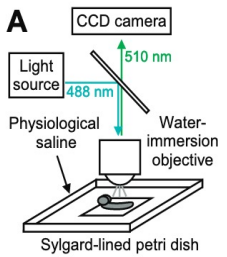


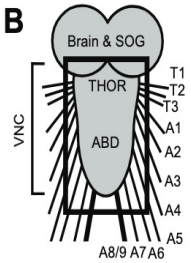
Wave-like oscillatory activity

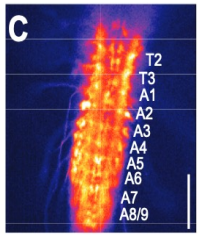


69

VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS





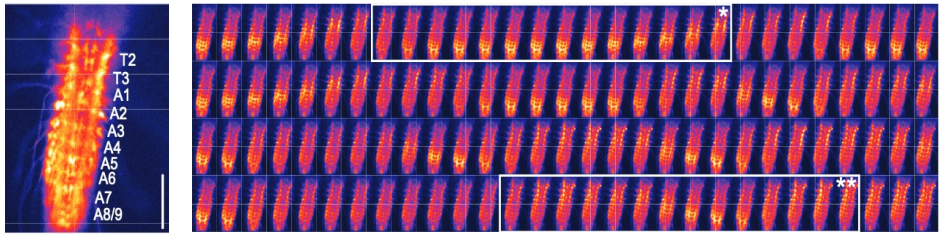


Pulver *et al.*, 2015

73

VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS

=> Wave of motoneurons activities could encode for forward or backward crawling (fictive behavior)

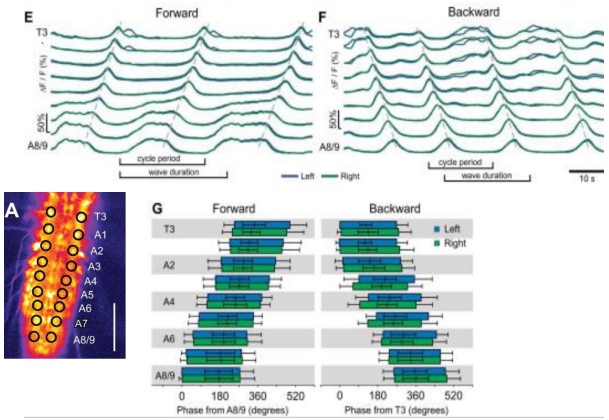


Stefan Pulver lab, St Andrews University, UK

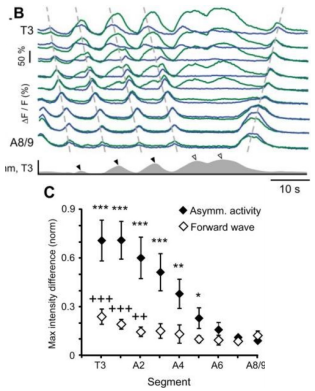
74

VISUALIZE CALCIUM ACTIVITY IN MOTOR NEURONS

1. Symmetric waves : forward *vs*. backward



2. Asymmetric waves



Pulver *et al*., 2015

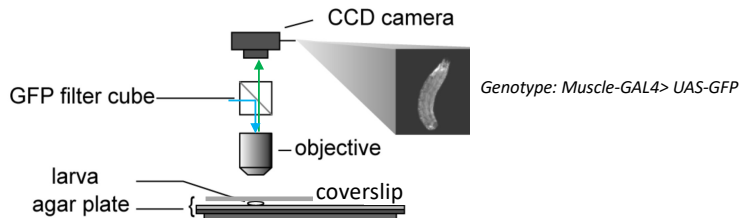
75

WHAT WILL WE DO ON THURSDAY?

76

1. Observe muscles with GFP & 2. Observe muscle contraction with GCaMP

Our setup!:

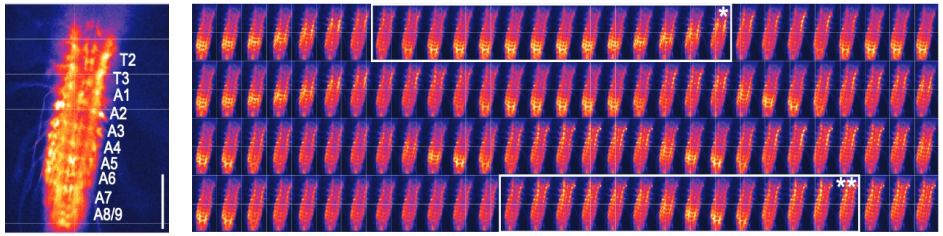


a. Describe your observations
b. Compare the fluorescence intensity of the 2 sensors
c. Acquire series of images on the microscope
d. Think critically about the tools and methodology

77

3. Analysis of Calcium imaging data using ImageJ

- Can you spot wave of activities? What behavior can they encode for (fictive behavior)?
- Choose 2 bouts of sequences from your recordings, each of them 10-20 frames long and work on them with ImageJ



Stefan Pulver lab, St Andrews University, UK

a. Using **ImageJ**, select Regions of Interest corresponding to segments T1/2 to A8/9 left and right
b. Pull the values of fluorescence for T3, A3 and A7 left and right.
c. Plot the normalized values in line plots ($\Delta F/F_0$ over time)

78