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i Publications

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PARCG, a protein linked to ciliary motility, mediates cellular signalling.
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Proceedings of the National Academy of Sciences of the United States of America. 2013, vol. 110 (2)

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iii Abbreviations

Worm genes unc-, itr-, dop described in abstract

AT2	Analysis Toolbox 2
CaM	Calmodulin
cDNA	Coding Deoxyribonucleic Acid
C.elegans	Caenorhabditis Elegans
CGC	Caenorhabditis Genetics Centre
Chrd2	Channel Rhodopsin 2
CTX	Chemotaxis
DEG/ENaC	Degenerin/Epithelium Sodium Channel
DNA	Deoxyribonucleic Acid
E.coli	Escherisha Coli
EEO	Electroendosmosis
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic Reticulum
FRET	Fluorescence Resonance Energy Transfer
GABA	*Gamma-Amino Butyric Acid*
gDNA	Genomic Deoxyribonucleic Acid
GECI	Genetically Encoded Calcium Indicator
GFP	Green Fluorescence Protein
LED	Light-Emitting Diode
NEB	New England Biolabs
NGM	Nematode Growth Medium
PCR	Polymerase Chain Reaction
RFP	Red Fluorescence Protein
RNAi	Ribonucleic Acid Interference
RT-PCR	Revserse Trancsciption-Polymerase Chain Reaction
SEM	Standard Error of the Mean
TYE	Tryptone Yeast Extract
WT1	Worm Tracker 1
WT2	Worm Tracker 2

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v Abstract

Even with its simple nervous system, the nematode worm *Caenorhabditis elegans* can display a range of complex behaviours. Movement can be viewed as the main output of the *C. elegans* nervous system, and aberrations in the worm's locomotion can be used as an indicator for genetic function in mutant strains of *C. elegans*. Automated tracking of *C. elegans* locomotion has been used to determine phenotypic fingerprints for ~300 mutant *C. elegans* strains. Two methods of creating phenotypic fingerprints were used. The first based on pre-determined micro-behaviours previously described in worms, but never before analysed using automated tracking. The second used the tracking data itself to determine micro-motifs, repeated sets of behaviours observed at least twice in at least two mutant or wild-type strains.

Both methods of clustering successfully grouped together strains with mutations in genes known to interact together, verifying that the technique is able to detect meaningful connections between mutant strains. The following step was to determine whether the technique can be used to establish connections between genes on unknown function. A pair of strains with mutations in DEG/ENaC subunit encoding genes clustered strongly together using the micro-motif method, due to similar defects in their behaviours upon turning. The function of these genes, *asic-2* and *acd-5*, was unknown. Upon further investigation it was found that the two genes are expressed in different classes of neurons, the IL2s in the case of *asic-2* and the ASIs in the case of *acd-5*. Following investigation into behaviours known to be modulated by these two neuron classes it was found that the mutant strains displayed mutant phenotypes in similar behaviours, but that their mutant phenotypes are opposing. Mutations in *asic-2* cause increased lifespan and healthspan and a reduction in dauer entry in response to exogenous, purified ascarosides. Mutations in *acd-5* cause decreased lifespan and healthspan and a reduction in dauer entry in response to crude dauer pheromone. This suggested that the two genes were unlikely to be working in the same pathway, but do function in similar pathways.

Calcium imaging is a technique used in *C. elegans* to measure responses in excitable cells, in this case in neurons. Many calcium indicators are available for use in this technique, one in particular is GCaMP. GCaMP has undergone many rounds of targeted mutations with the aim to increase the molecule's dynamic range and dissociation constant. At the time of commencing this project, new variants of GCaMP, known as GCaMP6s, became available, and had yet to be tested in *C. elegans* neurons. The effectiveness of a total of 6 new variants was tested in the gentle touch neurons of *C. elegans*. It was found that the alterations made to GCaMP5G in order to make the GCaMP6 variants did not result in improved dynamic range or dissociation constant in the PLM of *C. elegans*.

Chapter 1

1.1 Introduction

1.1.1 *Caenorhabditis elegans*

An interesting question in biological research, and one for which sufficient answers still evade scientists, is how gene products interact to create coordinated and complex behaviours.

As humans our thought processes and resultant actions are so complex that an explanation for this question seems beyond the realms of possibility. Even those processes for which no conscious thought is necessary are complex and multifaceted, with many genes interacting to perform some of the smallest of actions. Due to these factors human experimentation would be time-consuming and costly, not to mention unethical. For this reason researchers have, for many years, attempted to identify human homologies in the genetics of simpler organisms that are more genetically tractable.

