Time Restrictions of Calcium Imaging with GCaMP 6 in *Caenorhabditis Elegans*

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Abstract

Calcium imaging is a technique that aims at tracking the activity of neurons with the help of a calcium sensitive fluorescent molecule (calcium indicator). Calcium imaging with GCaMP6 performed on *C.elegans* is restricted to 15 to 30 minutes because fluorescence intensity from GCaMP6 decays exponentially. Three worms were imaged continuously with varied laser powers. Depending on the laser power, the neurons could not be distinguished anymore after 15 to 30 minutes. In order to extend this time period, imaging was interrupted with breaks in which the worms were not exposed to laser. This method was partly successful; we could image the worms for approximately 2 hours. The results will be used for studies that aim at tracking neural activity of *C. elegans* during its development, which will provide us with new information about decision-making.

Summary

A high calcium concentration inside a neuron indicates that the neuron is active. Calcium imaging is a technique that aims at detecting high calcium concentrations inside neurons with the help of fluorescent molecules that can respond to the binding of calcium ions by changing their fluorescence properties (calcium indicators). GCaMP6 is a genetically encoded calcium indicator, which means that the gene that expresses GCaMP6 is located on a plasmid that was inserted into the cell. Calcium imaging is often performed on *C. elegans* because it has only 302 neurons and is transparent. A confocal spinning disk laser scanning microscope is used to image C. elegans. However, previous imaging in our lab has revealed that GCaMP6 bleaches when it is exposed to laser light. Hence, we can only image the worm for 15-30 minutes, depending on the laser power. We tried to extend this time period by sporadic imaging; imaging was interrupted with breaks in which the worms were not exposed to laser. This method was partly successful; we could image the worms for approximately two hours, which is less than what we expected but longer than when we imaged the worm continuously. The results will be used for studies that aim at tracking neural activity of *C. elegans* during its development.

1 Introduction

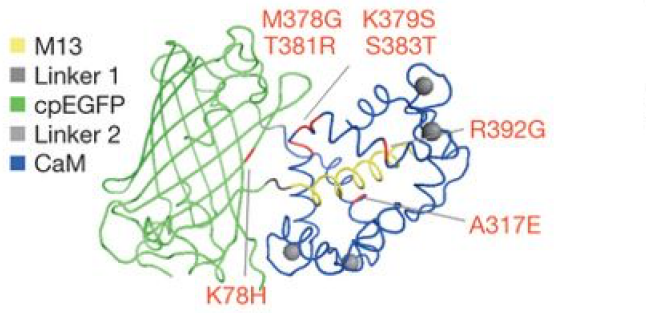
How do patterns of neural activity generate an animal's behavior? This is an important question, since it can help us understand neural circuits and decision-making. In order to answer it, it is important to find methods for recording neural activity in animals as they move and behave freely. Several methods like multi-electrode arrays [15] and fluorescence endoscopy [4] have been used. However, most of these methods are restricted to restrained behavior or small regions of the brain. Many of these issues are solved by using calcium sensitive fluorescent reporters to track the activity in individual neurons.

Calcium Imaging

Calcium regulates essential cellular signaling events [11]. In the brain, the calcium concentration inside a neuron undergoes large and rapid changes when an action potential passes by. Calcium imaging relies on this principle in order to track neural activity. A fluorescent calcium indicator inside the cell is used to detect high calcium concentration and therefore high cellular activity. There are synthetic calcium indicators and genetically encoded calcium indicators (GECI). GECIs can be easily targeted to specific cell types or subcellular compartments [2] and are produced by the cell itself.

GCaMP6

GCaMP6 is a single-fluorophore genetically encoded calcium indicator (sfGECI). Its structure is shown in Figure 1.



	cpEGFP	CaM					
GCaMP5G	K78	A317	M378	K379	T381	S383	R392
GCaMP6s	K78H				T381R	S383T	R392G
GCaMP6m			M378G	K379S	T381R	S383T	R392G
GCaMP6f		A317E			T381R	S383T	R392G

Figure 1: GCaMP6 consists of circularly permuted green fluorescent protein (cpGFP), the calcium binding protein calmodulin (CaM) and M13 peptide, which interacts with the calmodulin [2].

It consists of circularly permuted green fluorescent protein (cpGFP), the calcium binding protein calmodulin (CaM) and M13 peptide, which interacts with the calmodulin. When calcium binds to the CaM-M13 complex, it undergoes conformational changes that are associated with increased brightness. The most important characteristics of GCaMP6 and calcium indicators in general are signal-to-noise ratio (SNR), kinetics, response linearity, properly tuned calcium affinity, and stability. Previous studies have tried to and succeeded in improving those characteristics. For instance, protein structure-guided mutagenesis and semi-rational screening were used in order to develop new GCaMP variants [11]. The most promising result was GCaMP3, which is brighter, has greater protein stability and higher calcium affinity compared to GCaMP2. Using a similar approach, Chen et al. tried to improve GCaMP3 and developed GCaMP6. GCaMP6 has a higher $\Delta F/F$ ratio [1] and is more sensitive to small numbers of action potentials compared to GCaMP3.

[1]The $\Delta F/F$ ratio is the change in fluorescence over the fluorescence when the neuron is not active.

Caenorhabditis Elegans

Calcium imaging is often used to image the brain of *Caenorhabditis elegans* (*C. elegans*). *C. elegans* is ideally suited for calcium imaging because of its small nervous system (302 neurons), small size (1 mm), transparency, quick generation time, short lifespan (3 weeks) and ability to be frozen [8]. Furthermore, every neuron of *C. elegans* has been labeled. Calcium imaging has been used to study the behavior of freely moving C. elegans while tracking its brain activity [9]. The brains of four worms were imaged on the basis of GCaMP6 and RFP (a calcium insensitive protein) while their behavior was observed. RFP was used as an indicator for motion artifacts or noise. The imaging was used to investigate dynamics of those neurons whose activity correlates with forward, backward, and turning modes of locomotion. Of nearly 80 recorded neurons, 17 neurons of worm 1 were observed to correlate significantly with behavior. Such studies are very important to understand how population dynamics of a brain- sized neural network generates behavior [9].

ASK, AIA, AIB

ASK and AIB are both located in the lateral ganglia of the head, whereas AIA is located in the ventral ganglion of the head [1]. AIA and AIB are two of the four first layer amphid interneuron pairs that receive and process synaptic output from the amphid sensory neurons towards a behavioral response. AIA pair is suggested to sum inputs from various chemosensory neurons before passing the information on to AIB pair, which synapses onto motor neurons (AIA-AIB connections are likely to be inhibitory) [12]. ASK and AIB are two of the three neurons that trigger local search behavior [1]. Local search behavior consists of reversals and deep omega-shaped turns and is initiated after the worms are removed from bacterial food. Local search behavior is followed by dispersal approximately 30 minutes later, as reversals and turns are suppressed.

Time Limitations of Calcium Imaging

Calcium imaging is limited in time by weakening of the signal from the calcium indicator. This is mainly due to the fact that the fluorophore bleaches as a result of the exposure to laser light. Upon excitation caused by the photons of the laser, the fluorophore may interact with another molecule to produce irreversible covalent modifications [10]. A second cause may be the degradation of the fluorescent protein by proteasomes of the cell. Based on previous imaging, we assume that the signal of the GCaMP is too dim to be detected after about 15 minutes to 30 minutes, depending on the power of the laser. Ref [7] performed calcium imaging with GCaMP3 for approximately one hour, and did not have any issues regarding weakening of the signal or bleaching. In contrast, images that were taken in our lab suggest that the signal of the GCaMP is too dim after about 20 minutes (Figure 2.

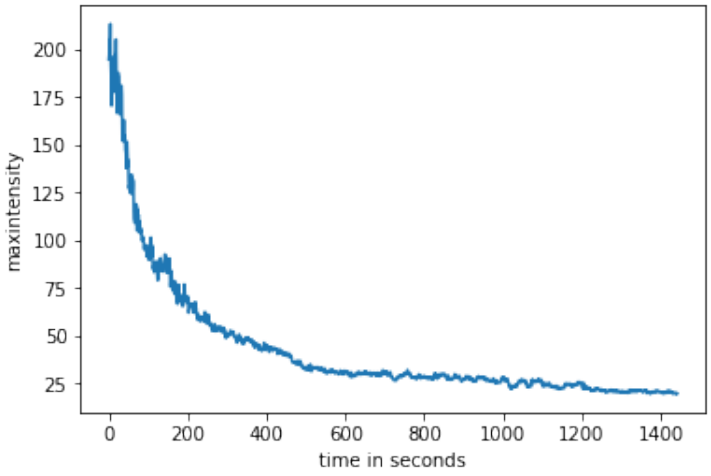


Figure 2: Shown is the average intensity of the 100 pixels of the highest intensity of the maximum projection as a function of time.

We assume that the bleaching rate depends on two factors. First, the chemical structure of the fluorophore affects protein stability. For example, GCaMP3 has greater protein stability than GCaMP2 [11]. Second, the power of the laser plays an important role. The more powerful the laser, the faster the bleaching of the fluorophore.

Sporadic Imaging

The main goal of this paper is to find out for how long the brain activity can be recorded with GCaMP6 in *C. elegans*. We imaged three neurons (namely ASK, AIA, and AIB) of *C. elegans* both continously and sporadically with breaks in which the worm was not exposed to laser. We think that the best way to maximize the time period for calcium imaging is by interrupting imaging periods with breaks in which the GCaMP is not exposed to laser light (Fig. 3.

