Generation and analysis of zebrafish expressing killerred to induce oxidative stress in zebrafish models of amyotrophic lateral sclerosis

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Declaration of Originality

I certify that the work in this thesis entitled "Generation and analysis of zebrafish expressing KillerRed to induce oxidative stress in zebrafish models of Amyotrophic lateral sclerosis" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by Macquarie University Animal Ethics Review Committee, reference number: 2012/050-8 on 31 January 2014.

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is an incurable neurodegenerative disorder characterized by the muscle wasting, paralysis, hyperreflexia, and spasticity. Zebrafish has emerged as a powerful model to study various diseases, including ALS. Moreover, ALS transgenic zebrafish have been developed to study ALS. Oxidative stress has been hypothesis as a cause of neuron death in neurodegenerative diseases including ALS. However, the lack of experimental tools to target oxidative stress in specific cells has prevented direct evaluation of the hypothesis. We attempted to use a KillerRed, a genetically-encoded protein that generates reactive oxygen species (ROS) upon light activation, to induce oxidative stress in the zebrafish motor neurons and ubiquitously. We have shown its efficacy in live zebrafish and have applied KillerRed to stress motor neurons. Thus, we established a method to address the role of oxidative stress in an ALS model Zebrafish.

Conflict of Interest Statement

We declare that there are no conflicts of interest and this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Dr. Nicholas Cole

Supervisor

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Abbreviations

ALS- Amyotrophic lateral sclerosis

E3 -Egg water

DPF- Day post fertilization

GFP -Green fluorescent protein

HPF -Hours post fertilization

mCherry-CAAX- Membrane targeted mCherry

mKillerRed -Membrane targeted KillerRed

MND- Motor neuron disease

ROS -Reactive oxygen species

SOD1- Superoxide dismutase type 1

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Section 1.0: Introduction

Amyotrophic Lateral Sclerosis (ALS), also referred as motor neuron disease (MND), or Lou Gehrig's disease, is an incurable neurodegenerative disease that is characterized by progressive degeneration of the both upper and lower motor neurons (Rowland and Schneider, 2001). This degeneration of the motor neurons initially starts form one group of motor neurons, but will spread to other motor neurons as the disease progresses (Barber and Shaw, 2010; Shaw and Eggett, 2000). Degeneration of the motor neurons will cause muscle atrophy, weakness, paralysis (lower motor neuron), hyperreflexia and spasticity (upper motor neuron) (Bruijn et al., 2004). ALS usually develops between 50 and 60 years of age. Although the rate of disease progression varies between individuals, patients usually die within 5 years following clinical onset of symptoms (Talbot, 2009).The major cause of death in ALS patients is respiratory failure (Bruijn et al., 2004). Riluzole, a glutamate antagonist, is the only disease modifying therapy available and it can extends life by only a few months (Miller et al., 2002).

The causes of ALS are likely to be multi-factorial and the only established cause of ALS is the mutation in genes. Research has already identified mutations in 13 different genes, such as superoxide dismutase type 1 (SOD1) (Rosen et al., 1993), TAR DNA-binding protein 43 (TDP-43) (Neumann et al., 2006) and fused in sarcoma/translated in liposarcoma (FUS/TLS) (Bosco et al., 2010), that can cause ALS. Although, an epidemiology study has found a strong family history for ALS among the patients with ALS mutant genes, not all people with this mutation develop ALS (Andersen and Al-Chalabi, 2011; Lagier-Tourenne et al., 2010; Mulder et al., 1986). Furthermore, only 5-10% of the people that develop ALS have a mutant in their disease causing genes (Gros-Louis et al., 2006;Byrne et al., 2011). Various factors, such as heavy metals (Mitchell, 1987), environmental (Mitchell, 2000;Armon, 2003), viral infection (Swanson et al., 1995;Walker et al., 2001), and smoking (Weisskopf et al., 2004), had been studies as a potential risk factor to trigger the pathology of ALS. Despite extensive research, there has been no convincing report in identifying a single trigger for developing ALS. Moreover, the pathology and clinical features between sporadic and familiar disease are similar (Bruijn et al., 2004). Therefore, the causes of ALS are likely to be complex and multifactorial and are not completely understood.

Moreover, the precise molecular pathways that cause the death of the motor neuron in ALS remain unknown. The possible primary mechanism that causes neuron deaths in ALS includes: oxidative stress (Beal, 1995; Lin and Beal, 2006), glutamate excitotoxicity (Rothstein et al., 1992), intracellular protein aggregation (Kopito, 2000;Ross and Poirier, 2004), mitochondrial dysfunction (Lin and Beal, 2006), neurofilaments disorganization (Côté et al., 1993), abnormal axonal transport (Williamson and Cleveland, 1999;LaMonte et al., 2002) and caspases enzyme activation (Friedlander, 2003). Among these proposed mechanisms, oxidative stress is highly thought-provoking because it has a twofold association with ALS. First, research has found the evidence to suggest the association between oxidative stress and ALS (Barber

and Shaw, 2010;D'Amico et al., 2013; Ferrante et al., 1997a; Lin and Beal, 2006; Valko, 2007). Second, oxidative stress is considered as secondary consequence of other primary mechanisms, such as glutamate excitotoxicity, mitochondrial dysfunction, and neurofilaments disorganization (Barber and Shaw, 2010).

Oxidative stress refers to the accumulation of excess reactive oxygen species (ROS) when the amount of ROS produced within the cells cannot be detoxified by its antioxidant defense system. ROS includes free radicals, such as superoxide anion ($O2^-$), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2) and nitric oxide(NO) (Dröge, 2002). The excess ROS can in a dose dependent manner cause damage to DNA, lipid membranes and proteins within the cells that ultimately results in the apoptosis of the cells (Kannan and Jain, 2000;Dröge, 2002). ROS-mediated oxidative stress has been associated with ALS in numerous studies (Barber and Shaw, 2010; D'Amico et al., 2013; Ferrante et al., 1997a; Lin and Beal, 2006; Valko, 2007). Examination of the post-mortem CNS tissue and cerebrospinal fluid form ALS patients has found more pronounced features of ROS mediated damage to DNA, lipids, proteins and an increased dROS-detoxification enzymatic activity compared with the control (Ferrante et al., 1997a;Smith et al., 1998;Shaw, 2005). Furthermore, studies from SOD1 mutant transgenic mouse and cell culture models of ALS have verified the ROS mediated damage of neuron (Ferrante et al., 1997b;Andrus et al., 1998;Liu et al., 1998). But, whether oxidative stress is a primary cause of the disease or a secondary consequence of the disease is yet to be understood.

The identification of mutations in the genes that can cause ALS has enabled the development of genetically engineered animal models, such as rodents (mice and rats), fruit fly (Drosophila melanogaster), and nematode worm (Caenorhabditis elegans), carrying ALS mutant protein (Hirth, 2010;Grice et al., 2011;McGoldrick et al., 2013). These animal models have been invaluable into understanding the molecular and cellular processes for ALS. However, there is no convincing evidence for mechanisms that lead to the onset of ALS. Moreover, the nervous system of non-vertebrate species, such as the fruit fly and worm, is very simple and far-distant form the human nervous system. On the other hand, overall the nervous system of zebrafish is similar to the human nervous system (Babin et al., 2014), which is a major advantage of zebrafish over other non-vertebrate species. Additionally, many of the relevant features of the nervous system start to develop within first day of development of the zebrafish embryo (Kabashi et al., 2010a). Compared to zebrafish, rodents are expensive and time consuming. Furthermore, zebrafish embryos develop externally and are transparent during this early stage of development, which makes it a great tool for live *in vivo* screening. By using fluorescent reporters in specific types of cells, proteins, and neurons can be easily identified and studied *in vivo* and in real time. These features of zebrafish are its' major advantage over rodent models. Additionally, genetic manipulations in zebrafish can be easily done *in vivo*. Transgenic zebrafish with different ALS mutations, such as TDP-43, SOD1, and FUS, have already been developed to study ALS (Lemmens et al., 2007;Bosco et al., 2010;Kabashi et al., 2010b). Therefore, zebrafish provide a powerful genetic model system to study ALS.

Even though, ALS mutant zebrafish have been developed to study ALS, the molecular pathway leading to cells' death in ALS has not been well understood. Since zebrafish have a tremendous capacity to regenerate their damaged neurons (Becker and Becker, 2008), any degeneration of the neurons in an ALS model zebrafish will be quickly regenerated. This capacity may limit the potential disease phenotype to be observed in the zebrafish models of neurodegenerative disease. Therefore, zebrafish will be an ideal tool to study nerves degeneration and regeneration. Research has shown that oxidative stress is highly associated with ALS (Valko, 2007, Ferrante et al., 1997a, Lin and Beal, 2006). Furthermore, oxidative stress can cause neuron death in ALS patients (Ferrante et al., 1997a), and neuronal death is considered as a pathological hallmark of ALS (Pasinelli and Brown, 2006). Therefore, we believe that generation of model which undergoes oxidative stress, such as zebrafish expressing KillerRed will help to understand the role of oxidative stress in ALS and may induce ALS phonotypical model.

ROS can be generated by various endogenous and exogenous sources. Endogenous sources of ROS include mitochondria, NADPH-cytochrome P450 reductase, xanthine oxidase, NADPH oxidase, lipoxygenase, myeloperoxidase and cyclooxygenase (Finkel and Holbrook, 2000;Barnham et al., 2004). Whereas, exogenous sources of ROS include physical agents such as gamma rays, x-rays, UV irradiation, and chemicals (Martin and Barrett, 2002).There are various well established agents to induce oxidative stress in zebrafish, such as hydrogen peroxide, paraquat, rotenone, menadione, and silver nanoparticles (Choi et al., 2010;Wang et al., 2011;Mugoni et al., 2014). Although, all these agents are well effective in producing oxidative stress in zebrafish, oxidative stress induced by these methods will produce ROS ubiquitously in the zebrafish. More importantly, real time manipulation and controlling for the desired level of ROS to be produced in just a desired part of the zebrafish, for example only at the desired motor neurons, is bit challenging with these agents. In contrast, KillerRed is a genetically engineered photosensitizer that can be incorporated in a desired cells to generate ROS upon light irradiation only within those cells (Bulina et al., 2005). In addition, the level and the duration of ROS can be controlled by manipulating the intensity and duration of the light.

KillerRed is a chromophore that is capable of generating reactive oxygen species (ROS) upon light irradiation. KillerRed has been engineered from the nonfluorescent and nonphototoxic chromoprotein anm2CP[20] from Hydrozoan jellyfish (Bulina et al., 2005). Upon irradiation by green light (fluorescence excitation maxima at 585 nm), KillerRed is efficiently photo bleached, which is accompanied by generation of the ROS. This generated ROS affects cells in a dose-dependent manner and may kill cells if used in high levels (Teh et al., 2010a). Applications of KillerRed have been demonstrated in Zebrafish (Del Bene et al., 2010b;Lee et al., 2010b;Teh et al., 2010a;Korzh et al., 2011a). In one study, Teh and colleagues expressed membrane targeted KillerRed in the hindbrain and heart of zebrafish. Then, irradiations with intense green light caused a twofold increases in the apoptosis cell death both in the hindbrain and heart, as was measured by TUNEL assay (Teh et al., 2010a). In a separate study, membrane targeted KillerRed was expressed in the habenula, a brain structure involved in avoidance

behaviors, the damage induced by KillerRed significantly reduced avoidance behaviors in the affected zebrafish (Lee et al., 2010b). Therefore, KillerRed will be a great method to address the role of oxidative stress in ALS using zebrafish.

Section 2.0: Hypothesis and Aim

We therefore hypothesis that

1) KillerRed can be used to induce oxidative stress in the motor neurons of zebrafish.
2) Exogenous oxidative stress in the motor neurons of ALS gene mutated zebrafish will enhance ALS phenotype.

AIMS

1) To create a membrane targeted KillerRed (KillerRed) plasmid and membrane targeted mCherry (mCherry-CAAX) plasmid as a control.
2) To generate membrane targeted KillerRed and mCherry transgenic zebrafish.
    a) To express membrane targeted KillerRed and mCherry in every cell of the zebrafish using an ubiquitin promoter.
    b) To express membrane targeted KillerRed and mCherry only in motor neurons using a HB9 promoter.
3) Demonstrate the efficacy of membrane KillerRed to the production of ROS using general ROS detection kit (CM-H2DCFDA; Life technologies, US)
4) Demonstrate the efficacy of membrane KillerRed to induce apoptosis using Acridine Orange and TUNEL assay.

Section 3.0: Materials and Methods

3.1 Zebrafish use and maintenance

The use and treatment of zebrafish in this project was approved by Macquarie University Animal Ethical Committee, and was done following the Australian code of practice for the care and use of animals for scientific purposes and the NSW Animal Research Act of 1985. Methods described here are the standard operating procedures in the Cole Lab.