Several organisms have been found to be ideal for just this, *Caenorhabditis elegans* (*C. elegans)* is one such organism. This soil dwelling nematode has become a favourite model species. Initially the appeal of the worm lay in its basic anatomy. Its small size (approx 1mm) and large brood size means it is easy to grow in large numbers in a relatively small amount of space. The worm is transparent and can be imaged under a light microscope, allowing for live observations.

The worm has simple nutritional needs, an agar plate seeded with bacteria is sufficient to keep dozens of individuals fed for several days (Brenner 1974). In this feeding environment, at 22°C, a single worm will develop, in 72 hours, from egg to adult (Maniatis et al. 1982). During this development the worm passes through 4 typical larval stages, L1 - L4 (Fig.1.1A) (Cassada & Russell 1975). *C. elegans* are easily staged by picking L4 larvae, noticeable due to their characteristic ‘saddle’, a clear semicircle on one side of the body formed by the invagination of the developing vulva (Fig.1.1B). One mature individual can lay approximately 300 viable eggs in a lifetime (LeDoux 2005). Since the worm is mainly hermaphroditic, with a low incidence of male births (approximately 0.2%) (Chaffey 2003), potentially huge numbers of identical, genetically stable worms can be grown in just two generations. Consequently, a large population of clones can be reached very quickly. When starved, *C. elegans* larvae will enter an alternate developmental pathway, forming what is known as a dauer larva. Dauers can survive for at least 6 months in starvation and will re-enter the normal path of development when reintroduced to food (Cassada & Russell 1975).

It was for these reasons that *C. elegans* was originally picked by Sydney Brenner as a candidate for research into developmental biology and neurology (Brenner 1974). Initial work on the worm involved the introduction of mutations into the worm’s genome using EMS (Ethyl Methane Sulphonate). Any worms that were exhibiting an obvious locomotory or morphological phenotypes were cultivated to produce colonies of mutants. Examples are dumpy, long and small for morphology (Fig.1.1C) and uncoordinated for locomotion. Using a

series of elegant genetic experiments a rudimentary genetic map of the mutated genes was made (Brenner 1974; Coulson et al. 1986), leading to a basic understanding of *C. elegans* genetics, and allowing the mutations responsible for these phenotypes to be mapped.

Following on from Brenner's work with *C. elegans*, many important discoveries have been made during experimentation with the worm, which have greatly impacted research in the wider research community. Most notable are those for which Nobel prizes have been won.

The first of these came in 2002 for the work carried out by Brenner, Sulston and Horvitz on the basics of organ development and programmed cell death. Sulston and co-workers discovered that during *C. elegans* development many cells undergo apoptosis in a predictable, identical pattern (Sulston and Horvitz 1976). This lead to the understanding that some cancers are caused by a fault in the cell's usual pathway: programmed cell death. Horvitz was able to decipher which genes within the worm were responsible for modulating apoptosis. He found that functioning *ced-3* and *ced-4* genes are required by all cells in *C. elegans* to properly undergo apoptosis (Ellis & Horvitz 1986). He was also able to determine that there is a *ced-3* like gene in the human genome, indicating that humans also have a particular gene required for proper cell death to take place (Yuan et al. 1993).

The second Nobel Prize for work with *C. elegans* was for the discovery of the technique RNAi (RNA interference) by Mello and Fire. This is an easy to use and effective method for silencing targeted genes by introducing double stranded RNA (dsRNA), either directly (e.g. by feeding) or by transgenic expression (Mello and Fire 1998). Since dsRNA usually hails from a viral source, the cell will recognise the RNA as pathogenic and destroy it. The mRNA from the targeted gene is blocked from ever becoming translated, hence it is silenced. Using cell specific promoters, the RNAi can be introduced into targeted cells, where it can silence a gene within this cell only. This technique has since been effectively used for gene silencing in a wide variety of organisms. Clinically, RNAi has been effectively used in treatment of Macular Degeneration (Kaiser et al. 2010) and human respiratory syncitial virus (Alvarez et al. 2009).