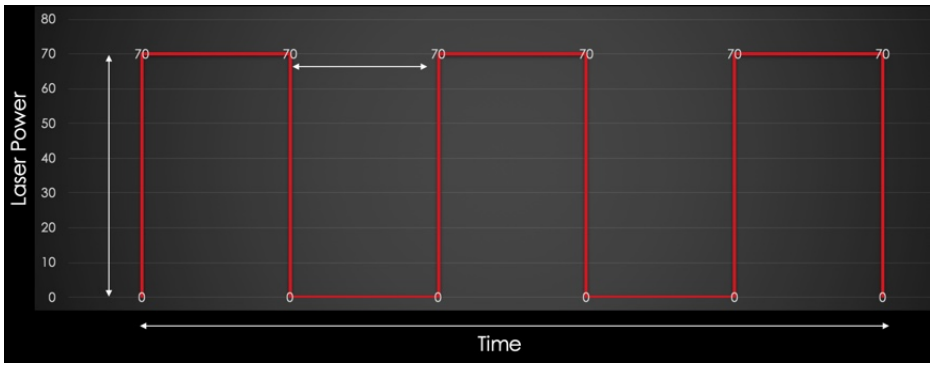


Figure 3: Laser exposure pattern to extend the time period for imaging. The white line is the laser power.

To maximize lifetime, we want to find optimal values for the three parameters that are represented by the arrows; the total time that is covered, the power of the laser, and the time period of the breaks. The fourth parameter, namely the time period of continuous imaging, was chosen to be as short as possible so that the frequency of imaging can be maximised. However, it has to be sufficiently long so that we get enough information to interpret the results. We chose the time period to be one minute, which makes it possible to observe several action potentials.

The total time in which the fluorophore is detectable should be maximized. Theoretically, the total time is infinite if the GCaMP can completely regenerate during the time it is not exposed to laser. We assume that the regeneration of the GCaMP is caused by the cell

producing new GCaMP. We think that the rate of GCaMP production depends on several factors like the age of the worm, the concentration of GCaMP, and especially the food availability. The worms have no access to food while imaging. This might have a strong influence on the gene expression. The results, especially the form of the regeneration curve, might reveal some information about the transcription and translation of GCaMP.

We hope to find values for all three parameters by analyzing images that focus on one of the three parameters. For example, we used various laser powers to find out how the laser power affects the bleaching rate. This helped us design an optimal imaging strategy to prolong the time in which calcium imaging can be carried out in *C. elegans*.

2 Methods

Strain

We cultivated transgenic worms on agar plates with OP50 bacteria. Each agar plate was seeded with 250μL of a suspension of OP50 bacteria in lysogeny broth. We tried to select L4 C. elegans for imaging according to their size. The cells contain several copies of a plasmid that has the gene for GCaMP on it[2]. mCherry is a calcium insensitive protein that marks worms that have the plasmid. The number of plasmids inside the neurons may vary.

Sample Preparation

A suspension of 10% agarose in water was heated until it melted. We then ad ded a single drop of the liquid onto a glass slide and covered it with a second glass slide. As soon as the agarose solidified, the upper glass slide was removed. The agarose sheet was cropped into a square of approximately 1 cm^2. The square was then enclosed with Vaseline with the help

[2][sra-9p::GCaMP6s::SL2::mCherry::H2B (ASK), gcy-28.dp::GCaMP6s::SL2::mCherry::H2B (AIA), npr-9p::GCaMP6s::SL2::mCherry::H2B (AIB), ofm-1p::RFP (co-injection marker)]

of a syringe and 2.00 μL of 0.1 μm diameter polystyrene microspheres were added onto the agarose square. We then transferred the worm onto the polystyrene microspheres suspension. Finally, we added a second coverslip on top. The worm was not able to move because of the normal force compressing the worm between the pad and coverslip and the friction between the worm, agarose pad, and coverslip[3]. However, small movements with the head or the tail were still possible.

Microscope

A confocal spinning disk laser microscope was used for imaging the neurons. It was custom built in our lab. Its structure is shown in Figure 4.

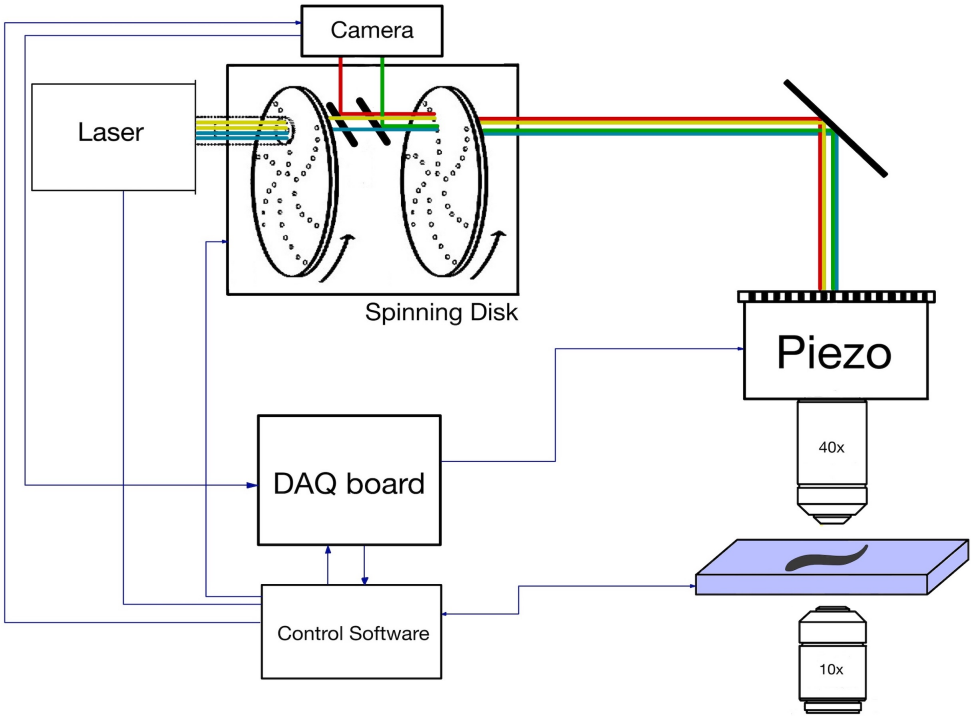


Figure 4: Structure of the confocal spinning disk laser microscope.

Images of the head of a worm were taken through a 40x objective using 488 nm laser light (100% power corresponds to 150 milliwatt). The microscope operates in three dimensions.

The piezo moves the 40x objective up and down to get volumetric images. In order to increase optical resolution and contrast, the light has to pass through small pinholes in the spinning disk. The pinholes are arranged in a specific pattern and the spinning disk rotates very quickly in order to increase the frame rate. The *C. elegans* hermaphrodites are immobilized.

In order to construct accurate volumetric images, the microscope scans twenty planes. The distance between the planes is constant. While images of the 20 planes are taken, the hermaphrodite should not move more than 0.1 millimeter horizontally, otherwise the image will be warped. The hermaphrodites can reach a maximal speed of 1 mm/s. Hence, the pictures of the 20 planes should be taken within 0.1 seconds; the picture of one plane should be taken in 0.005 seconds. This corresponds to a frame rate of 200 fps. The spinning disks allow a frame rate of more than 200 fps. However, the camera can only take approximately 160 fps. This rate is increased to the required 200 fps by reducing the number of pixels from 1024x1024 to 256x512 pixels.

Program for Analysis

The volumetric images of the microscope are analyzed by a program written in Python.

Maximum intensity projection

Unless otherwise specified, the projection axis of the maximum intensity projection is the z axis.

Graphs "Intensity versus time"

The average intensity of the hundred pixels with the highest intensity of the maximum intensity projection as a function of time is used as an approximation for the average intensity of the neurons inside the image. Based on previous images, we estimate that the distinguishable area of a neuron consists of approximately 100 pixels, although this number

might be subject to some fluctuations, depending on the age and size of the worm or the orientation of the worm relative to the camera. The hundred pixels with the highest intensity might all be from the same neuron, which does not correctly describe the average intensity of all neurons inside the image. However, this is of no further importance, because the images that contain several neurons are only used to show the decay of the GCaMP signal. In cropped images, the average intensity of the fifty pixels with the highest intensity is used as an approximation for the average intensity of the neuron.

Graphs "x/y versus time"

Because the neurons can move from image to image, the program has to be able to follow the neuron so that the neuron stays fully inside the the cropped image. The program calculates the centroid of the 30 pixels with the highest intensities and uses this point as the center of the next image. This algorithm has a certain susceptibility to errors; if a second neuron enters the cropped image, the program may start to follow that neuron. However, we neglect this case because the worm is immobilized, although slight movements of the head are still possible. Besides that, we neglect pixels with relatively high intensity that are not due to fluorescence. Those pixels are relatively rare but become more important as the intensity of the neuron decreases.

Curve fitting

A function of the form

$$f(x) = A + B * e^{-C*t}$$

is fitted to the graphs that display the decay of the fluorescence intensity as a function of time. A function of the form

$$f(x) = y_0 + m * t$$

is fitted to the regeneration curves. t denotes the time. The program returns values for the parameters A, B, and C, and y_0 and m, respectively. Furthermore, it calculates the ratio of the measured intensity over the intensity of the curve fitting and plots it as a function of time.

Images and Graphs

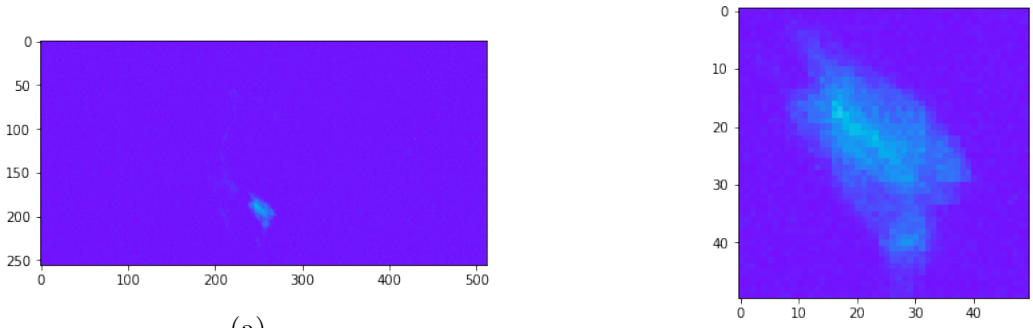
Three different worms were imaged with 40%, 50%, and 60% laser power. The time period varied; we stopped the imaging as soon as the fluorophore was not distinguishable anymore. The same worms were used to find the regeneration rate of the GCaMP; the laser was turned off for varied periods of time (between 10 and 40 minutes) and then turned on again for approximately 15 seconds to image the worm. This was repeated several times. Graphs for the intensity and the movement of the neurons were plotted with the help of the analysis program. Based on the first results, one worm was imaged according to the pattern that is shown in Figure 6. The value of the parameter is shown in Table 1.