All adult zebrafish were exposed to a light/dark cycle of 14 hours light and 10 hours dark. The adult fish were feed small pellets and live artemia twice a day. The temperature (28ºC), pH (7) and conductivity (800µS/m) were constantly maintained.

3.2 Embryo generation

Adult male and female zebrafish were placed in a mesh bottom breeding tank separated from each other with a divider in between them. Fish were left in the tank with the divider in-situ for overnight. The divider was removed early in the morning to allow the fish to spawn. The tanks were later screened for the presence of eggs and then the tank that contains the eggs were run through the tea strainer to collect the embryos. The eggs in the strainer were rinsed with the egg water (E3) (0.6g/L aquarium salt in distilled water + 0.01mg/L methylene blue) to remove any faeces and unwanted residue. Then, the tea strainer with the embryos was inverted over a petri dish and run with E3 to collect the egg in the petri dish. The faeces and other unwanted residue were further removed by plastic disposable pipette. About 20-30 fertilized eggs were kept in 85-mm per petri dish with about 30 ml of E3. The collected embryos were either used for injection or were raised by placing in an incubator at 28°C.

3.3 Generating stable transgenic lines

The DNA construct, RNA transposase and phenol red were injected into early one-cell stage embryos. At 24hpf, injected embryos (F0) were screened and those with mKillerRed or mCherry expression were kept and grown to adulthood. Since integration into the germline is random, these F0 fish were raised to adulthood and outcrossed with the wild type to identify those that incorporated the construct into their germline. F1 larvae from these outcrosses were then screened for mKillerRed or mCherry expression and those with mKillerRed or mCherry expression were kept and grown to adulthood. It was these fish (F1) that were used to establish stable transgenic lines (F2).

3.4 Construction injected

We injected a set of mKillerRed and mCherry-CAAX fusion constructs. The constructs were injected into wild type zebrafish. The plasmid injected has been summarized in the table below.

Promoter	Middle entry vector	Destination vector
HB9	mKillerRed	pTol2
HB9	mcherry-CAAX	pTol2
ubiquitin	mKillerRed	pTol2
ubiquitin	mcherry-CAAX	pTol2
Gal 4	Hb9	pTol2 + eye with EGFP
4 x UAS	mKillerRed	pTol2 + eye with EGFP

Table 3.1: Plasmids injected into zebrafish eggs

3.4 Microinjections

The injections were done under Nikon SMZ-745T dissection stereomicroscope using a three-dimensional micromanipulator and a picospritzer III. Initially, an injection tray was made by pouring hot 1.5% agarose in 85-mm diameter petri dish and then a plastic mold was placed on top of the agarose to make wedge shaped troughs within the agarose. The agarose was then allowed to cool down before removing the plastic mold. The advantage of using agarose is that the pipette tip will generally not break while touching its surface and, at the same time, can hold 100's of embryos for injection.

Microinjection needles were made by pulling fine glass capillaries (Harvard Apparatus, UK, 1.0-mm OD, 0.58 ID, and 15 cm length) with a vertical micropipette puller (David Kopf Instruments Tujunga, CA, Model 700D). The tip of the micropipette was about 0.05mm. If the pipette tip is too thin, it can easily withdraw from the chorion without dragging the embryo out of the trough, but may lack tensile strength to penetrate into the chorion and may even bend while trying to penetrate the chorion. On the other hand, a thicker pipette can easily penetrate into the chorion but does not easily slip out of the chorion. Needles were then subsequently loaded with a DNA construct, RNA transposes

and phenol red (Sigma-Aldrich) using a microloader (Eppendorf, Germany). Phenol red was used to verify the injection site. The capillary reaction with in the micropipette draws the solution to the tip of the micropipette. Finally, the tip of the micropipette was broken using a pair of forceps for the solution to come out of the micropipette while injecting the embryos.

The micropipette with the DNA construct was attached to a needle holder in the micromanipulator (Narishige, Tokyo, Japan). The needle holder was also connected to the Picospritzer III (Parker Hannifin Corp, USA), which delivers pressure using the air it gets from the compressed air cylinder. The amount of DNA construct to be injected was calibrated by manipulating the pulse pressure and duration (e.g., 50 psi and 20 ms).

A wide-mouth pipette was used to transfer the fertilized embryos into the troughs of the injection tray. The tray was then tilted to remove excess water. With a pair of blunt forceps, embryos were oriented into the desired position. The injection can be done through the chorion into the cytoplasm or directly into the cytoplasm. By manipulating the micromanipulator, a micropipette was introduced into the cytoplasm of the one-cell stage of fertilized embryos and by using a foot-paddle the desired volume of DNA construct was injected. Following injection, the embryos were transferred into a clean dish and incubated at 28.5ºC in the incubator.

3.5 Raising embryos to adulthood

About 20-30 embryos per petri dish with about 30 ml of E3 were kept in the incubator at 28.5ºC. The embryos were screened daily for 5 days. During screening, dead embryos were removed and the E3 changed. On the fifth day, larvae were moved into larva tanks with about 2cm of E3 and were fed live paramecium cultures twice daily. From 10 days post fertilization (dpf) onwards, live artemia were fed twice daily. At approximately 21 dpf, the fish were transferred to the main system and fed small pellets and live artemia twice daily.

3.6 Euthanasia

A stoke solution of 4% Tricaine (3-amino benzoic acid ethyl ester also called ethyl 3-aminobenzoate) solution was made by dissolving 400 mg tricaine powder in 97.9 ml of deuterium-depleted water and ~2.1 ml 1 M Tris (pH 9) and pH of tricaine was adjusted to 7 (Westerfield, 1994) . Euthanasia of the zebrafish embryos was done by overdosing with tricaine (0.2-0.3 mg/ml). Fish were left in the tricaine for at least 10 minutes following cessation of opercular movement.

3.7 Chorion removal

Embryos were dechorionated manually using a pair of Dumont #5 watchmaker forceps (Sigma-Aldrich) if only a few embryos were required. For this, with a pair of forceps a gentle tear was made in the chorion and then the chorion was turned upside down so that the embryos could fall out. Special care was taken when removing the chorion, so that no damage was caused to embryos. On the other hand, if a large quantity of embryos were required, the embryos were first treated in pronase (2mg/ml in E3) for 1 minute at 28.5ºC to make the chorions brittle and easy to remove. Then, embryos were rinsed 3 times in E3 to remove all the pronase. The embryos usually fall out of their chorions with gentle swishing during the rinse.

3.8 Mounting and removing live zebrafish in 1% agarose

To use tricaine as the anesthetic, 4.2ml of 4% tricaine was added in 100ml of E3, to make a final tricaine concentration of 0.168mg/ml. 1% Agar for mounting was made by adding 1g of low-melting-point agarose (Sigma-Aldrich) in 100ml of deionized water. The solution was then microwaved until the agarose was dissolved in water. The agarose was put in the warm water bath (35-40ºC) to cool down but not harden. Once, the agarose had settled down, tricaine was added.

Firstly, zebrafish were anesthetized in 0.168mg/ml of tricaine. Once the zebrafish had stopped there movement, the zebrafish were transferred into glass-bottom petri dish or glass slide, as required by the procedure, with as little solution as possible. A few drops of warm agarose, the temperature about 35-40ºC, was poured on top of the zebrafish until it was completely submersed in agarose. If the agar is too hot, it can kill the fish, so only warm agarose was used. The agarose was allowed to cool down and solidify for about a minute to avoid embryos shifting their positions while manipulating the dish later on. Once agarose was solidified, a few drops of anesthetic tricaine were added on top of the agarose to keep it moist.

To remove the zebrafish from the agarose, several drops of E3 were added on top of the agarose. First, with a pointed scalpel, a “V” shaped cut was made in the agar with the point of the “V” aiming towards the embryo’s head. Then, a tip of the closed sharp forceps was plunged into the agar between the point of the “V” cut and the head of the embryo. Then, the closed forceps were gently opened to make a crack in the agar, which runs along the length of the zebrafish. Finally, the zebrafish will float out of the agarose into the E3.

3.9 Construction of membrane targeted KillerRed (mkillerRed) plasmid

3.9.1 Restriction enzyme digests

The construct containing the pKillerRed membrane vector was purchased from Evrogen (Waterloo, Australia). The pKillerRed member vector form Evrogen had cytomegalovirus (CMV) promoter. But, we planned to use Hb9 and Ubiquitin promoter.

Therefore, the membrane and KillerRed sequence were cut out of the pdoner KillerRed-membrane vector from Evrogen, at the same time in-house middle entry vector (PTNOR-22) was also cut to make room for membrane-KillerRed to be inserted. Digests were done using restriction enzymes from NEB. In short, BamHI and Not1 restricted enzyme (0.5 unit each) were used to cut out membrane and KillerRed sequence from the pdoner KillerRed-mem vector (5µg). And BamHi and EcoRI restricted enzymes (0.5 unit each) were used to cut PTNOR-22 (3.5µg). The DNA fragments were digested with restricted enzymes for 3 hours at 37ºC.

The pKillerRed membrane vector and in-house middle entry vector (PTNOR-22) map is as follows.

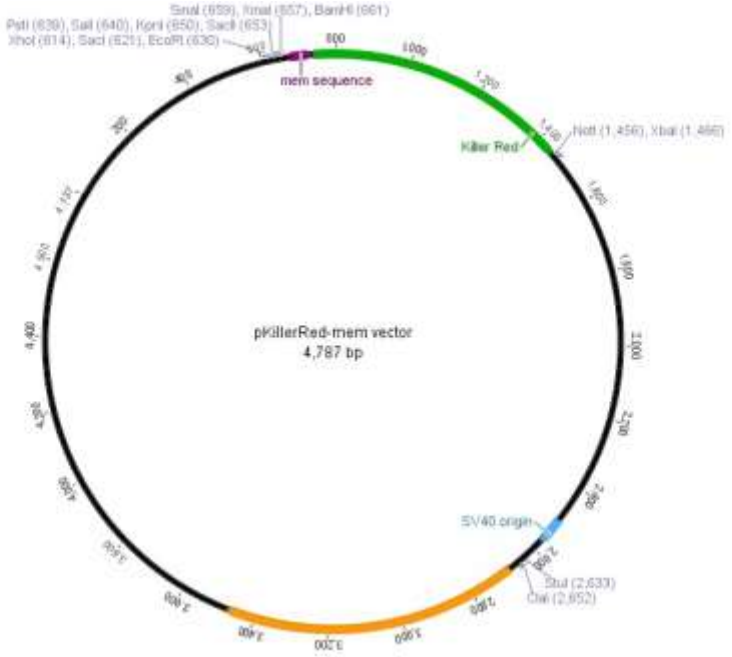

Figure 2.1: pKillerRed membrane vector map.

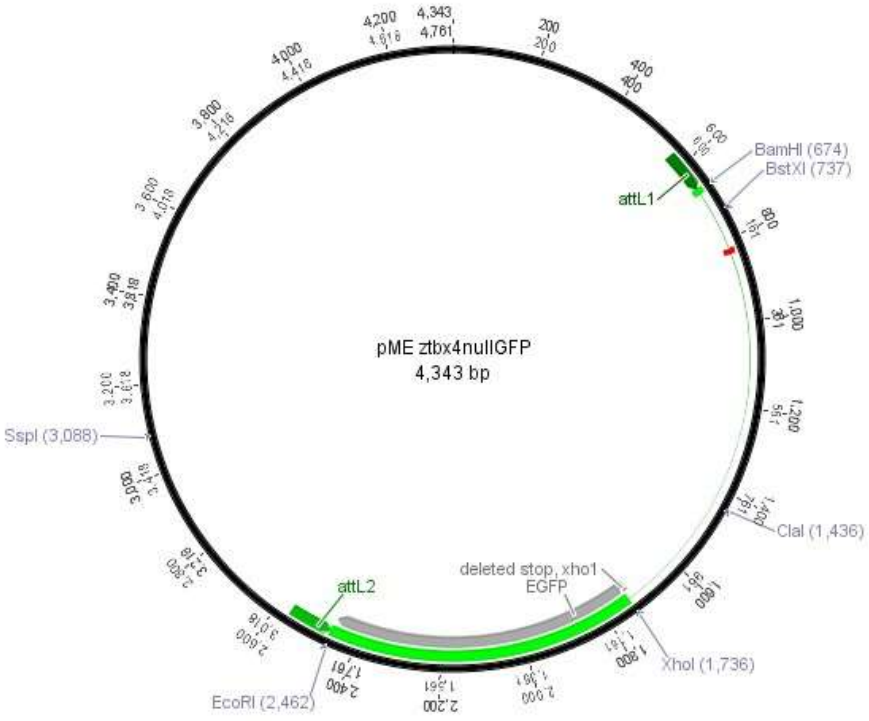

Figure 2.2: In-house middle entry vector (PTNOR-22) map

3.9.2 Klenow blunting

While the plasmid vector was cut with different enzymes and did not produce blunt ends, a klenow blunting protocol was employed. The klenow blunting of sticky-ended DNA fragments was performed with 43.5µl of the DNA template, 1µl of klenow (NEB) and 0.5µl of dNTP. Reactions were incubated at 25ºC for 15 minutes for blunting, then incubated to 75ºC for 20 minutes for heat inactivation and finally for 5 minutes at 4ºC.