Third and finally, a Nobel Prize was won in 2008 by Chalfie, Shimomura and Tsien for the discovery and development GFP (Green Fluorescence Protein) (Chalfie et al 1994, Shimomura 1979; Heim et al. 1994). Chalfie and co-workers were responsible for developing GFP for use in *C. elegans*. Promoter-GFP fusions are incredibly useful in *C. elegans* research as they offer a quick and relatively reliable method of determining the expression pattern of specific genes. One merely has to identify a promoter region for the gene of interest, fuse it to a green fluorescent protein and express it in the worm (Chalfie et al 1994).

The GFP molecule has since been manipulated in many ways to offer a variety of colours of fluorescent protein and has been combined with various protein functional domains to give rise to fluorescent indicators. Indicators exist for a number of different ions; Outside of *C. elegans* research fluorescent indicators are used for a wide variety of applications. Detection of low levels of heavy metals in solutions and environmental samples can be achieved using indicators that bind the target metal (Prestel et al. 2000). In neuronal research the most common indicators used detect changes in sodium or calcium ion concentration. In mammalian research sodium indicators have many uses (Rose & Konnerth 2001; Moore & Fay 1993), but they are of limited use in research with the worm, as *C. elegans* does not have recognised sodium

transients. In *C. elegans* the most commonly used indicators are the calcium indicators: Cameleon, GCaMP and G-GECO, used for measuring calcium transients in excitable cells. In recent years a wider variety of colours of fluorescent indicators has become available, the red-shifted RCaMP and R-GECO and blue-shifted B-GECO (Akerboom et al. 2013; Zhao et al. 2011).

In recent years more elaborate techniques for researching with *C. elegans* have been developed. It is possible to stimulate specific neurons with blue light, using cell specific expression of channelrhodopsin (ChR), a functional ion channel activated by UV light (Nagel 2002). Emissions from red-shifted calcium indicators, expressed in cells postulated to be downstream of ChR containing cells, can then be tracked to elucidate neuron circuitry (Akerboom et al. 2013). We can accurately measure electrical gradients across a cell membrane (Goodman et al. 2012); and, most importantly for the work I have been conducting, we can measure the calcium transients within specific neurons in response to a variety of stimuli (Suzuki et al. 2003; Hilliard et al. 2005; Kimura et al. 2004).

During my study I have been exploiting a number of the techniques known to *C. elegans* researchers, both old and new, to determine the function of unknown genes.

1.1.2. *C. elegans* anatomy

The *C. elegans* hermaphrodite has an un-segmented cylindrical body with a recognisable head and tail at either end. The body is formed of an outer and inner tube held apart by internal hydrostatic pressure. The outer tube consists of the cuticle, hypodermis, excretory system, neurons and muscles (Fig 1.2A). The inner tube is made up of pharynx, intestine and gonad, which produces both egg and sperm (Brenner 1988) (Fig 1.2B and 2D).

The male anatomy is similar, but the male is smaller in comparison to age-matched hermaphrodites and can be identified by their characteristic fanned tail (Fig.1.2C). The male has no ovaries and instead has a single j shaped arm that produces sperm only. Males and hermaphrodites can mate to generate cross-progeny of around 50% of each sex (Ward & Carrel 1979). The *C. elegans* male has 385 neurons in comparison to the 302 in hermaphrodites. 91 of the male neurons are sex specific, while hermaphrodites have only 8 sex specific neurons. The majority of male sex specific neurons are found in the tail and many have specific roles in male mating behaviour. Sex specific neurons in the head are involved in pheromone sensation and mate detection (Sulston and Horvitz 1997).