Parameters	*Worm 1*
Laser power	50%
Time on	60 seconds
Time off	1740 seconds
Total time	4 hours

3 Results

Continuous imaging

Figure 5 shows the 50% laser power maximum intensity projection at t=0. Like the maximum intensity projection of 40% laser power, it is very dim. In contrast, the maximum intensity projection of 60% laser power is much brighter.



(a) (b)

Figure 5: Full-size (a) and cropped (b) image of the 50% laser power maximum intensity projection at t=0.

Figures 6 and 7 show the maximum intensity image of 60% laser power at t=0 and at t≈150 s. After 150 s, only the left neuron remains. This indicates that the left neuron had a higher initial GCaMP concentration or is producing more GCaMP.

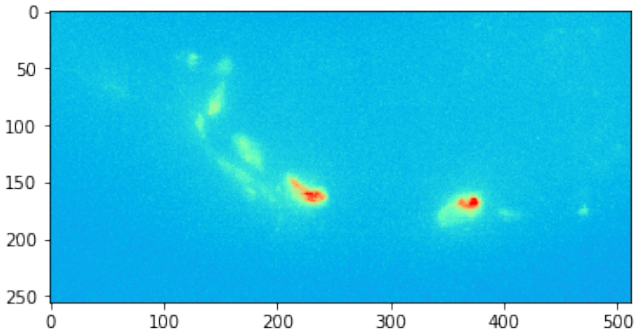


Figure 6: 60% laser power maximum intensity projection at t=0.

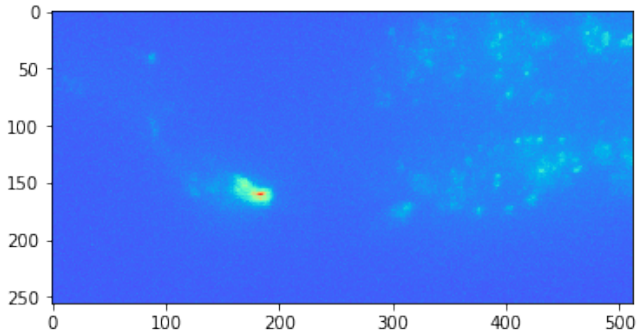


Figure 7: 60% laser power maximum intensity projection after 150 s.

The average intensity of the 50% laser power neuron as a function of time with a curve fit is plotted in Figure 8. The graphs for 40% and 60% laser power look similar in shape, but they have different initial intensities and decay rates.

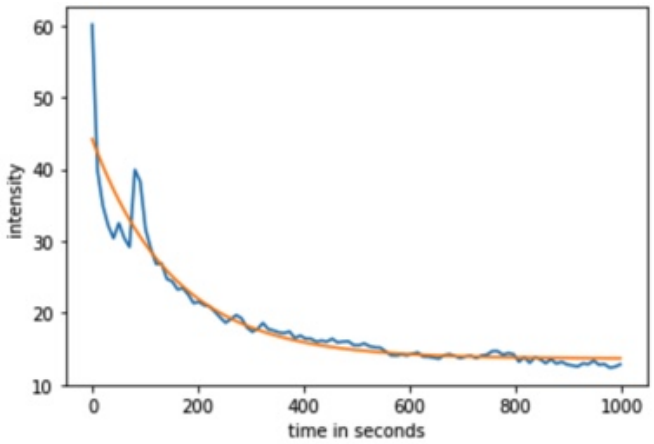


Figure 8: Average intensity of the 50% laser power neuron as a function of time with curve fit.

The parameters of the exponential decay curve fit are listed in Table 2. The sum of A and B is the initial intensity, B is the final intensity, and C is the decay rate. The initial intensities increase in proportion to the laser power, but the initial intensity of 60% laser power is unexpectedly high. The decay rate shows no clear dependency on the laser power; the decay rate of 60% laser power is unexpectedly small.

Parameters	*40% LP*	*50% LP*	*60% LP*
A	$1.16 * 10^1$	$1.37 * 10^1$	$5.52 * 10^1$
B	$1.91 * 10^1$	$3.05 * 10^1$	$1.59 * 10^2$
C	$2.91 * 10^{-3} s^{-1}$	$6.49 * 10^{-3} s^{-1}$	$3.43 * 10^{-3} s^{-1}$

The intensity that we observe is determined by the amount of GCaMP6 inside the cell and the brightness of each GCaMP molecule. If we assume that the amount of GCaMP6 decays exponentially, the fluctuations around the curve fit have to be due to fluctuations in the calcium concentration inside the neuron. Hence, the ratio of the measured intensity over the expected intensity indicates neural activity (Fig. 9 and Fig. 10).

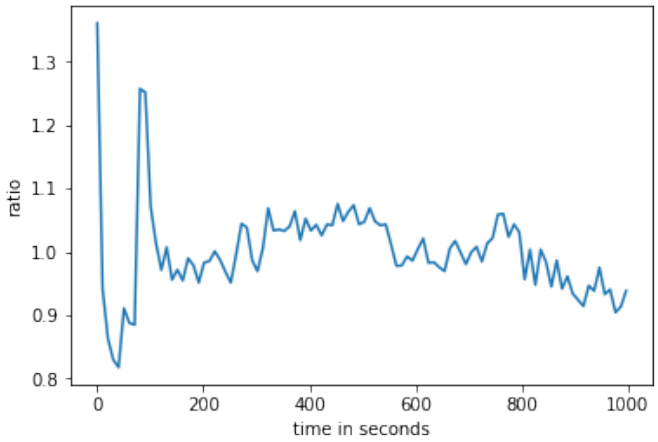


Figure 9: Ratio of the measured intensity over the predicted intensity of the 50% laser power neuron.

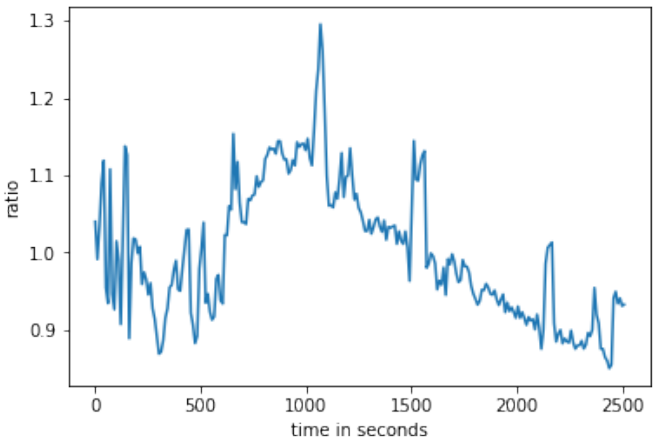
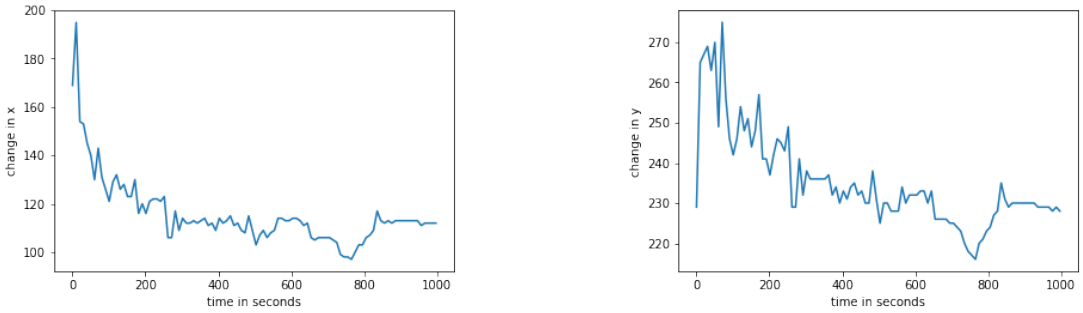


Figure 10: Ratio of the measured intensity over the predicted intensity of the left neuron of the 60% laser power images.

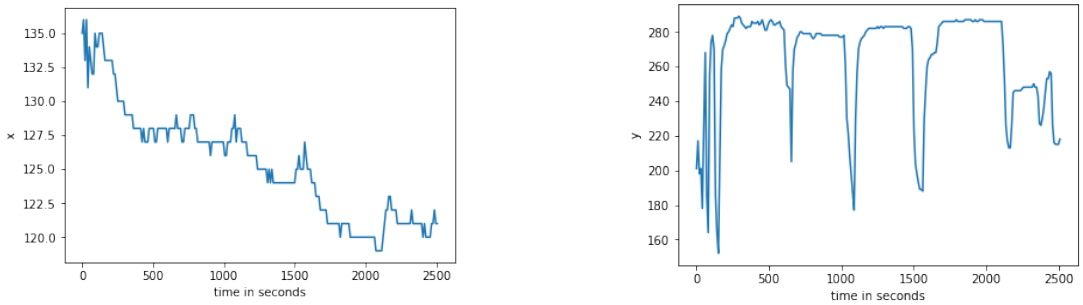
The movement of the 50% laser power neuron along the x axis and the y axis is plotted in Figure 11. The movement of the worm is important because of two reasons. First, to find out whether there is any correlation between the movement and activity of one or several of the tracked neurons. The movement along the y axis (Fig. 12b) seems to correlate with the ratio of the measured intensity over the predicted intensity (Fig. 10). Both figures have four peaks at t≈700 s, t≈1050 s, t≈1500 s, and t≈2100 s. In addition, both curves oscillate strongly within the first 200 seconds. In Figure 11 and Figure 8, we can observe a similar phenomenon. The peak in Figure 8 is correlated to a drop in y. Second, an abnormal shape (like a straight horizontal line) can indicate that the program has lost the neuron, or it could also mean that the worm is dead.