3.9.3 Dephosphorylation Vector

Dephosphorylation of the linearized vectors was performed to avoid self-ligation during subsequent ligation reactions. 2 µl of Antarctic phospatase (NEB) was utilised to dephosphorylate approximately 5 µg of vector template in 6µl 10x Antarctic phospatase buffer (NEB). Reactions were incubated at 37ºC for 30 minutes for dephosphorylate and then 65ºC for 5 minute for heat inactivation.

3.9.4 Phosphorylation of insert

For ligation to occur, at least one of the DNA ends (insert or vector) should contain a 5' phosphate. As the vector has already been dephosphorylated, phosphorylation of the insert was done so that the insert will be able to bind with the vector.

Phosphorylation of the insert was done by mixing 75ng of insert with 0.5µl polynucleotide kinase (NEB) and 1µl of ligation buffer (NEB). The reaction was incubated for 37ºC for 20 minutes for phosphorylation of the insert, then incubated at 65ºC for 10 minutes for heat inactivation and finally 4ºC for 2 minutes.

3.9.5 Ligations

The amount of insert needed was determined according the following formula, with an insert vector molar ration of 3:1

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb of insert}}{\text{kb of vector}} \times \text{insert/vector molar ratio}$$

Therefore, the required phosphorylated insert and de-phosphorylated vector were mixed with 0.5ul DNA ligase (NEB) in ligation buffer (NEB). The solution was then incubated overnight at 16◦C.

3.9.6 Multisite Gateway cloning

The MultiSite Gateway® LR recombination reaction was performed according to the manufacturer's instructions using 60 ng of each vector and 2 µl of LR clonase II plus. Reactions were allowed to incubate for 24 hours at 25ºC. Reactions were stopped at 10 minutes with Protinase K (Invitrogen™, Life Technologies™) digestion at 37ºC before proceeding to transformation.

3.9.7 Transformation of vector into bacteria

Plasmids were transformed into competent bacteria using OneShot® Top10 chemically competent *E.coli* (Invitrogen™, Life Technologies™). In 25µl of chemical competent cells, 2µl of plasmid DNA was added. Initially, the reaction was first incubated on ice for 5 minutes, then heat-shocked at 42ºC for 30 seconds and immediately returned to

ice. A 250 µl S.O.C. medium at room temperature was added to the cells which were then incubated for 1 hour at 37ºC at 200 rpm in a shaking incubator. After incubation, 50 µl of plasmid DNA cells were spread over the surface of an LB agar plate with an antibiotic. Plates were inverted and incubated at 37ºC overnight.

3.9.8 Mini Preparation

Mini preparation was done to isolate the plasmid from DNA using a Bioline mini prep kit. Any growth of an E-coli colony was looked for in all the plates. A few well grown colonies were selected, marked, and numbered for mini preparation. While selecting the colony, the colonies where there was mixing and joining of more than 2 colonies were not selected. Using the fine pipette tip, the marked colony of E-coli was gently touched and the pipette tip was dropped into the falcon tube which had LB-broth with kanamycin. Falcon tubes were shaken at 37◦c at 200 rpm overnight. The next day, 1.5ml of a saturated E-coli LB culture from the falcon tube was centrifuged for 30 seconds at 11,000 g. Then the supernatant was discarded and cells were subsequently lysed by treating with the buffer P1, buffer P2 and buffer P3. The lysate was clarified by centrifuging for 5 minutes at 11,000 x g at room temperature. The silica membrane was washed with buffer PW2 and dried by centrifuging for 2 minutes at 11,000 x g. Finally, the DNA was eluted with mili Q water, after incubating for 1 minute at room temperature and centrifuging for 1 minute at 11,000 X g.

3.10 Nano Drop DNA.

To measure the DNA concentration, a nano-drop was done by using the Thermo scientific Nano-Drop spectrophotometers following the manufacturer's protocol.

3.11 Linearization of the plasmid

Digests were done using Ssp1 restriction enzymes from NEB. To linearize 500ng of DNA, we used ssp1 enzyme x 0.5, buffer x 10, BSA x 1000 times and finally added RNAse-free water to make the final concentration of solution of 20ml. The solution was incubated for 1 hour at room temperature.

3.12 Agarose gel electrophoresis

Agarose gels were prepared by dissolving 1% of agarose in 50ml of TBE buffer. The solution was heated in the microwave until all the agarose gel was dissolved. The solution was then allowed to cool prior to the addition of 5ul of SYBR green which binds and detects nucleic acids. The solution was allowed to set in a Bio-Rad gel apparatus with a comb. Typically, the Bioline Easy Ladder II was used to analyse the

size, concentration, and quality of bands. Before loading the DNA for electrophoresis, we added an orange loading buffer (5ul) in the samples.
Following the loading of product to be analysed, the gel was run at 120V for approximately 30 minutes from a black colour to red. To make sure that the gel was running, we checked to see if the bubbles were coming from the black coloured side.

3.12 Sequencing

The sequencing of DNA was sent to Australian Genome Research Facility Ltd.

3.13 Generation of Transposase mRNA

The transposase messenger RNA (mRNA) was generated from the PCS2-vector. Approximately 5 µg of the plasmid was linearized by the digestion of 0.5µl NotI restriction enzyme and incubating for 2 hours at 37ºC. To verify that the plasmid has been linearized, a gel extraction was done. Once verified, the linearized plasmid template was purified with the Qiagen Gel Purification Kit following the manufacturer's instructions. The linearized plasmid template was eluted twice into 30 µl milliQ water and nano-drop was done to determine concentration. Transposase mRNA was generated using a mMessage machine SP6 kit (Ambion®, Life Technologies™). Approximately 1 µg of linear plasmid template was utilised in the reaction which was assembled according to the manufacturer's instructions. The reaction was incubated for two hours at 37ºC. The Transposase mRNA was then run through gel electrophoresis to make sure that mRNA has not been degraded. The mRNA was then purified using an RNeasy mini kit (Qiagen) following the manufacturer's instructions for RNA purification. The transposase mRNA was stored at -80ºC until needed.

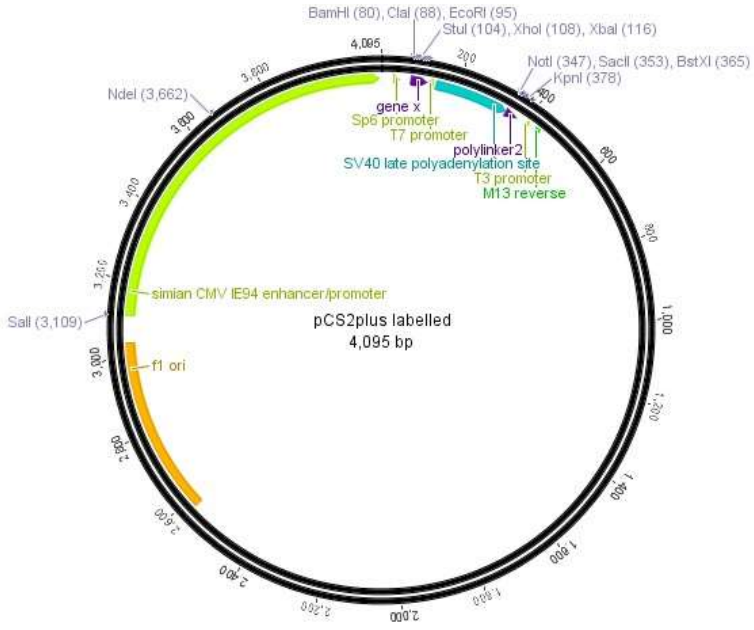

Figure 2.3: The vector map of PCS2-vector

3.14 Purification of DNA construct

DNA products were purified by column with a QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions with slight modifications. Five times the reaction volume of buffer QG was added to the DNA construct and incubated at 50ºC for 10 minutes. One time the DNA construction volume of isopropanol was added to the above solutiosn and centrifuged for 1 minute. After discarding the flow-through, 0.5ml of buffer QG was added, centrifuged, and the flow-through discarded. Then, 0.75 ml of buffer PE was added, centrifuged, and the flow-through discarded. Finally, DNA was eluted by washing it twice with MilliQ water. To measure the DNA concentration a nanodrop was done.

3.15 Sequence analysis and alignments

Geneious® 6.0.3 software (Biomatters) was utilised to perform sequence analysis and alignments.

3.16 Light illumination

Embryos at the one cell stage were injected with tol2 plasmid (either mKillerRed or mCherry -CAAX). The next day, positive embryos were screened under fluorescent microscopy (Leica M165FC) using a mCherry filter. At 30 hours post fertilization (hpf),

one positive embryo per glass bottom petri dish (35mm diameter, 0 coverslip thickness, and 20mm microwell diameter; Mat Tek corporation, USA) was mounted with a warm 1% low melt agarose (Sigma-Aldrich). Once the agarose was solidified, 2 ml of egg water was added on top of the agarose to avoid dryness during light irritation. A mercury lamp was pre-heated at least 15 minutes before stating the experiment. The desired area of the embryo was first brought under focus with X 40 objective lens and then light irritation was done under a Leica DM 3000B inverted microscope with full light intensity. The duration of illumination depended on the experiment. For example, to detect the reactive oxygen species (ROS) embryos were illuminated until complete photo bleaching of KillerRed occurred, which usually tokes 5-10 minutes. Whereas, to detect the apoptosis, illumination was done until double the time it needed for complete photo bleaching of KillerRed, which usually takes 10-20 minutes.

3.17 Reactive oxygen species detection

ROS was detected using 5-(and-6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Life technologies, US).

Larvae were treated with 5µM CM-H2DCFDA for 60 minutes in the dark and were washed for 15 minutes in 5ml of E3 3 times. Then, the embryos were anesthetized with tricaine, mounted in a 1% low melt agarose and fluorescence was visualized on a Leica M165 FC fluorescence dissecting stereomicroscope (Leica) under GFP filter.

3.18 Hydrogen peroxide (H_2O_2) exposure to assessed ROS production

Due to the high reactive nature of H_2O_2, exposure was carried out in three different times. The zebrafish larvae were exposed to 30 ml of H_2O_2 in E3 water at 0 mM, 1mM, 2 mM, 2.5mM, 3.0 mM, 3.5mM, 4mM and 5mM concentrations of H_2O_2 for 24 hours starting from 72 hpf. The toxicity was assessed by mortality and phenotype abnormality. The ROS was detect with CM-H2DCFDA (Life Technologies, US) on live zebrafish embryos only. The viability at 24 hours was scored and plotted to form an average of 3 clutches (n=30 larvae per treatment, mean= s.e.m).

3.19 Acridine orange

Acridine orange (acridinium chloride hemi-[zinc chloride], Sigma-Aldrich) straining was done to detect apoptosis. First, optimization for the amount and time needed by acridine orange staining to detect neuron death was done. For this, 48hpf zebrafish larvae were anesthetizde, mounted in 1% low melt agarose, and picked with a 24-gauge needle on the lateral-plate/ segmental plate at the level of urogenital opening. After removing embryos from the agarose and recovering for 15 minutes, the live zebrafish larvae were stained in the dark with acridine orange at 0.5µg/ml, 2.5µg/ml, and 5µg/ml for 5 minutes, 15 minutes, and 30 minutes for each concentration. After staining, the

larvae were washed for 5 minutes in 5ml of E3 3 times, and for 30 minutes in 20ml of E3. Then, the embryos were anesthetized with tricaine, mounted in 1% low melt agarose, and fluorescence was visualized on a Leica M165 FC fluorescence dissecting stereomicroscope.

Once optimized, for the remainder of the experiments, the zebrafish were stained with 2.5µg/ml acridine orange for 30 minutes. The larvae were then washed for 5 minutes in 3ml of E3 3 times, and for 30 minutes in 20ml of E3.

3.20 TUNEL assay

FIXATION

All zebrafish were euthanized using tricaine (0.2-0.3 mg/ml, Sigma-Aldrich), and were then washed twice in phosphate buffered saline with 0.01% tween-20 (PBST; Amresco) for 5minutes each. Samples were fixed overnight in 4% paraformaldehyde (PFA, Amresco) in PBS at room temperature.

DEHYDRATION

After fixation, the embryos were washed 3 times in PBST for 5 minutes each time and dehydrated by a methanol (Amresco) series.

1) In 25:75 MEOH/PBS for 5 minutes at RT.

2) In 50-50 MEOH/PBS for 5 minutes at RT.

3) In 75-25 MEOH/PBS for 5 minutes at RT.

4) In 100% MEOH for 5 minutes at RT.

5) replaced with fresh 100% methanol after 5 minutes and kept in the freezer overnight or until needed.