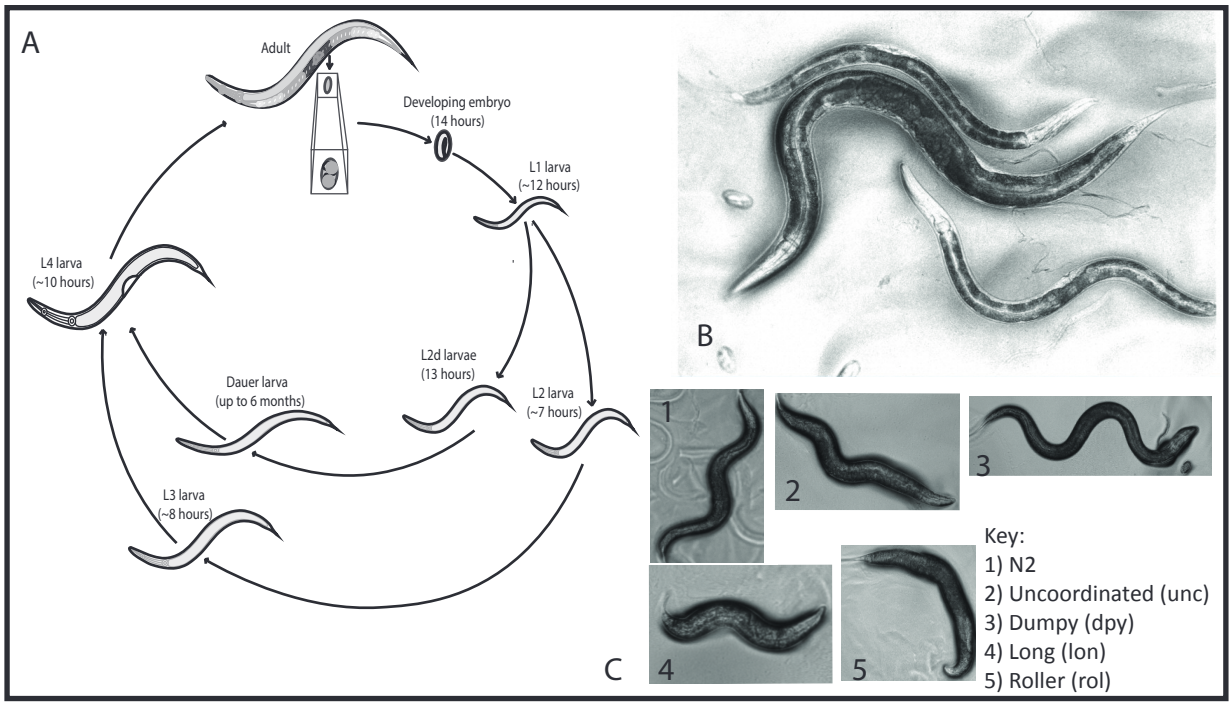

Fig.1.1: The Lifecycle and Anatomy Of *C. elegans.* A: The life-cycle of *C. elegans*, eggs are released from gravid adults, eggs hatch after ~26 hours as L1 larvae, in favourable conditions the worm progresses through larval stage 2 and 3, then become L4 larvae. In low or poor food conditions the worm enters the dauer pathway. B: A larger, adult individual flanked by two L4 larvae. The larvae display the characteristic semicircular arc midway down the body (Genome research limited). C: 1, shows an N2 adult, 2, an uncoordinated adult, 3, a long adult and 4 a dumpy adult and 5, a roller.