(a) (b)

Figure 11: Movement of the 50% laser power neuron along the x-axis (a) and the y-axis(b).

Figure 12 shows the movement of the left neuron of the 60% laser power images. Strong fluctuations in y but not in x indicate that worm moves its head from side to side.



(a) (b)

Figure 12: Movement of the left neuron of the 60% laser power images along the x-axis (a) and the y-axis(b).

The regeneration graph of the worm that was imaged with 50% laser power is plotted in Figure 13. The laser was turned off for varied periods of time (between 10 and 40 minutes) and then turned on again for approximately 15 seconds to image the worm. This was repeated several times. The vertical lines show when the laser was turned on.

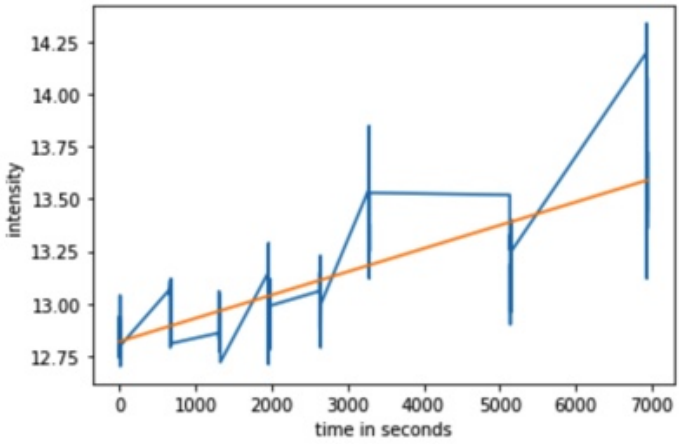


Figure 13: Regeneration curve with linear regression.

Table 3 shows the regeneration rate (m) and the intensity when we stopped exposing the worm to laser light (y_0) for all three worms. The worm that was imaged with 60% laser power was the only worm that showed significant regeneration of GCaMP. Its regeneration rate is more than 10x larger than the regeneration rate of the worms imaged with 40% and 50% laser power.

Parameters	*40% LP*	*50% LP*	*60% LP*
m	$5.44 * 10^{-7} s^{-1}$	$1.2 * 10^{-4} s^{-1}$	$2.1 * 10^{-3} s^{-1}$
y_0	13.0	12.8	44.1

Sporadic imaging

Figure 14 shows the average intensity of the 100 pixels with the highest intensity of the maximum intensity projection as a function of time.The neurons are not distinguishable anymore after about 2 hours. Except for the first break, the GCaMP does not regenerate significantly.

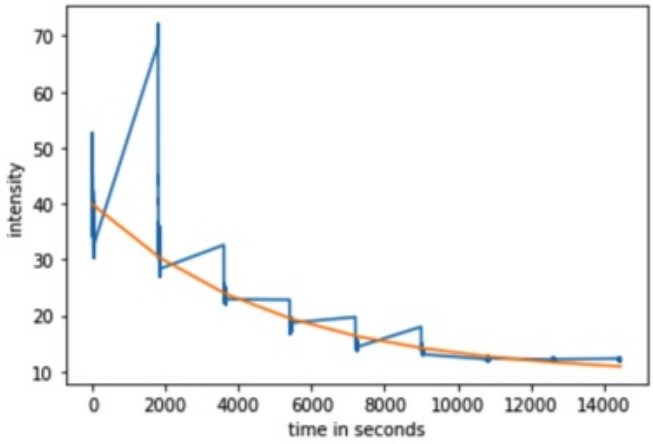


Figure 14: Graph of the average intensity of the 100 pixels with the highest intensity of the maximum intensity projection as a function of time.

4 Discussion

Regeneration rate

Only the worm that was imaged with 60% laser power showed significant regeneration of the GCaMP6. Its regeneration rate was $2.1 * 10^{-3}$, which is more than 10 times larger than the regeneration rates of the other two worms. Hendriks et al. have revealed extensive periodic gene expression during *C. elegans* larval development from L3 to yound adult stage; the expression of a fifth of all expressed genes oscillated at an eight hour period. For instance, expression patterns at t = 21 hr were noticeably more similar to expression patterns at t = 27 hr and 28 hr than to those at 24 hr. This results in periodic translation. Hence, small differences in the age of the worm could account for different GCaMP production/regeneration rates.

Initial intensities

The initial intensities of the neurons varied a lot. The average intensity of the 100 pixels with the highest intensities of the maximum projection is around 30 for the 40% laser power images, 60 for the 50% laser power images, and 225 for the 60% laser power images. This is what we expected, since high laser power increases the fluorescence intensity. However, the initial intensity of 60% laser power seems disproportionately high. This indicates that the calcium concentration was unusually high in the 60% laser power condition. Figures 6 and 7 reveal that the left neuron of the 60% laser power maximum intensity projection had a higher concentration of GCaMP6, because the right neuron is not visible anymore after about 150s.

The difference between GCaMP production and GCaMP degradation is the net turnover in GCaMP concentration. We think that the GCaMP concentration inside a neuron develops as follows. At a certain stage of the worm's development, presumably L2 or L3, the net turnover increases strongly. At some point, it reaches its maximum and starts falling. The point where it changes from positive to negative designates the highest GCaMP concentration inside the neuron. After this point, the GCaMP concentration decreases as the worm gets older, and eventually reaches zero. This is supported by the fact that several studies have shown that gene expression pattern strongly depends on the age [6]. The worms that we selected did probably not have the same age, even though we tried to select worms of approximately the same size, which is strongly correlated to the age. As mentioned above, it is possible that the expression of the gene for GCaMP6 oscillates at an eight hour period. This would also account for oscillations in the GCaMP concentration inside the neurons.

Other factors that might have changed the expression pattern in general are food availability, temperature, or population density. Those are also the factors that trigger the dauer stage (Appendix B). The dauer stage is correlated to significant changes in the gene expression pattern [13]. The expression pattern also differs from cell to cell. This could explain why

there is only one neuron on the 50% laser power images and also why one of the neurons of the 60% laser power images disappears already after approximately 150s.

Finally, a unusually high number of plasmids could explain why the GCaMP level of a particular neuron is higher than another. However, we know that the number of plasmids inside the cells of the transgenic *C. elegans* is very high. Statistically, it is very unlikely that the number of plasmids is subject to relatively large fluctuations.

Decay rate

The decay rate (denoted by the parameter C) is composed of the bleaching rate and the regeneration rate. We expect the bleaching rate of 40% laser power to be smallest, the one of 60% laser power to be greatest, and the one of 50% laser power to be in between, because higher laser power will excite more GCaMP. Based on the assumption that the value of the bleaching rate is linear proportional to the laser power, the difference of the bleaching rate of 40% laser power to the one of 50% laser power should be equal to the difference of the bleaching rate of 50% laser power to the one of 60% laser power. The regeneration rate of 60% laser power has the greatest value (Table 3). This explains why the decay rate of 60% is smaller than the decay rate of 50% laser power. Using the difference between the 40% and the 50% laser power decay rates, we can approximate the effect of 10% laser power increase as an increase of $3.58 * 10^{-3} s^{-1}$ in the decay rate. We neglect the regeneration rates of 40% and 50% laser power since they are very small.

Neural activity

The ratio of measured intensity over predicted intensity ranges from approximately 0.8 to 1.6. A high ratio indicates that the neuron is active. Motion artifacts can be ruled out because the worm is immobilized. However, it is still not clear how accurate this method is.

For example, we assumed that the GCaMP decays exponentially because of photo-bleaching. However, the processes inside the cell are very complex. Therefore, we think that other factors lead to a decay curve that is not perfectly exponential, which causes errors in the graph of ratio over time. The only behavior that we observe are slight movement of the head, because the worm is immobilized. Figures 12 and 10 and Figures 11 and 8 suggest a correlation of the activity of the neurons to their movement. This is supported by the fact that the tracked neurons affect behavior. The observed activity might be the initiation of local search behavior, since the worm was removed from bacterial food for imaging. However, we do not know the exact time between removal from food and the actual start of imaging. There is a lot of neural activity that can not be correlated to movement of the neurons or the worm. In order to find out whether this activity affects behavior, the worm has to be able to move freely. This could be the goal of a further study.

Sporadic imaging

Imaging interrupted with breaks in which the worm was not exposed to laser was partly successful. Under the assumption that the neurons are too dim when the fluorescence intensity falls below 15, we could image the worm for approximately two hours. The worm that was imaged continuously with 50% laser power could only be imaged for 600 seconds. Hence, we could extend the time period by a factor 12. However, we expected to be able to image the worm for five hours, because the worm was only exposed to laser for one minute every thirty minutes. Figure 14 suggests that the bleaching of GCaMP as a response to laser exposure was higher when we imaged the worm sporadically than continuously.

Future directions

For further research, it is important to make sure that every neuron of an imaged worm has approximately the same concentration of GCaMP. This means that we have to find a way to control the number of plasmids in a certain neuron and we have to be able to select worms that have the same age.

Besides that, it would be helpful to find out how fast pure GCaMP bleaches, because the complexity of the processes inside the cell makes it difficult to isolate one parameter. Furthermore, there should be more information about the transcription and translation of GCaMP and how they are regulated; which factors affect the expression of the gene that codes for GCaMP.