REHYDRATION

The next day, samples were rehydrated into PBS through a methanol: PBS series.

1) In 75:25 MEOH/PBS for 5 minutes at RT
2) In 50:50 MEOH/PBS for 5 minutes at RT
3) In 25:75 MEOH/PBS for 5 minutes at RT
4) In 5: 95 MEOH/PBS for 5 minutes at RT
5) 2 x 5 minutes in PBST.

PERMEABILIZATION

Permeabilization was done by incubating in proteinase K diluted in PBST at room temperature with continuous rocking. The duration and the amount of Proteinase K of permeabilization depended on the age of the zebrafish.

a) 16 hpf - 1 minute with 1µg/ml of Proteinase K in PBST.
b) 24 hpf -2 minutes with 1µg/ml of Proteinase K in PBST.
c) 36 hpf – 10 minutes with 1µg/ml of Proteinase K in PBST.
d) 48 hpf – 15 minutes with 2µg/ml of Proteinase K in PBST.
e) 72 hpf – 20 minutes with 3µg/ml of Proteinase K in PBST.
f) 96 hpf or adult tissue – 30 minutes with 4µg/ml of Proteinase K in PBST.

After permeabilization, the sample was washed twice in PBST for 7 minutes each time, fixed with 4% paraformaldehyde in PBS for 15 minutes, and again washed twice in PBST for 5 minutes each time.

STAINING IN TUNEL REACTION

The preparation of TUNEL reaction (In Situ Cell Death Detection Kit, POD; Roche, Indiananapolis, IN) was done according to the manufacturer's protocol on ice. We added 50µL of Enzyme solution (vial 1) to the 450µL labeled solution in vial 2 to obtain 500µL of TUNEL reaction mixture. About 125µl of TUNEL enzyme was added per eppendorf tube which contains about 4-6 embryos and incubated overnight at room temperature. The next day, the embryos were rinsed 3 times in PBST for 5 minute each time. The sample was analysed under fluorescence microscopy at about 500 excitation spectrum (GFP filter).

3.21 Microscopy

Light microscopy
The images were taken on a Leica M165 FC fluorescence dissecting stereomicroscope (Leica) using a ProgRes CJ cool camera (Jenoptic).

Inverted microscopy
The image and light irritation was taken on Leica DM 3000B inverted microscope (leica) using a mercury lamp and ProgRes CJ cool camera (Jenoptic).

4.0 RESULTS

4.1 Verification of membrane targeted KillerRed (mkillerRed) plasmid.

To test the efficiency of KillerRed as a photosensitizer we replaced the CMV promoter from then pKillerRed-membrane vector from Evrogen with either ubiquitin or HB9 promotor. For this, we cut the membrane and KillerRed sequence out of pKillerRed-membrane vector from Evrogen, and incorporated into our in-house middle entry vector (PTNOR-22). To verify that the membrane-KillerRed sequence had been incorporated into middle entry vector, first we measured the concentration of the DNA in each tube we got from mini prep by Nano drop (Table 4.1). Then, the DNA from all the tubes was run through gel electrophoresis. The result of our gel electrophoresis shows that DNA of sample A2, A3, A5, A6 and A8 was longer than the DNA of our control, middle entry vector (PTNOR-22) (Figure 4.1). This suggests that the middle entry vector in the tubes A2, A3, A5, A6 and A8 had incorporated membrane-KillerRed sequence. To, verify that the membrane-KillerRed sequence had been incorporated in right order, samples A2, A3, A5, A6 and A8 were sent for sequencing at the Australian Genome Research Facility Ltd. The results of the sequencing from all 5 tubes were analysed using Geneious® 6.0.3 software (Biomatters) in order to determine if the membrane-KillerRed sequence is incorporated in correct sequence and order. Of those 5 samples sent for sequence, samples A6 and A8 were found to be in the correct order and were used for making our DNA plasmid.

Tube	DNA concentration
A1	55ng/ul
A2	326 ng/ul
A3	220 ng/ul
A4	40ng/ul
A5	357 ng/ul
A6	352 ng/ul
A7	57 ng/ul
A8	299 ng/ul

Table 4.1: DNA concentration form Nanodrop**.**

Figure 4.1 Gel electrophoresis after mini prep for middle entry vector. (C) Control, (A1-A8) middle entry vector. Sample A2, A3, A5, A6 and A8 is longer than control plasmid.

4.2 Generation of Transposase mRNA

Transposase messenger RNA (mRNA) was generated from the PCS2-vector (Figure 2.3 in section 2.13). First, the vector was linearized by 0.5µl NotI restriction enzyme. The linearization of the vector was confirmed by gel extraction (Figure 4.2). Once the linearization was confirmed, a nano-drop showed that concentration of DNA was 100µg/µl. Finally, transposase mRNA was generated using a mMessage machine SP6 kit (Ambion®, Life Technologies™). After transposase mRNA was generated, the gel extraction showed that the final mRNA was not degraded (Figure 4.3). The final concentration for mRNA by nano-drop was 2000µg/µl. The transpose mRNA was then diluted to the concentration of 160µg/ul and kept at -80 c until further use.

Figure 4.2- Gel electrophoresis to conform linearization of vector. (A) Uncut vector and (B) Linearized plasmid.

Figure 4.3 - Gel electrophoresis conform that mRNA has not been degraded.

4.3 Optimization of Acridine orange dose to assessed apoptosis

In order to optimize the dose and time needed for acridine orange to stain dead cells in zebrafish, the tissues of zebrafish were subjected to death by picking the zebrafish larvae at 48hpf with a needle at the lateral-plate/ segmental plate at the dorsal to the level of urogenital opening (figure 4.4).The acridine orange was able to stain dead cells induced by picking with any of the three different doses (0.5µg/ml, 2.5µg/ml, and 5µg/ml) and with any of the three different times (5 minutes, 15 minutes, and 30 minutes) (figure 4.5- figure 4.7). This is because, picking will create a superficial wound which will allow acridine orange to easily penetrate through the wound and detect dead cells around the wound site.

By using above the method, we were not able to know dose and timing needed to stain the deep tissues such as neurons and muscles, natural apoptosis present at 24 hpf embryos were analysed, to verify the dose and timing needed for acridine orange staining. We were especially analysing the ability of the acridine orange to stain natural apoptosis present in the eyes and Rohon-Beard neurons in 24 hpf embryos (Cole and Ross, 2001). Staining with 0.5µg/ml of acridine orange for just 5 minutes at first seems to stain the natural apoptosis of both the Rohon-Beard neurons and eyes (figure 4.8; A, D and G, white arrow head). But, as the staining time of acridine orange was increased to 15 minutes, the natural apoptosis at Rohon-Beard neurons was not stained. In contrast, the amount of apoptosis cells detected after staining for 15 minutes in the eyes were increased compared to that of 5 minutes of staining (figure 4.8; B, E and H, white arrow head). Then, when the staining was done for 30 minutes, apoptosis in Rohon-Beard neurons again re-appeared and apoptosis cells in the eyes were most compared to other staining times (figure 4.8; C, F and I, white arrow head). This observation suggested that apoptosis detected along the segmental plate at the 5 minute stain were superficial cells and not the deep tissue cells including Rohon-Beard neurons. As, rapid turnover of death cells usually happen in epithelia cells including in the skin, this further verified that the staining observed by a low amount of time was superficial cells rather that deep seated tissue, such as neuron and muscles.

Similar observations were found when embryos were stained with higher doses, of 2.5µg/ml and 5µg/ml, for different time periods (figure 4.9 and figure 4.10). However, staining with the concentration of 2.5µg/ml and 5µg/ml of acridine orange for 30 minutes was able to stain more natural apoptosis of the both Rohon-Beard neurons and eyes compared to 0.5µg/ml stain for 30 minutes. Although with 5µg/ml, we able to stain natural apoptosis of the both Rohon-Beard neurons and eyes, to avoid toxicity, high back ground and non-specific staining usually found with the acridine orange staining (Rodriguez and Driever, 1997), we decided to use 2. 5µg/ml for 30 minutes for staining apoptosis in the neuron and deep seated muscle during the rest of our experiment (figure 4.11).

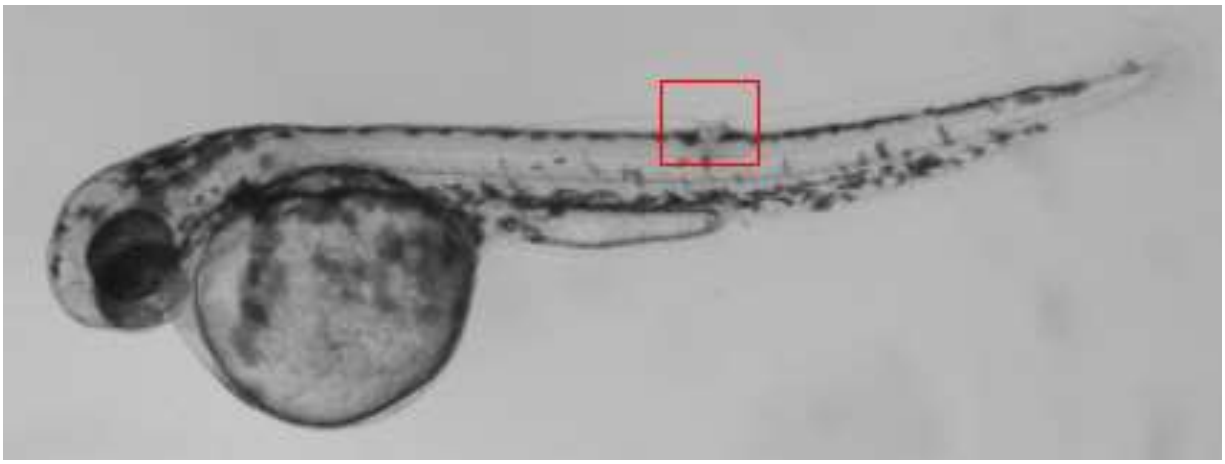

Figure 4.4: Image of zebrafish after picking with the needle. Red square was the position of the embryos where picking with the needle was done to induced cell death

0.5ul/mg of Acridine orange

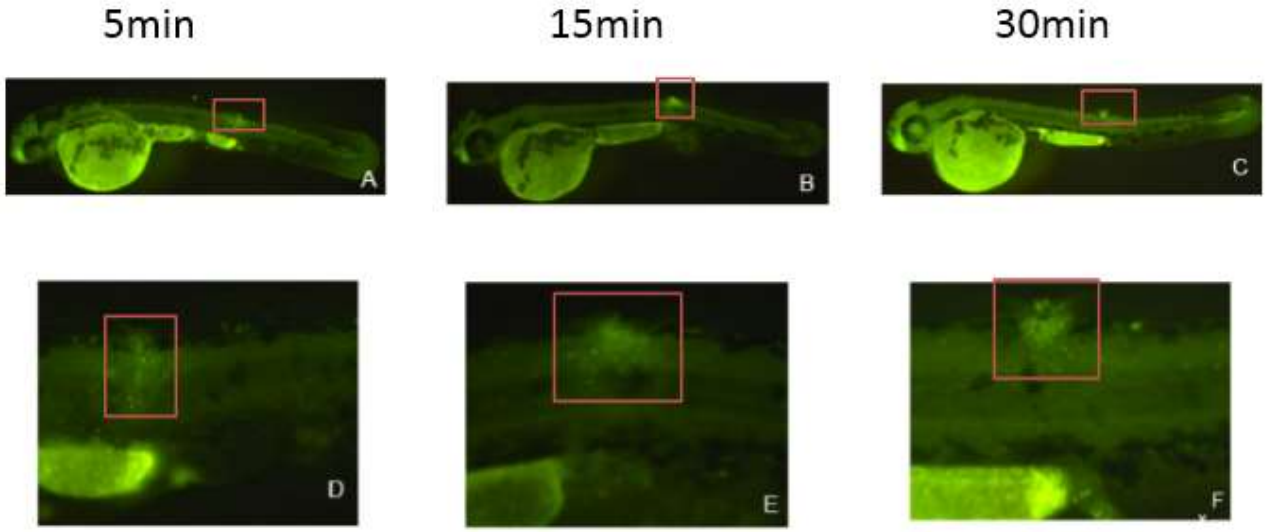

Figure 4.5- Acridine orange staining after picking with 0.5µg/ml concentration for different times; for 5 minute-(A) whole view and (D) higher magnification view of same embryo; for 15 minute- (B) whole view and (E) higher magnification in view of same embryo; and for 30 minute-(C) whole view and (F) higher magnification view of same embryo. Dead cells detected by acridine orange after picking are under red square.