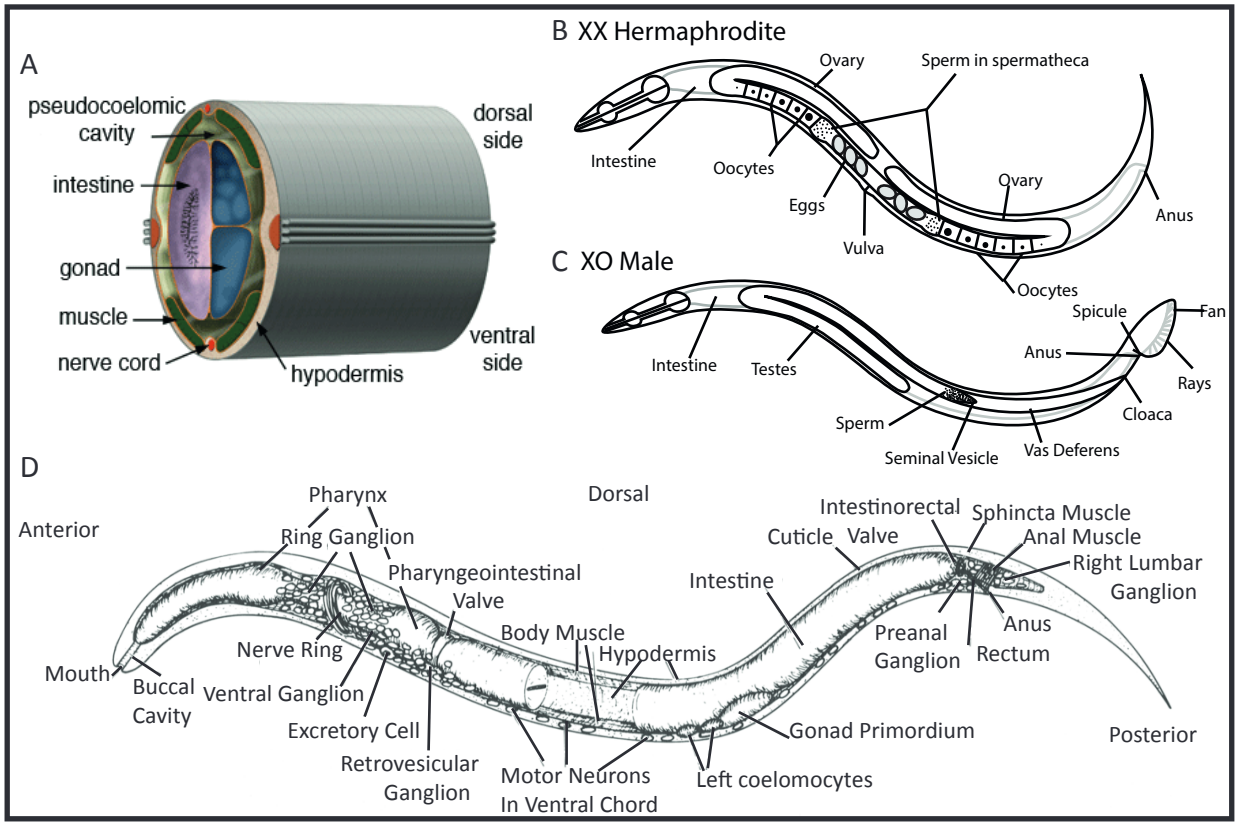

Fig.1.2. The internal anatomy of *C. elegans*. A: A cross section of the *C. elegans* body (Altun and Hall wormatlas). B: The basic bodily structure of the *C. elegans* hermaphrodite (Wormatlas) C: The basic bodily structure of the *C. elegans* male D: A detailed schematic of the *C. elegans* hermaphrodite physiology (Sulston et al. 1983).

1.1.3 *C. elegans* Genetics

Although *C. elegans* has long been a popular organism for genetic research, its appeal increased in 1998 when it became the first multi-cellular organism to have its full genome sequenced (*C. elegans* Sequencing Consortium 1998). Consequently knowledge of the worm's 100MB genome has had somewhat of a head start over other popular model species.

The worm's genome contains around 20,470 protein coding genes (WS277 release letter WormBase). It is thought that there are around 1,200 gene families containing two or more paralogues in *C. elegans* (Cavalcanti et al. 2003; Gu et al. 2002) accounting for 32% of their genes.

A number of genes in the worm are highly conserved with lower complexity organisms; these tend to be encoding essential proteins or are genes that function in processes that are evolutionarily, strongly conserved. Around 300 of the worm's genes are essential to its survival (Kamath et al. 2003). These essential and conserved genes tend to cluster to the centre of the autosomes, while the arms contain fewer, sparser and less vital genes (*C. elegans* Sequencing Consortium 1998; Hutter et al. 2000). At least some of these clusters are thought to act as operons (Blumenthal et al. 2002).

Around 35% of *C. elegans* genes have human orthologs (Shaye et al. 2011), which is incredibly useful for researchers. Particular human disease genes have already come to be much better understood by studying their homologous gene in the worm. An example of this is in the study of the inherited skin fragility disease Kindler syndrome, caused by a mutation in KIND1. KIND1 has a *C. elegans* ortholog, *unc-112*. Since *unc-112* is implicated in linking the actin cytoskeleton to the extracellular matrix (ECM) in worms, it was theorised that kindler syndrome was the result of an actin-ECM defect, rather than the more common keratin-ECM defect. Further investigation in the direction of this theory proved it to be correct (Siegel et al. 2003). Many mammalian orthologues can rescue mutations in their worm counterparts. For example the *C. elegans* mutant, *nlg-1*, an ortholog of the human synaptic cell adhesion protein neuroligin, have their atypical gentle touch and osmotic responses rescued by expression of human NLGN1 or rat *Nlgn1* (Calahorro et al. 2012) suggesting functional conservation of human orthologues.

The worm has 6 chromosome pairs I, II, III, IV, V and X, the final being the sex chromosome. A configuration of XX will cause the individual to be hermaphrodite, and XO will create a male (Riddle 1997). When males are lacking in a population the hermaphrodites will self fertilise and create a colony of clones, which is useful when propagating mutant recessive lines. Crossing with males allows for the introduction of extrachromosomal arrays or integrated genes into the hermaphrodite genome.