The idea of imaging interrupted with breaks in which the worm is not exposed to laser light seems reasonable, even though we are still not able to image the worm for as long as we want. Sporadic imaging can easily be applied to freely moving and behaving worms. However, if the worm can move freely, we have to find an automated way to move the stage of the microscope according to the worm's position, so that it stays in the field of view of the 40x magnification objective. Besides that, we need to find a method with which we can reliably image the brain activity. The final goal will be to image the brain activity of *C. elegans* when it enters the dauer stage, because this might reveal new information about decision-making.

5 Acknowledgments

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teaching assistants for helping me with the paper and the presentation.

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Appendix A

Continuous imaging

40% laser power

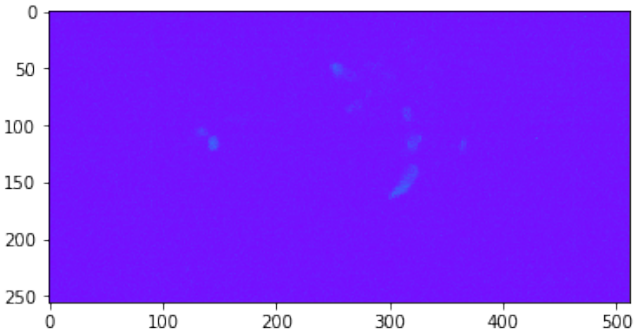


Figure 15: Maximum intensity projection at t=0.

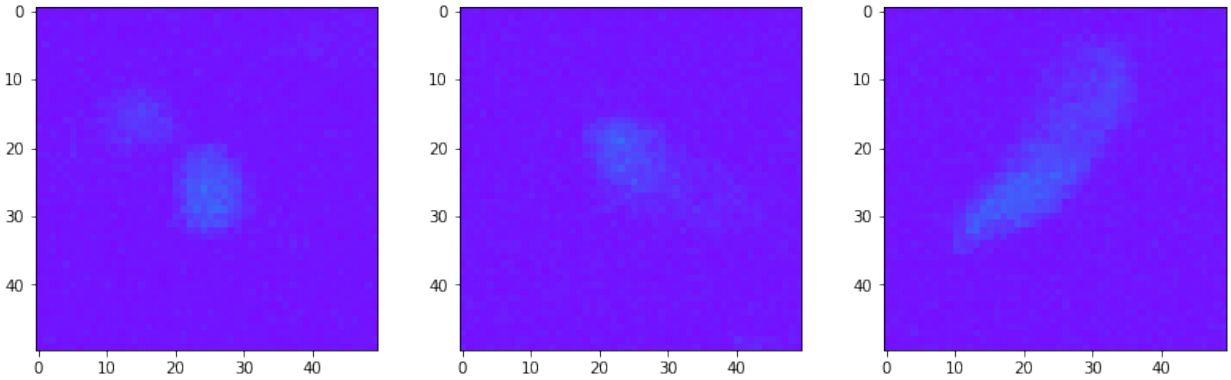
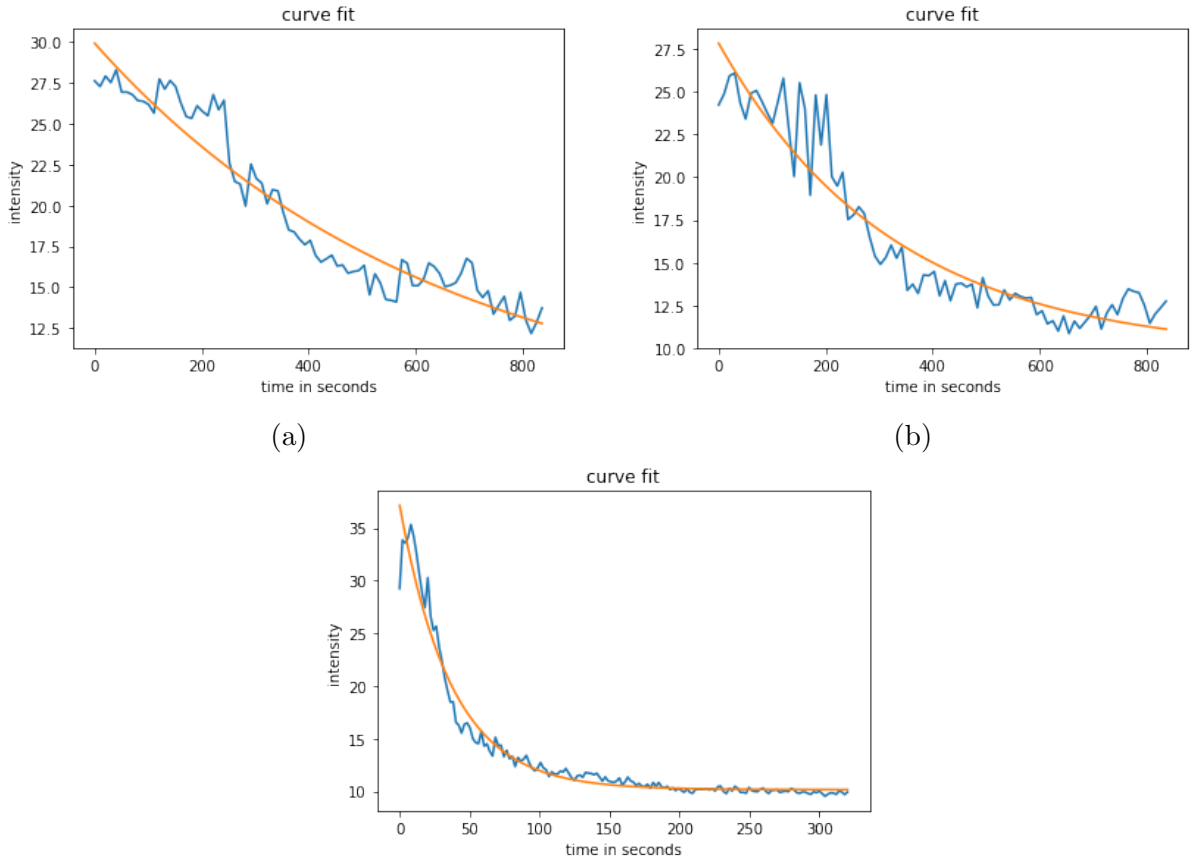


Figure 16: Cropped images of the left, the middle, and the right neuron of Figure 13.



(a) (b)

(c)

Figure 17: Average intensity with curve fit of the left (a), the middle (b), and the right (c) neuron of Figure 13.

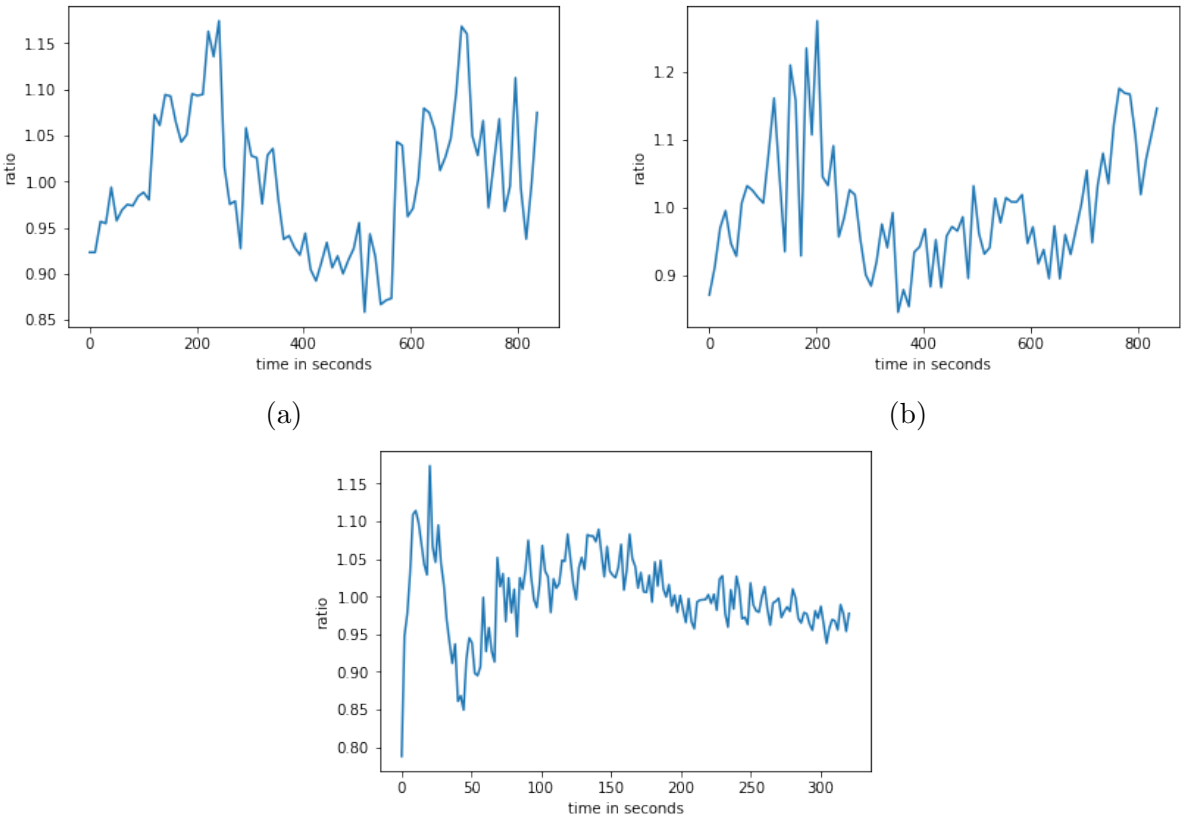


Figure 18: Ratios of the measured intensity over the predicted intensity of the left (a), the middle (b), and the right (c) neuron of Figure 13.