2.5ul/mg of Acridine orange

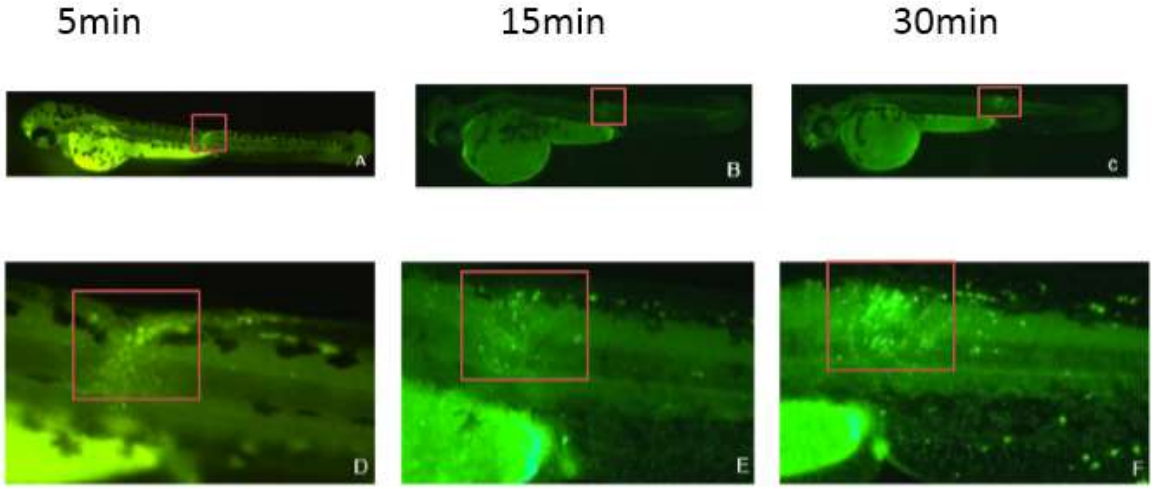

Figure 4.6- Acridine orange staining after picking with 2.5µg/ml concentration for different times; for 5 minute-(A) whole view and (D) higher magnification view of same embryo; for 15 minute- (B) whole view and (E) higher magnification view of same embryo; and for 30 minute-(C) whole view and (F) higher magnification view of same embryo. Dead cells detected by acridine orange after picking are under red square.

5.0ul/mg of Acridine orange

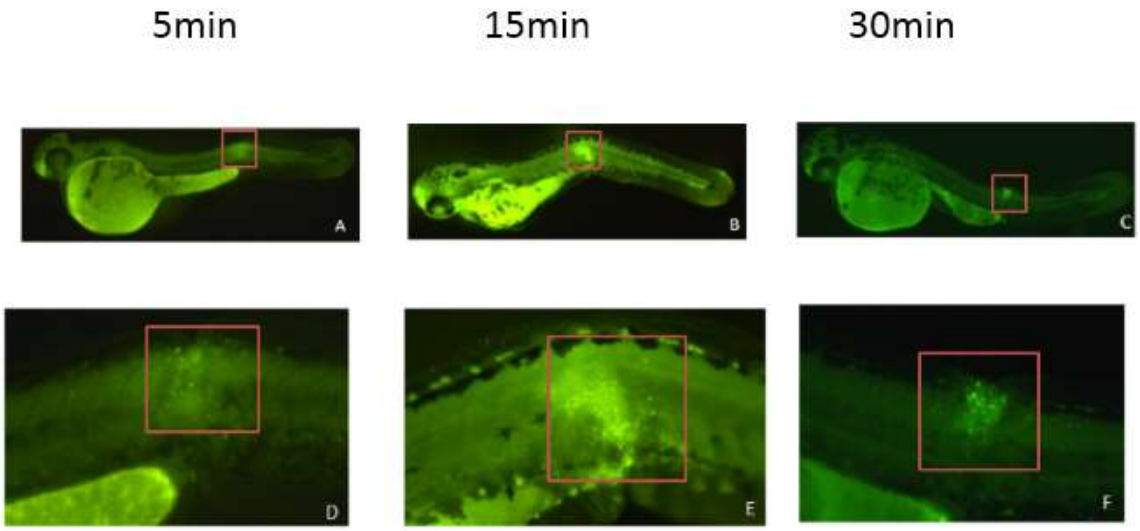

Figure 4.7- Acridine orange staining after picking with 5µg/ml concentration for different times; for 5 minute-(A) whole view and (D) higher magnification view of same embryo, for 15 minute- (B) whole view and (E) higher magnification view of same embryo, and for 30 minute-(C) whole view and (F) higher magnification view of same embryo. Dead cells detected by acridine orange after picking are under red square

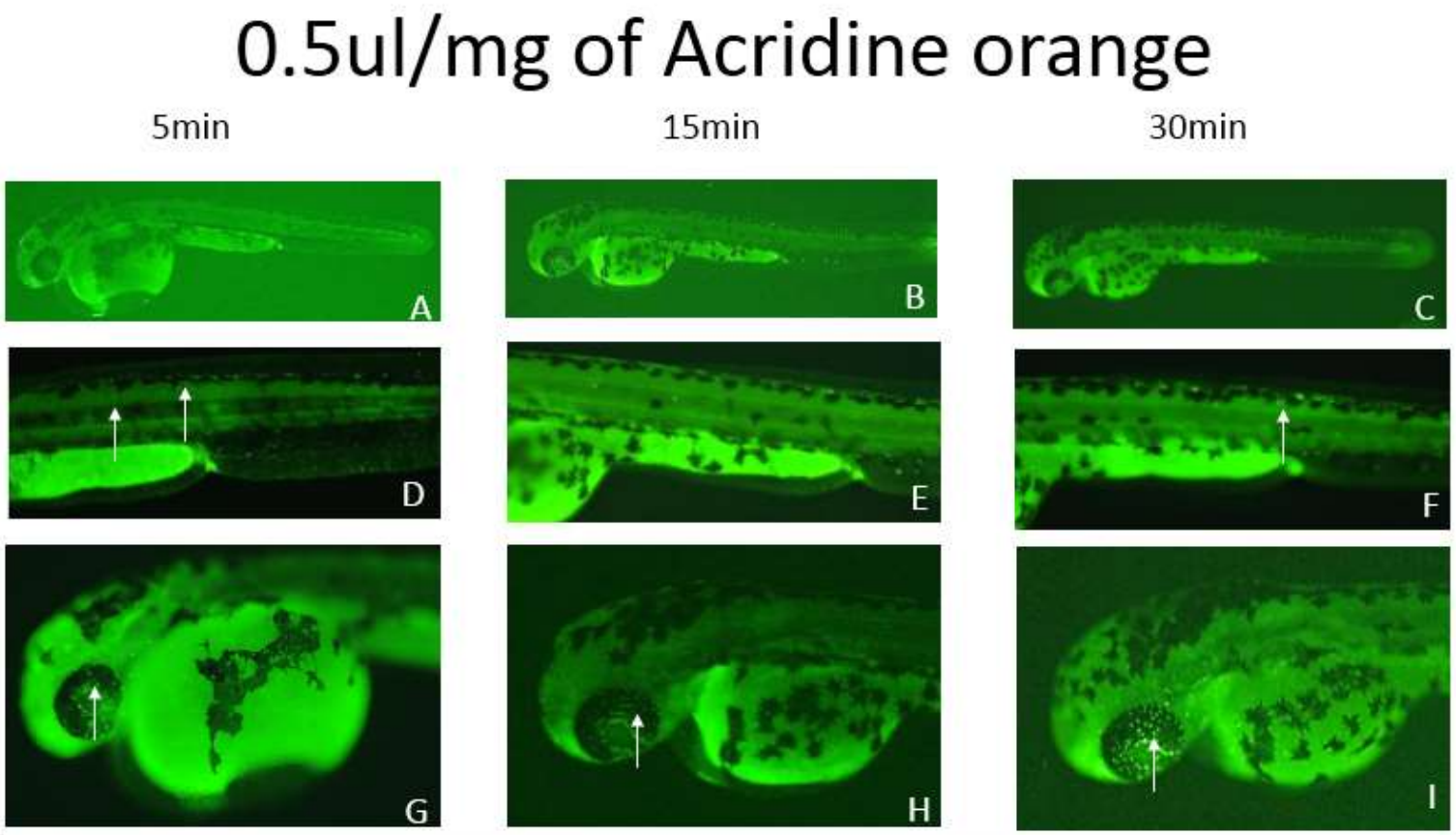

Figure 4.8- Acridine orange staining with 0.5µg/ml concentration for different times looking for natural apoptosis; for 5 minute-(A) whole view of the embryo and (D, G) higher magnification view; for 15 minute- (B) whole view and (E, F) higher magnification view; and for 30 minute-(C) whole view and (F, I) higher magnification view. White arrow head apoptosis cells.

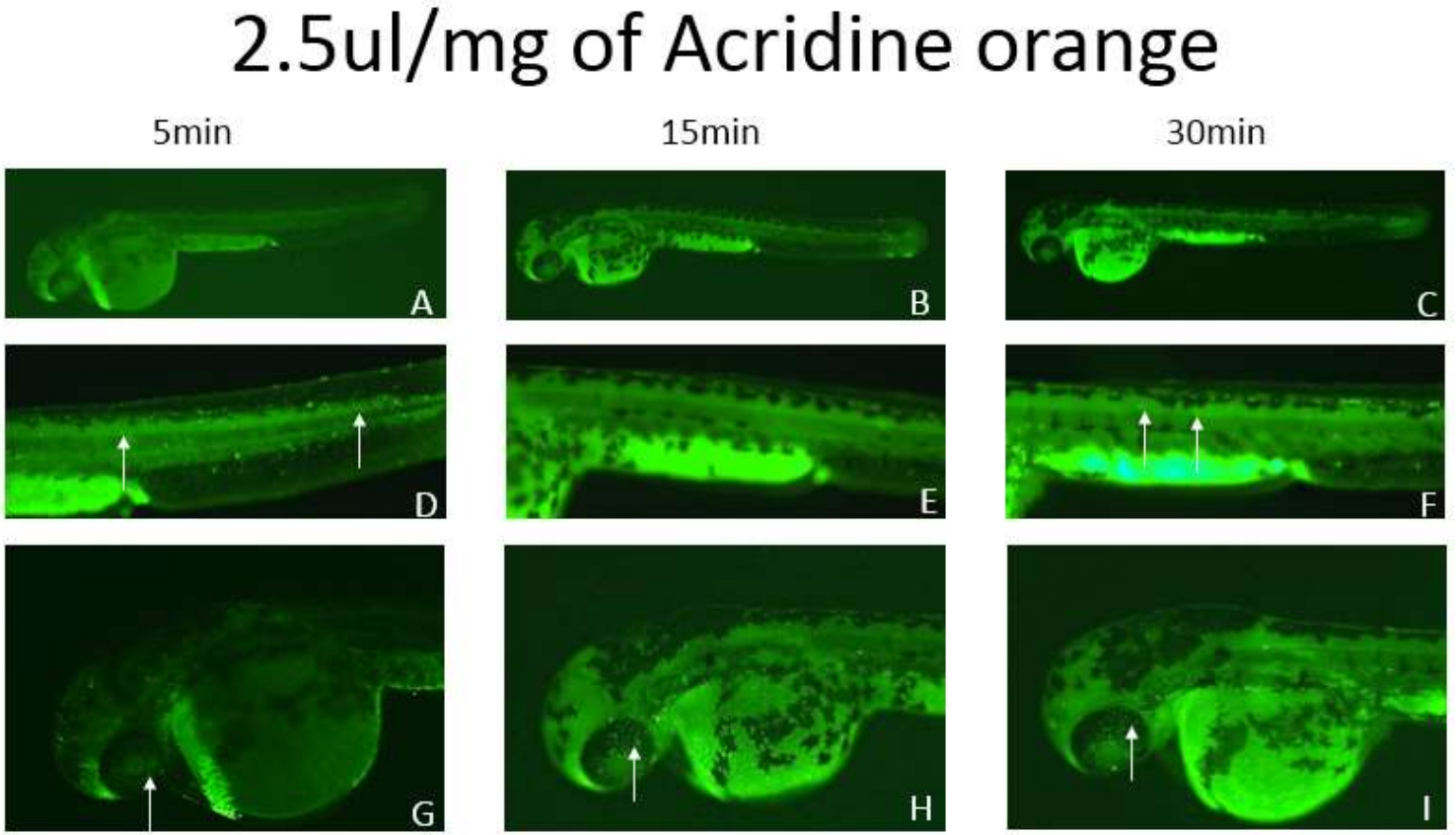

Figure 4.9- Acridine orange staining with 2.5µg/ml concentration for different times looking for natural apoptosis; for 5 minute-(A) whole view of the embryo and (D, G) higher magnification; for 15 minute- (B) whole view and (E, F) higher magnification view; and for 30 minute-(C) whole view and (F, I) higher magnification view. White arrow head apoptotic cells .