1.1.3.1 Introducing mutations and genetic screening

Genetic mutations can be introduced into the worms' genome in a number of ways, for a number of screening techniques. The method by which a gene is mutated largely depends on the experimental requirements of the researcher, and which type of screen is to be done.

Forward genetic screening is a method used to attribute a randomly mutated gene to a specific, observed phenotype, while a reverse genetic screen involves specifically mutating a gene, or genes of interest, and observing the resultant phenotype. Thus, forward genetic screening allows for wide-scale mutagenesis, while reverse screening requires a more specific, targeted method of mutagenising.

Chemical mutagenesis with EMS, Formaldehyde, DES (Diethylsulfate) and DEB (Dipoxybutane) can be used for forward genetic screens. Short wave UV, IR and 32P decay are other useful techniques for creating genome wide mutations.

In reverse genetics both MosTIC (Robert 2012) (see 4.1.9) and CRISPR (Friedland et al. 2013) (see 3.3.3) have proved to be incredibly useful methods for targeting mutations to specific parts of the genome.

1.1.4 *C. elegans* Neurobiology

An adult hermaphrodite has 302 neurons that form two distinctly different systems; the significantly larger of the two is the somatic system which consists of 282 neurons, only the somatic neuronal system contains support cells, of which there are 56. The other, smaller group of 20 neurons comprises the pharyngeal system. The two are connected by just two inter-neurons, the RIP pair (White et al. 1986). During my research with *C. elegans* I have concentrated only on the neurons forming parts of the somatic system.

The worm has two clusters of ganglia and cell bodies; one is located in the head and known as the nerve ring, the other, less populated cluster can be found in the tail. A full map of the connections between all the neurons, labelled the connectome, has been produced (White et al. 1986; Chen et al. 2006; Varshney et al. 2011). Neurons in *C. elegans* can confer responses via both chemical and electrical connections (white et al 1986). In the worm, gap junctions are formed by innexins, for which there are 25 different genes. Innexins encoded by different genes appear to be able to form functional channels with each other, and each forms a distinctive expression pattern (Altun et al. 2009). *C. elegans* chemical synapses release numerous neurotransmitters, depending on the function of the neuron; Table 1.1 shows the neurotransmitters and the neurons from which they are released. When acting upon muscle cells acetylcholine causes excitation, leading to contraction while GABA acts to relax muscle.

Table 1.1 The Neurotransmitters Released by *C. elegans* Neurons.

Neurotransmitter	Neurons
Acetylcholine	ADF, AIA, AIM, AIN, AIY, AS1, ASJ, AVA, AVB, AVD, AVE, AVG, AWB, CEM, DA1, DB1-2, IL2, RIB, RIF, RIH, RIR, RIV, RMD, RMF, RMH, SAA, SAB, SIA, SIB, SMB, SMD, URA, URB, URX, VA1, VB1-2, I1, I3, M1, M2, M4, M5, MC, AS2-10, DA2-7, DB3-7, HSN, VA2-11, VB3-11, VCn, ALN, AS11, DA8-9, DVA, DVE, DVF, HOB, PCB, PCC, PDA, PDB, PDC, PGA, PLN, PVN, PVP, PVV, PVX, PVY, PVZ, R1A, R2A, R3A, R4A, R6A, SPC, SPV, VA12
Dopamine	ADE, CEP, PDE, Male only: R5A, R7A, R9A, SPSo
Octopamine	RIC
Tyramine	RIM
Serotonin (5HT)	ADF, AIM, RIH, NSM, HSN, VC4-5, CP1-6, R1B, R3B, R9B
GABA	AVL, DDL, RIS, RME, VD1-2, DD2-5, VD3-11, DD6, DVB, VD12-13
Glutamate	ADA, ADL ,AFD, AIB, AIM, AIZ, ASE, ASG, ASH, ASK, AQR, AUA, AWC, BAG, FLP, OLL, OLQ, RIA, RIG, RIM, URY, M3, MI, I2, I5, ALM, AVM, DVA, LUA, PHA, PHB, PHC, PLM, PVD, PVQ, PQR, PVR, PVV, R6A

C. elegans do not have recognised sodium action potentials, as observed in mammals, but instead rely solely on opposing calcium and potassium currents across the cell membrane (Lockery & Goodman 2009). It is for this reason that calcium indicators are the only form of fluorescent indicator