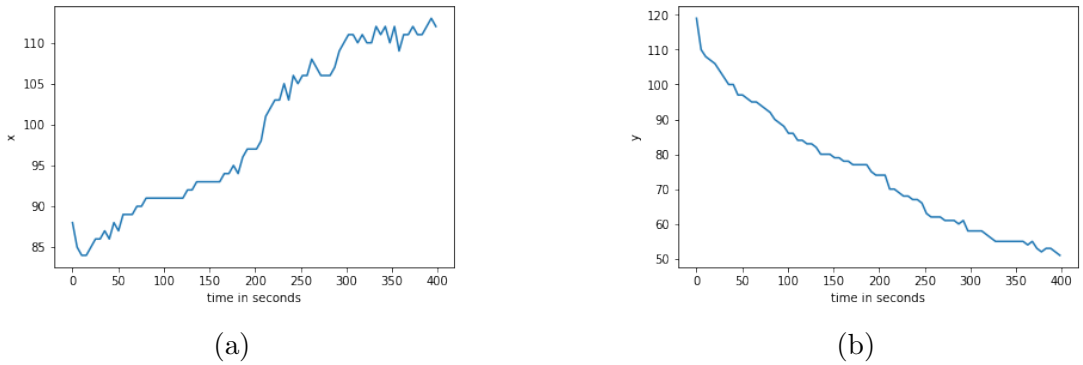
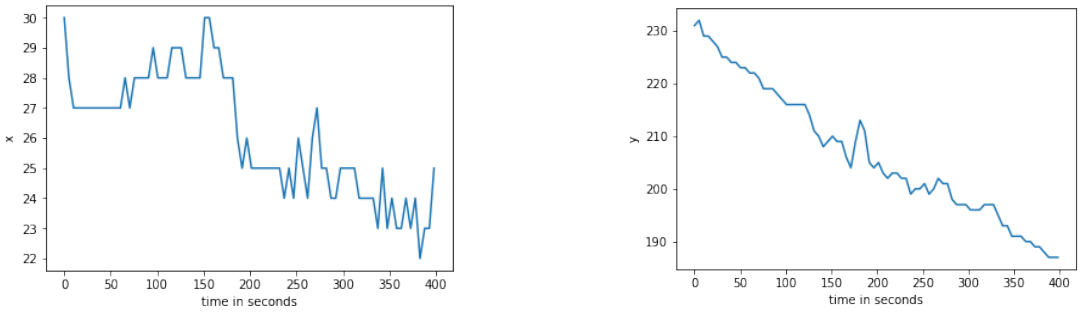
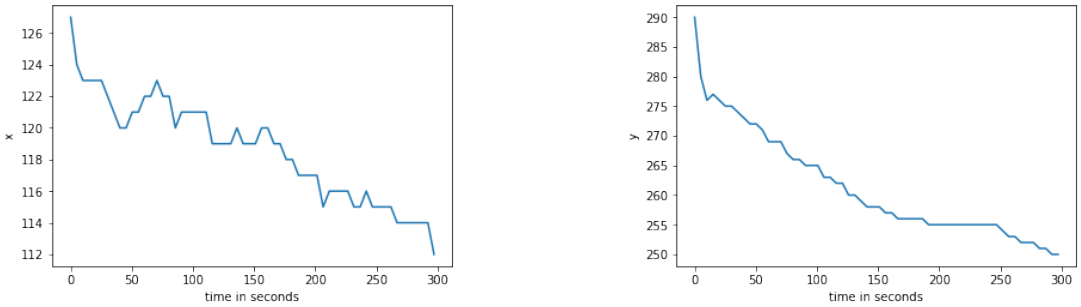


Figure 19: Movement of the left neuron of Figure 13 along the x-axis (a) and the y-axis(b).



(a) (b)

Figure 20: Movement of the middle neuron of Figure 13 along the x-axis (a) and the y-axis(b).



(a) (b)

Figure 21: Movement of the right neuron of Figure 13 along the x-axis (a) and the y-axis(b).

50% laser power

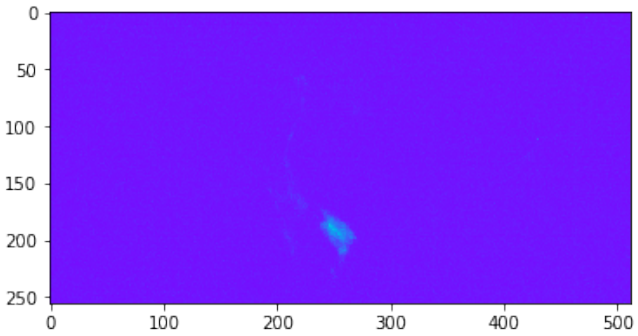
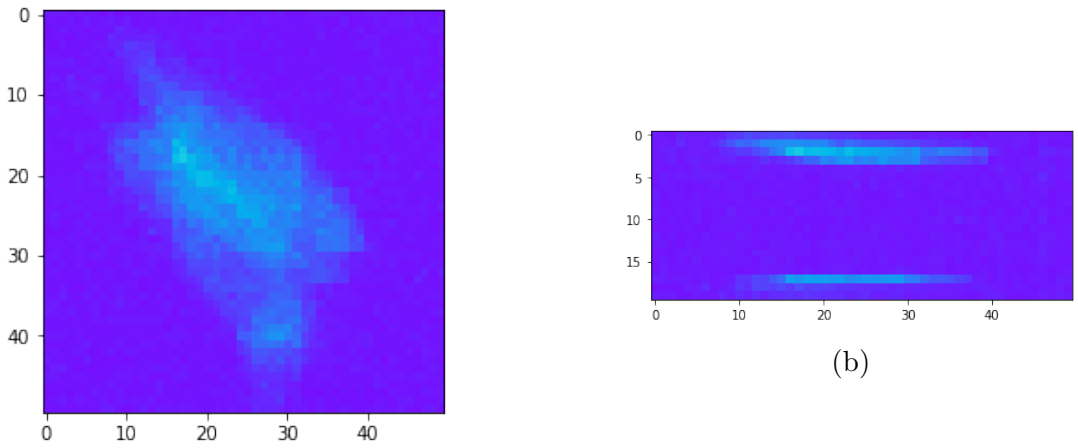


Figure 22: Maximum intensity projection at t=0.



(a) (b)

Figure 23: Maximum projection of the neuron (a) and along the x-axis (b) at t=0.

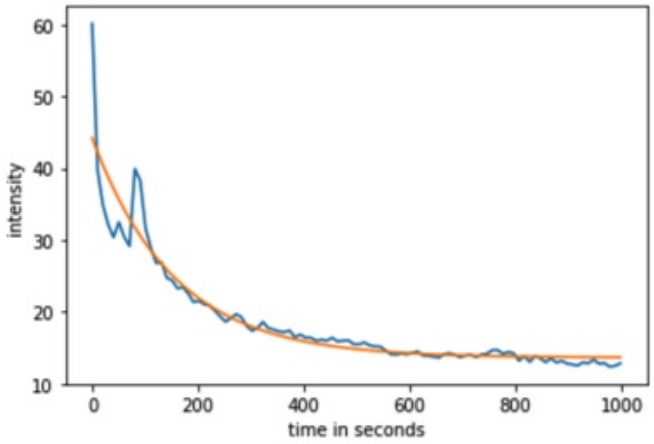


Figure 24: Average intensity of the neuron as a function of time with curve fit.

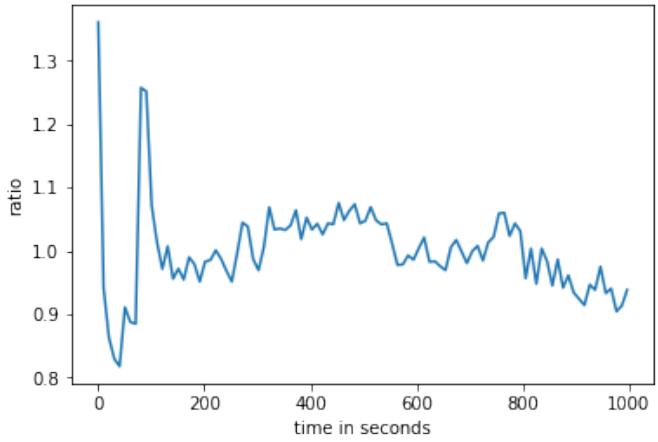
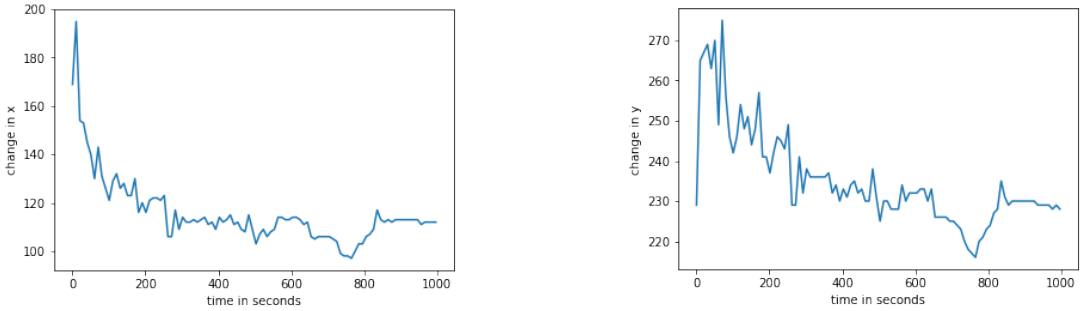


Figure 25: Ratio of the measured intensity over the predicted intensity.



(a) (b)

Figure 26: Movement of the neuron along the x-axis (a) and the y-axis(b).