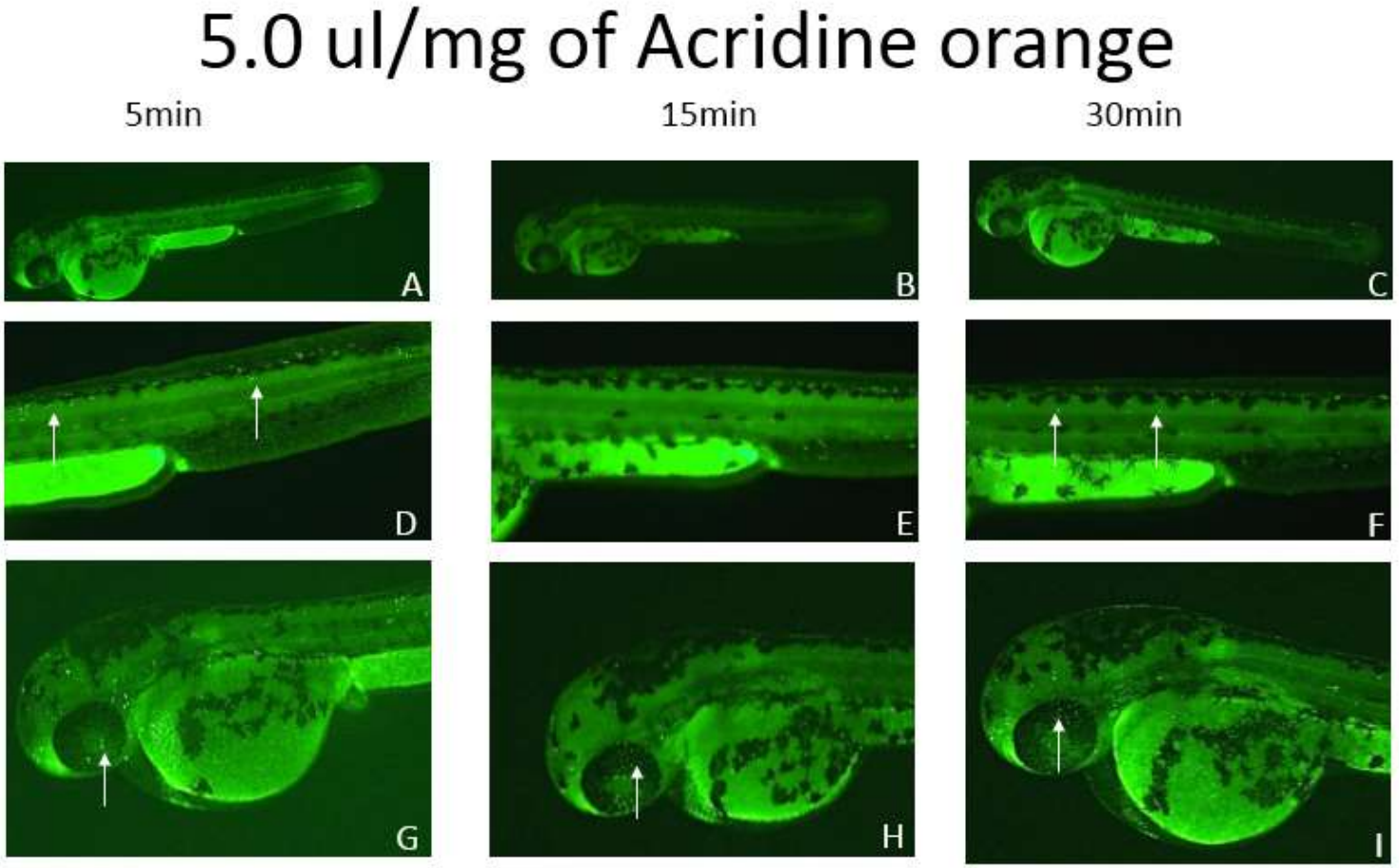

Figure 4.10- Acridine orange staining with 5µg/ml concentration for different times; for 5 minute-(A) whole view of the embryo and (D, G) higher magnification view , for 15 minute- (B) whole view and (E, F) higher magnification in view; and for 30 minute-(C) whole view and (F, I) higher magnification view. White arrow head apoptosis cells.

Figure 4.11- Acridine orange staining with 2.5µg/ml concentration for 30 minutes. Arrow head apoptosis bodies in the eye and Rohon-Beard neurons.

4.4 Hydrogen peroxide (H_2O_2) exposure to assessed ROS production

The toxicity of H_2O_2 was analysed by exposing the zebrafish to different doses of H_2O_2. In our experiment, 2.0mM was the lowest dose that introduced mortality. Whereas, no zebrafish survived at and above 4.0mM (figure 4.12). Interestingly, none of the survivors on any dose exhibited any phenotypic abnormalities. Initially, the larvae at doses higher than 2.5 mm of H_2O_2 after being removed from H_2O_2 and rising in E3, maintained their motionless position for a few minutes before resuming normal swimming behaviours. The generation of ROS of the whole larvae was detected in 1mM, 2 mM, 2.5 mM, 3.0 mM, 3.5 mM by CM-H2DCFDA, with the higher the dose of H_2O_2, the presence of more ROS was detected (Figure 4.13a and Figure 4.13b). In our study, in zebrafish exposed to 1mM and 2 mM of H_2O_2 presence of ROS was detected only in the gill area. As increasing doses of H_2O_2 presence of ROS became more prominent in the gill area, some ROS was also noted in other regions of the head. Even with the highest tolerable dose of H2O2, ROS were not detected in other parts of the body, except for the head region.

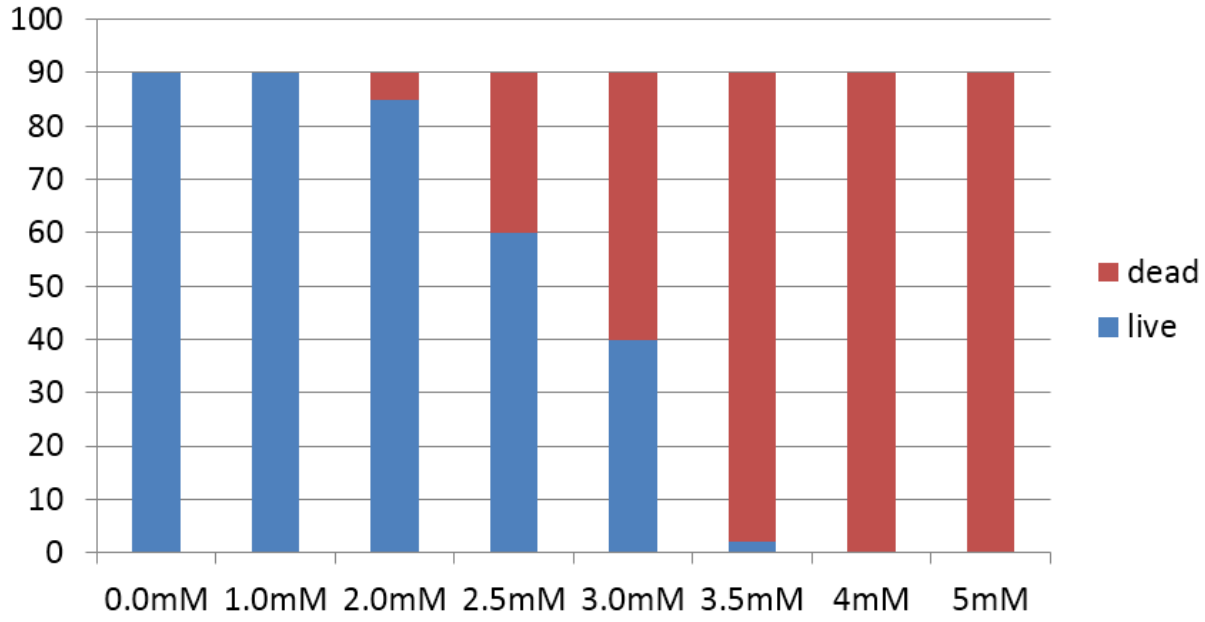

Figure 4.12 – Hydrogen peroxide survival analysis after exposing larvae to hydrogen peroxide to the concentration of 0mM , 1.0mM, 2.0mm, 2.5mM, 3.0mM, 4.0mM and 5.0mm for 24 hours**.**

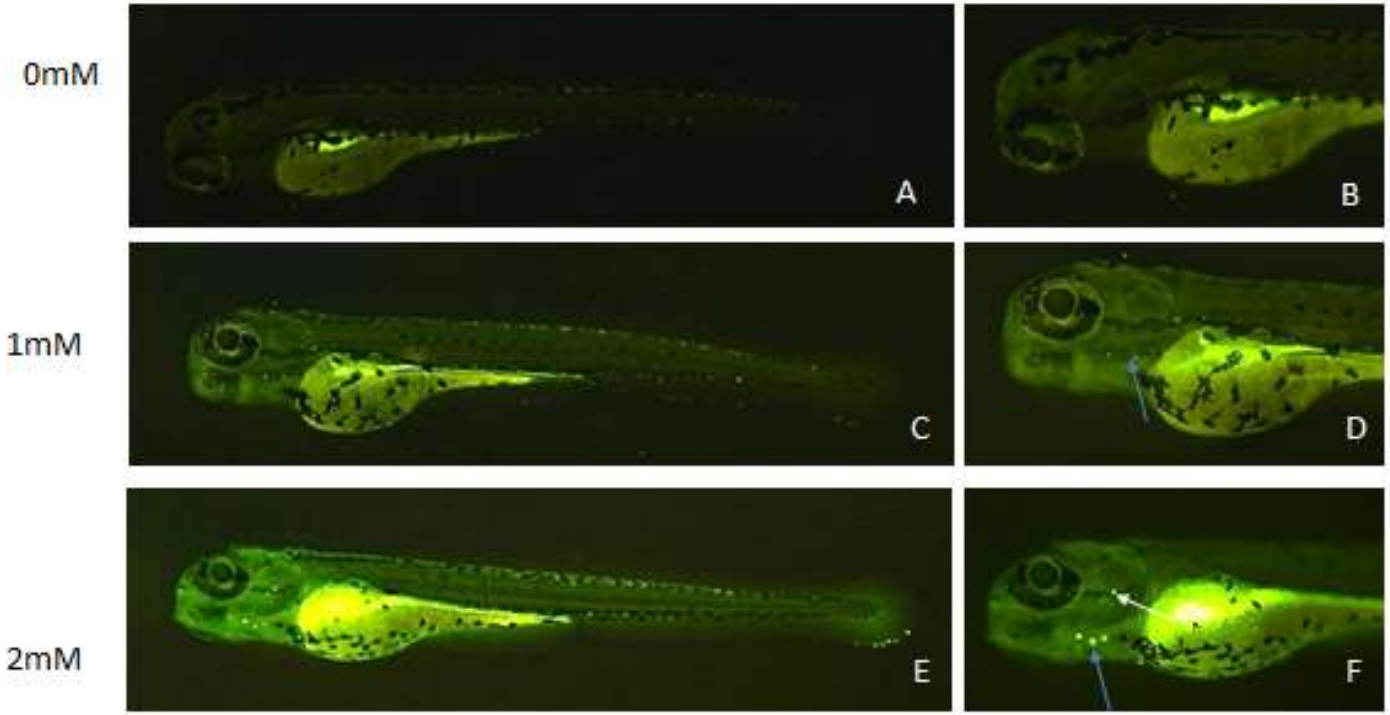

Figure 4.13a- Image of ROS detected by CM-H2DCFDA (Life technologies, US) after exposure of larvae to the concentration of 0mM , 1.0mM, and 2.0mm of hydrogen peroxide for 24 hours. White arrow head is ROS detected by CM-H2DCFDA. Blue arrow head ROS in the gills.