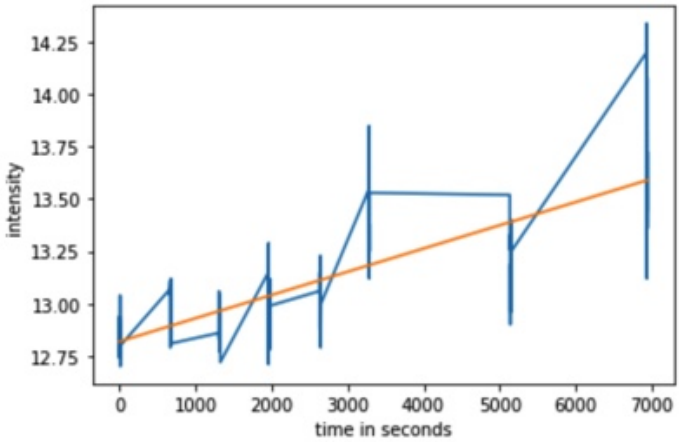


Figure 27: Regeneration curve with linear regression.

60% laser power

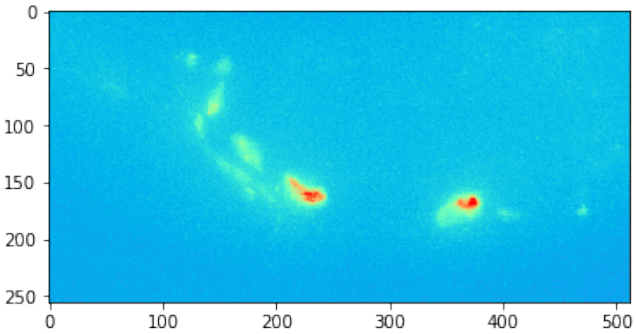


Figure 28: 60% laser power maximum intensity projection at t=0.

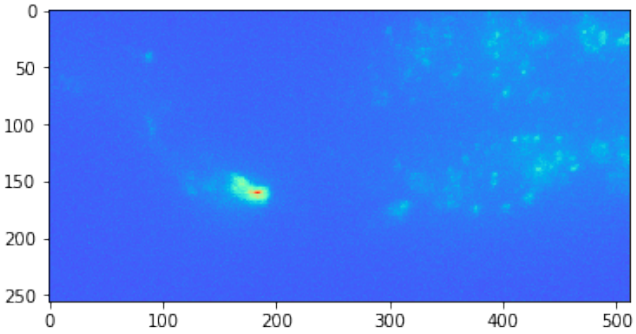
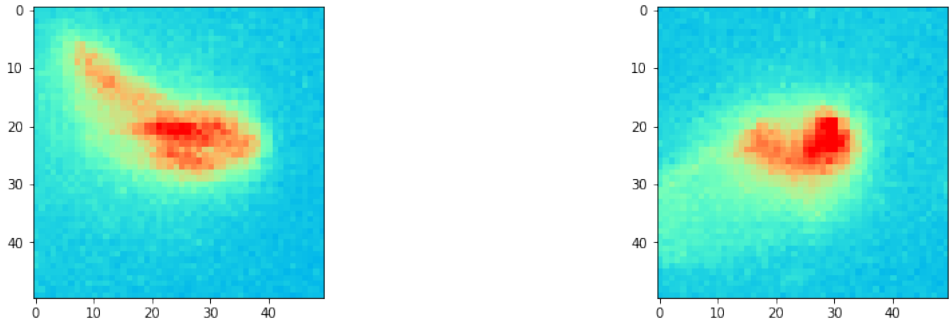


Figure 29: 60% laser power maximum intensity projection after 1500 volumes.



(a) (b)

Figure 30: Cropped images of the left (a) and the right (b) neuron of Figure 13.

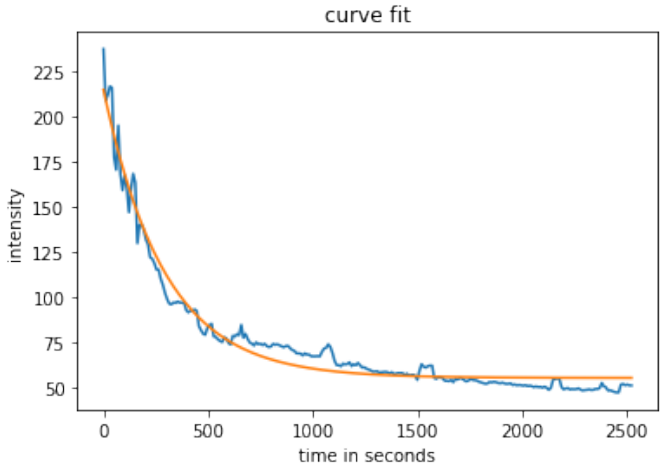
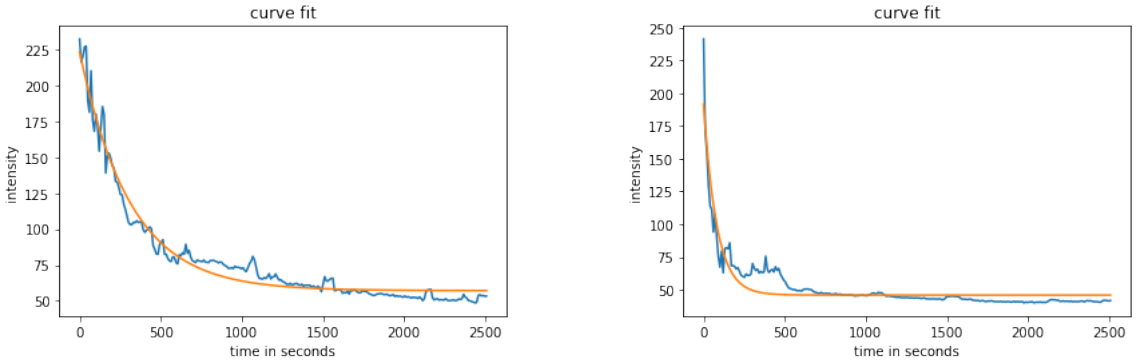
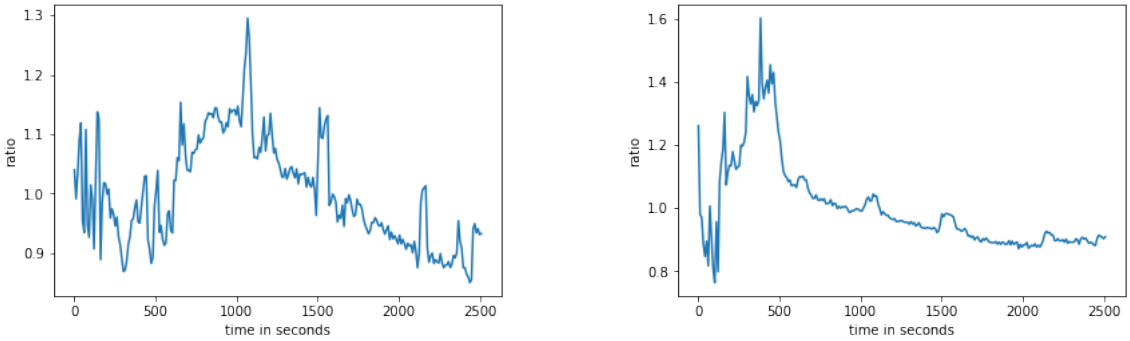


Figure 31: Average intensity of the 100 pixels with the highest intensity of the maximum projection as a function of time.



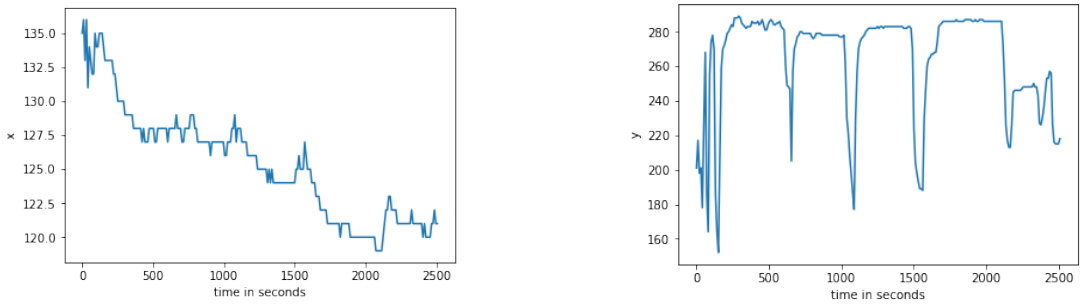
(a) (b)

Figure 32: Average intensity of the 50 pixels with the highest intensity of the left (a) and the right (b) neuron of Figure 13 as a function of time.



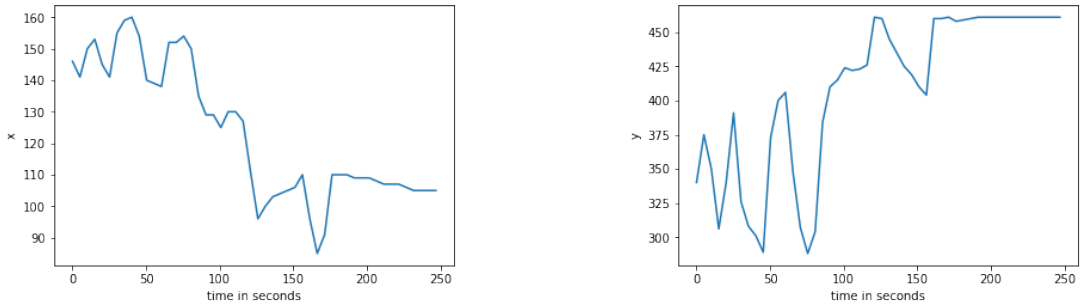
(a) (b)

Figure 33: Ratio of the measured intensity over the expected intensity for the left (a) and the right (b) neuron of Figure 13.



(a) (b)

Figure 34: Movement of the left neuron of Figure 13 along the x-axis (a) and the y-axis(b).



(a) (b)

Figure 35: Movement of the right neuron of Figure 13 along the x-axis (a) and the y-axis(b).

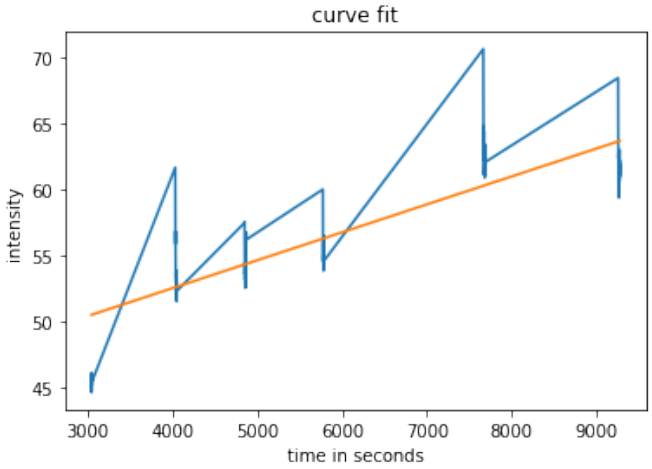


Figure 36: Curve fit of the regeneration curve.

Interrupted imaging

50% laser power

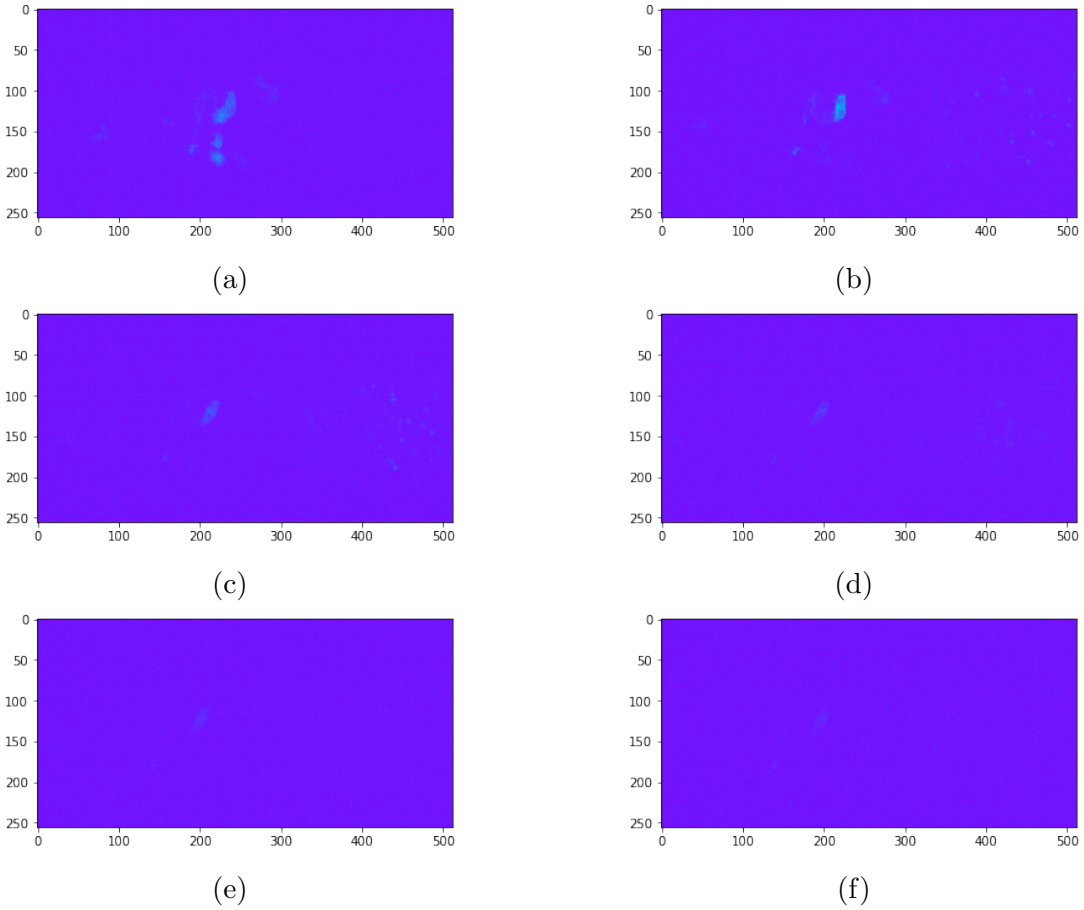


Figure 37: Maximum intensity projections at t=0, t=1800s, t=3600s, t=5400s, t=7200s, and t=9000s.

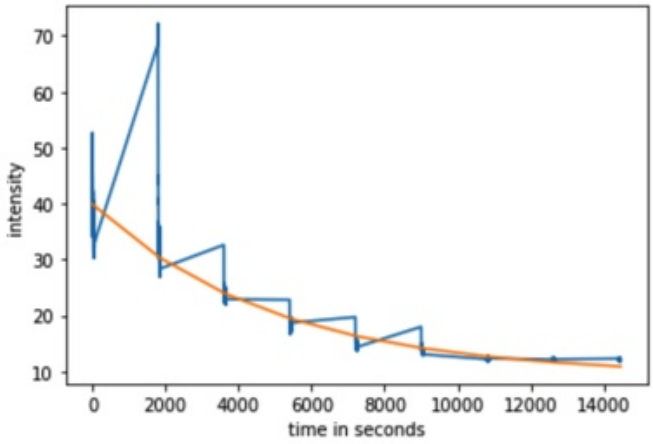


Figure 38: Graph of the average intensity of the 100 pixels with the highest intensity of the maximum intensity projection as a function of time.

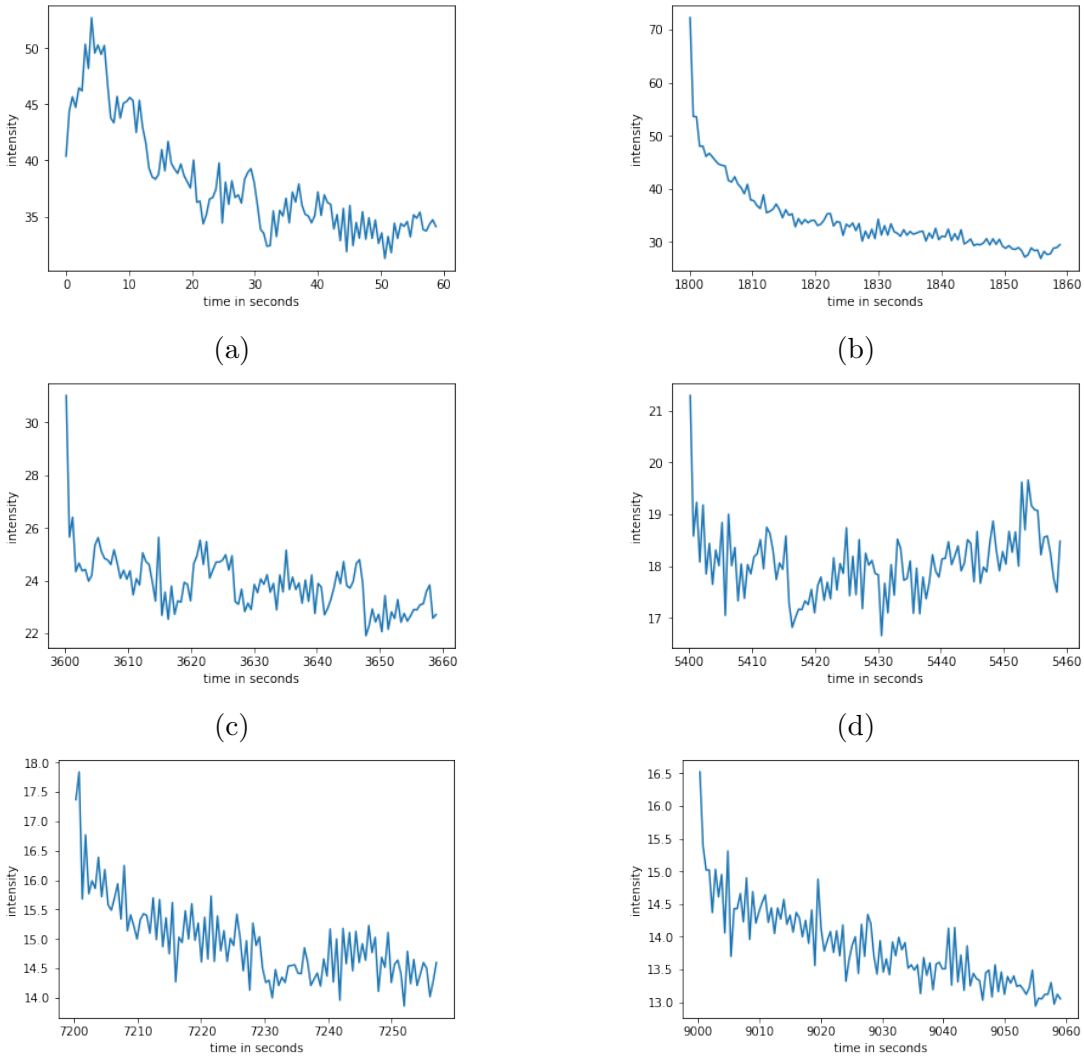


Figure 39: Intensity of 100 pixels with the highest intensity of maximum intensity projections for one minute continuous imaging at t=0, t=1800s, t=3600s, t=5400s, t=7200s, and t=9000s.

Appendix B

The dauer stage is one of two alternative pathways in the development of C. elegans[14]. Choosing one of these pathways is the only decision in the development of C. elegans that is not genetically predetermined[14]. Hence, it could help us understand the process of decision-

making and its dependency on environmental factors. The dauer stage is entered under harsh conditions with limited food availability, high temperatures, or high population density[14]. The three neurons that we image (AIK, AIA, AIB) play an important role in the onset of the dauer stage.