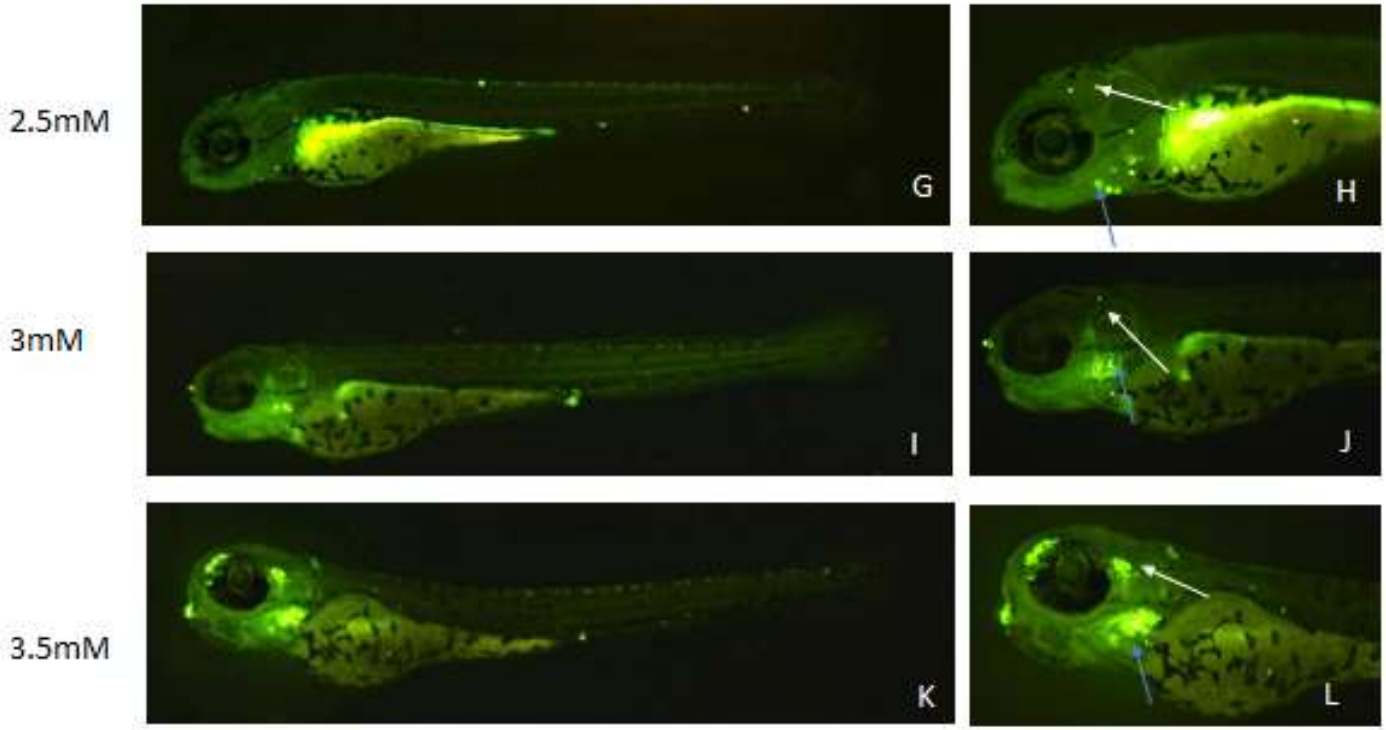

Figure 4.13b- Image of ROS detected by CM-H2DCFDA (Life technologies, US) after exposure of larvae to the concentration of 2.5mM , 3.0mM, and 3.5mm of hydrogen peroxide for 24 hours. White arrow head is ROS detected by CM-H2DCFDA. Blue arrow head ROS in the gills.

4.5 Selection of embryos for experiment

Due to time constraints of my Master of Research course, all the experiments were performed in the mosaic (F0) generation, while waiting for the transgenic line to grow sexually mature. We injected either mKillerRed plasmid or mCherry-CAAX plasmid (as a negative control) into the one-cell stage of the zebrafish embryos. The next day, the embryos that were expressing either mKillerRed (figure-4.14 a) or mCherry-CAAX (figure 4.14 b) were chosen for further experiment. Each experiment was done in average of 4 times with minimal of 3 larvae per treatment.

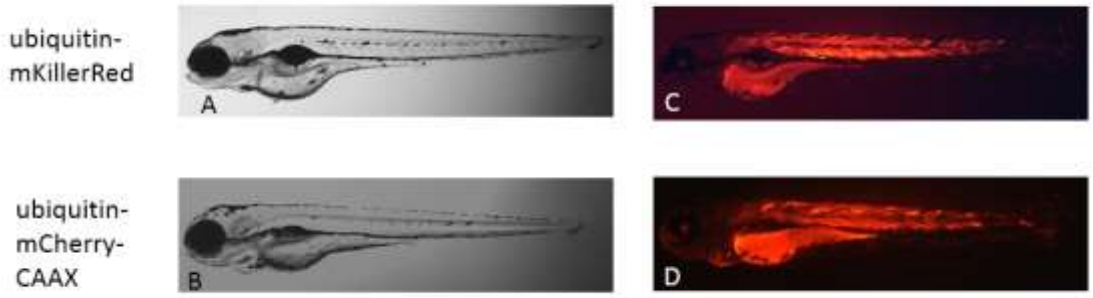

Figure 4.14- Image of mKillerRed and mCherry-CAAX mosaic (F0) generation. Ubiquitin-mKillerRed in figure (A) bright field and figure (C) mCherry filter. Ubiquitin-mCherry-CAAX in figure (B) bright field and figure (D) in mCherry filter.

4.6 ROS production of KillerRed

We have studied to the extent of KillerRed to generate the ROS in live zebrafish after illuminating with green fluorescent light. To rule out the possibility that the heat or/and laser is not causing necrosis or cell death that might lead to the release of ROS. We also subjected the zebrafish expressing mCherry in the cell membrane with the exact same duration, intensity and wave length of light illumination.

The phototoxicity of KillerRed is associated with intense photobleaching (Teh et al., 2010a) . Therefore, the KillerRed expressing cells were illuminated with the green light until it got completely photobleached. We were able to completely photobleach the KillerRed expressing cells usually within 5 to 10 minutes of light illumination (Figure 4.16a, E and F; Figure 4.16b, E and F). On the other hand, we were not able to photobleach the mCherry expressing cells even after 15 minutes of light illumination (figure 4.15 E and F).

We then studied if photobleaching of the KillerRed will produce ROS using CM-H2DCFDA. Since ROS can diffuse across membranes and spread across the cells (Halliwell and Gutteridge, 1985), their effect may spread outside the areas of the KillerRed expression cells . Therefore, CM-H2DCFDA can detect some ROS outside the photobleached areas. In our study, exposure of the KillerRed -positive cells to green light for 5-10 minutes causes increased production of ROS which was detected by a CM-H2DCFDA probe (Figure 4.16a, G and H; Figure 4.16b, G and H). However, ROS could not be detected in the mCherry- positive embryos after light illuminated (figure 4.15 G and H). Moreover, ROS was detected only in the light illuminated part of the zebrafish. ROS was not detected in the parts of the zebrafish where the KillerRed – positive cells were not illuminated with the light, except the small area just outside the light illuminated part of the embryos (Figure 4.16a and Figure 4.16b). This increase in ROS just outside the photobleached area was most likely due to the diffusion of ROS from the photobleached cells that were producing ROS, and not from the KillerRed-positive cells that were not illuminated with the light. This observation suggests that the KillerRed produces ROS after light illumination in zebrafish tissues. Furthermore, it also suggests that without light illumination, KillerRed is not toxic to the cells.

ROS produced by the KillerRed is directly related to the intensity of the light source and the duration of the light (Bulina et al., 2006b;Waldeck et al., 2012). Even by keeping the duration of the light constant, the intensity of the light source might vary per different experiment. Beside these, there might be a number of unknown bios and variations between two same experimental setups. Therefore, to avoid any bias and to further solidify our finding, we illuminated both the KillerRed-positive embryo and the mCherry- positive fish at the same time in one setting. For this, one KillerRed-positive embryo and one mCherry positive embryo were first anesthetized with tricaine. Then they were put in a glass-bottom petri dish (Mat Tek Corporation, USA) with a CM-H2DCFDA probe within it. Finally, both the embryos, KillerRed and mCherry, were exposed to the green light using Leica M165 FC fluorescence dissecting stereomicroscope (Leica) at the same time.

After 30 minutes of light illumination, there was almost a complete photobleaching of the KillerRed- positive cells in the KillerRed-positive embryos (figure 4.17a B and C, top fish). Whereas, we did not notice any significant photobleaching of mCherry-positive cells in the mCherry-positive embryos (figure 4.17a B and C, bottom fish). Moreover, we were able to detect ROS in the KillerRed positive embryos after photobleaching (figure 4.17a E and figure 4.17b G, top fish), which were not present in the mCherry positive embryos (figure 4.17a E and G, bottom fish). Furthermore, the ROS detected by the CM-H2DCFDA probe in the KillerRed positive cells after light illumination were not present before light illumination (figure 4.17a D and F). This again, suggested that KillerRed can produce ROS after light illumination and without light illumination KillerRed is not toxic to the cells. Moreover, we also noticed that it took longer time (30 minutes) to photobleach the KillerRed using the fluorescence dissecting stereomicroscope compared to the inverted microscope, which usually takes 5- 10 minutes.

Finally, we evaluate to see if KillerRed can produce ROS in neurons. For this, we injected Gal4-Hb9 plasmid combined with 4x UAS-mKillerRed plasmid with GFP eye reporter into the one cell stage of zebrafish embryos. The next day, when the mem-KillerRed- positive neuron was illuminated with the green light, the KillerRed was photobleached in 15 minutes (Figure 4.18 D). The time taken by the neurons to photobleach was comparatively longer than the time taken by muscle cells to photobleach. Furthermore, staining with CM-H2DCFDA identified the presence of ROS within the photobleached neuron (Figure 4.18 E and F).

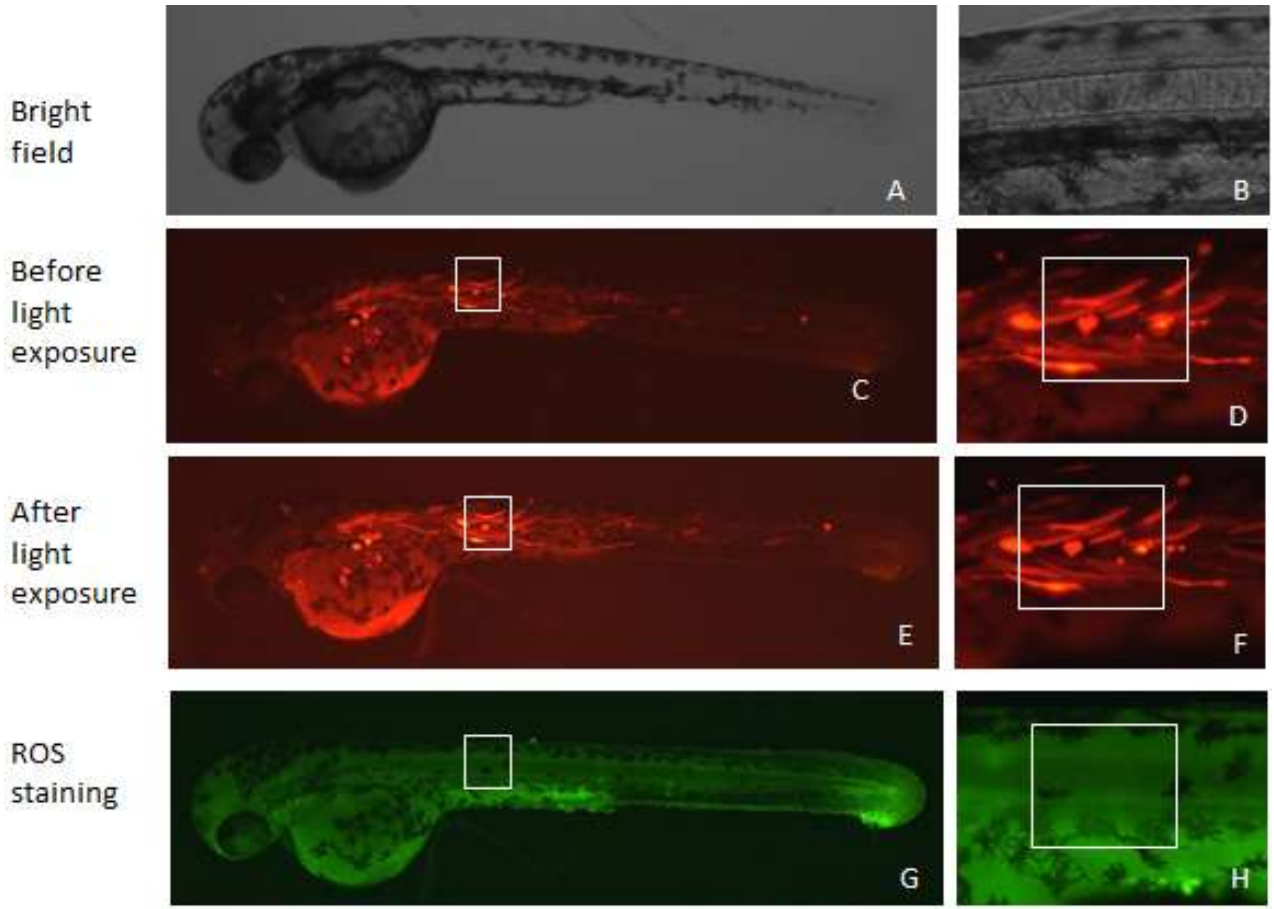

Figure 4.15- Light illumination of the mCherry-CAA followed by CM-H2DCFDA staining. (A and B) bright field image of whole embryo and focus image on the light illumination part. (C and D) fluorescent image of before light illumination of whole embryo and focus image on the light illumination part. (E and F) fluorescent image after light illumination of whole embryo and focus image on the light illumination part. (G and H) fluorescent image of CM-H2DCFDA staining after light illumination of whole embryo and focus image on the light illumination part. White square box correspond to the light illumination part

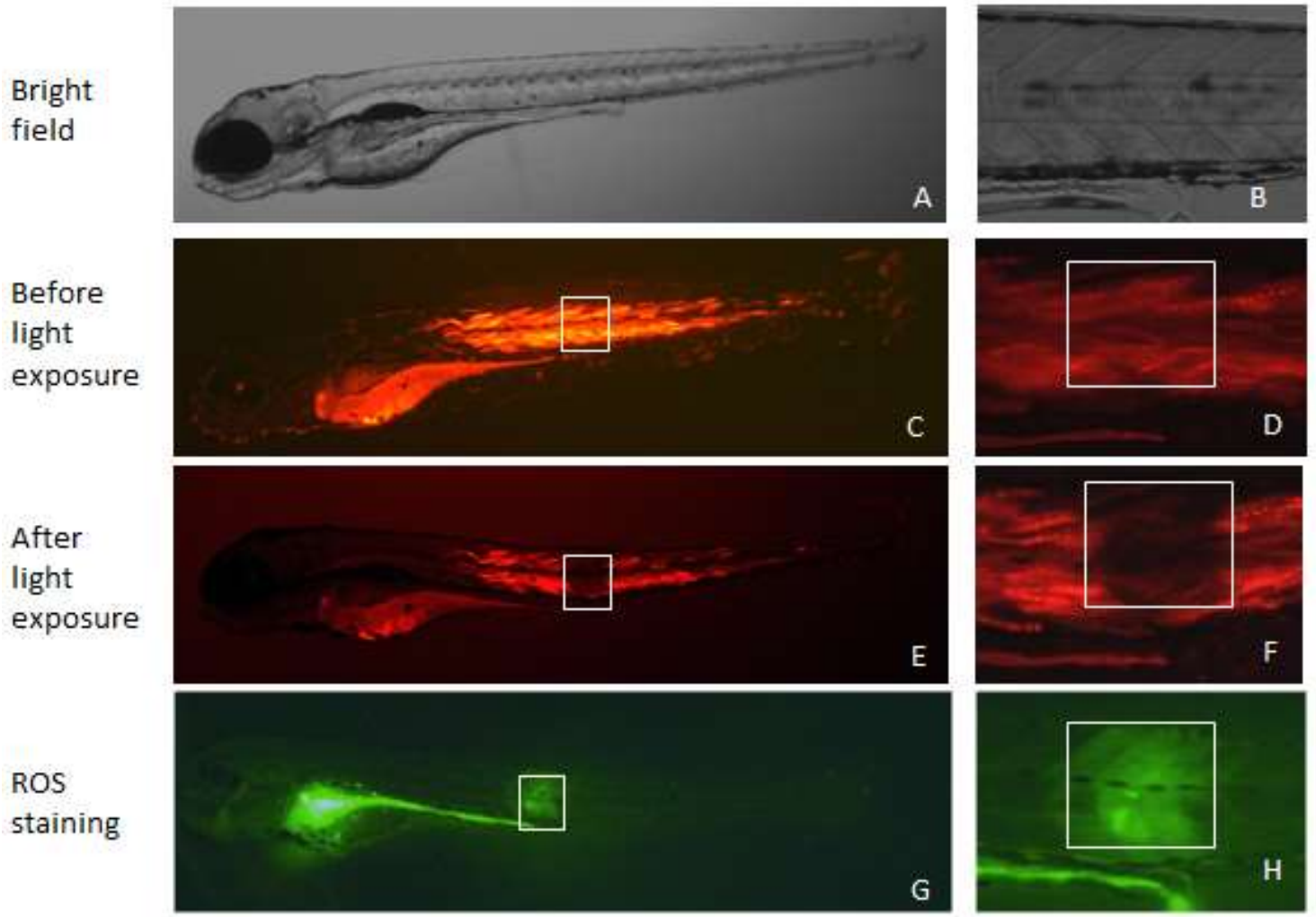

Figure 4.16 a- Light illumination of the mKillerRed-positive zebrafish followed by photobleaching and CM-H2DCFDA staining positive. (A and B) bright field image; (A) of whole embryo and (B) image focus on the light illumination part. (C and D) fluorescent image of before light illumination; (C) of whole embryo and (D) image focused on the light illumination part. (E and F) fluorescent image after photobleaching; (E) of whole embryo and (F) image focus on the photobleaching part of the zebrafish. (G and H) fluorescent image of CM-H2DCFDA staining after light illumination; (G) of whole embryo and (H) focus on the light illumination part. White square box correspond to the light illumination part of zebrafish.

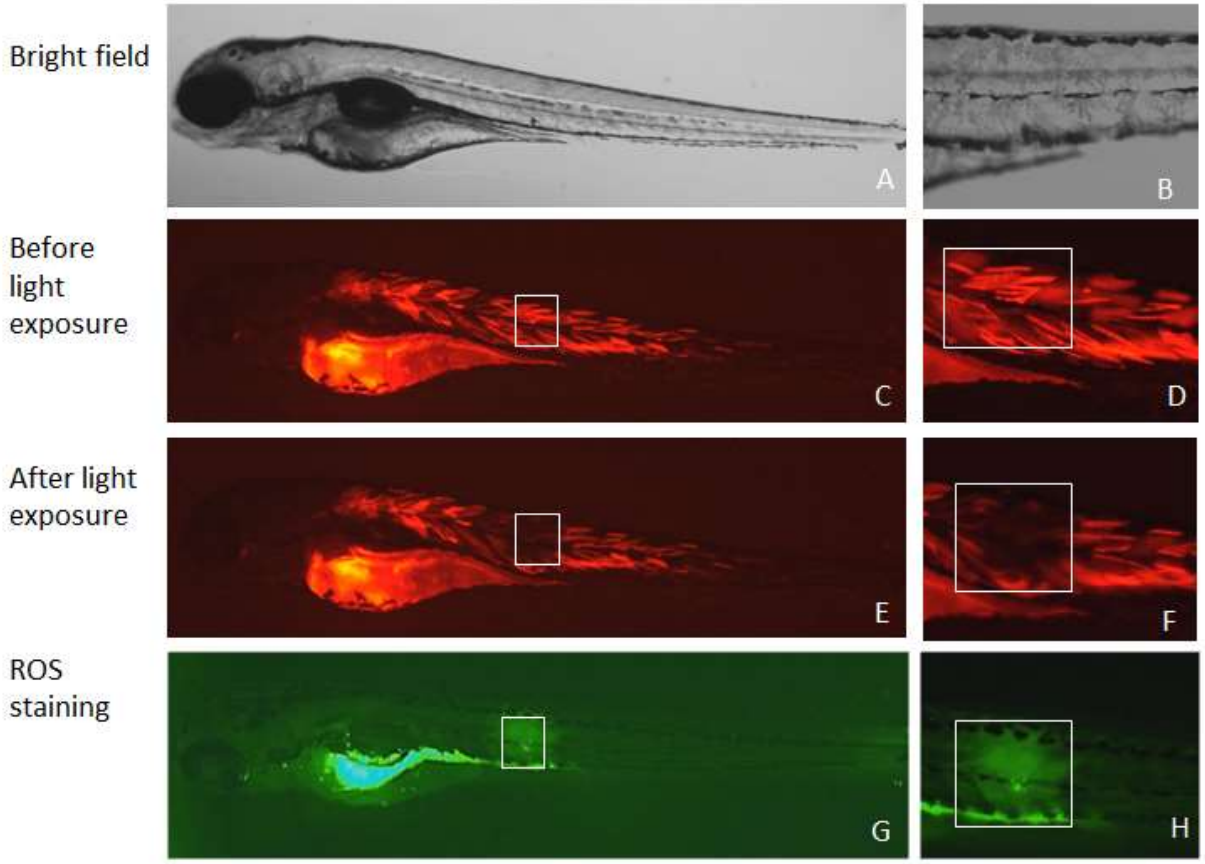

Figure 4.16 b- Light illumination of the mKillerRed-positive zebrafish followed by photobleaching and CM-H2DCFDA staining positive. (A and B) bright field image; (A) of whole embryo and (B) image focus on the light illumination part. (C and D) fluorescent image of before light illumination; (C) of whole embryo and (D) image focused on the light illumination part. (E and F) fluorescent image after photobleaching; (E) of whole embryo and (F) image focus on the photobleaching part of the zebrafish. (G and H) fluorescent image of CM-H2DCFDA staining after light illumination; (G) of whole embryo and (H) focus on the light illumination part. White square box correspond to the light illumination part of the zebrafish.

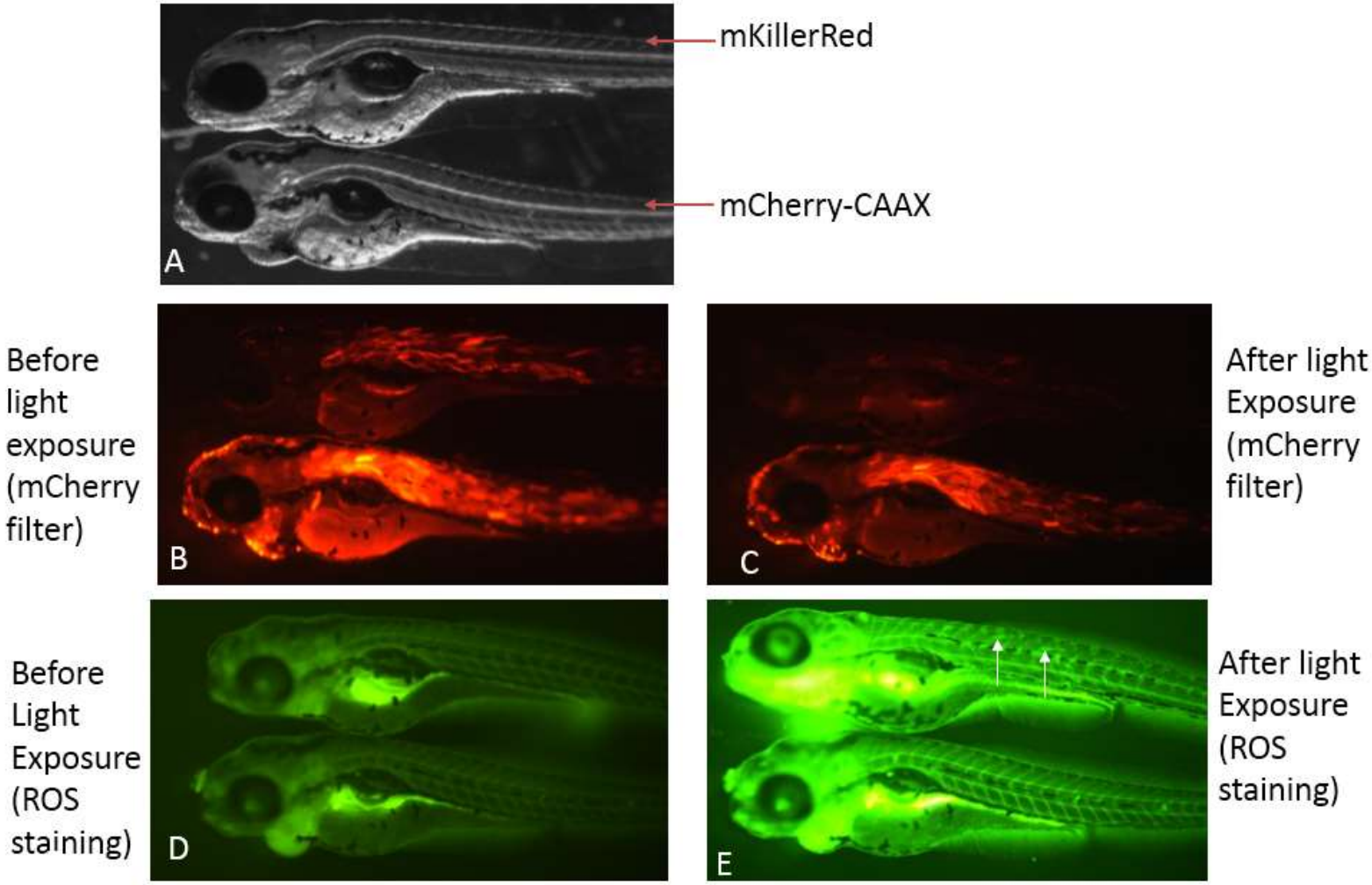

Figure 4.17a- Light illumination of the mKillerRed and mCherry at the same time and same setting, which was follow by photobleaching of mKillerRed cells with positive CM-H2DCFDA staining. (A) Bright field image, with mKillerRed on the top and mCherry at below. (B) Fluorescent image before light illumination of the embryo, again top one is mKillerRed and bottom one is mCherry. (C) Fluorescent image after photobleaching of the embryo, there is almost complete photobleaching of the mKillerRed embryo, top one, whereas as there is not significant photobleaching of mCherry, bottom one. (D and E) fluorescent image of CM-H2DCFDA staining before (D) and after (E) light illumination of the embryo. White arrow head is the CM-H2DCFDA staining positive cells

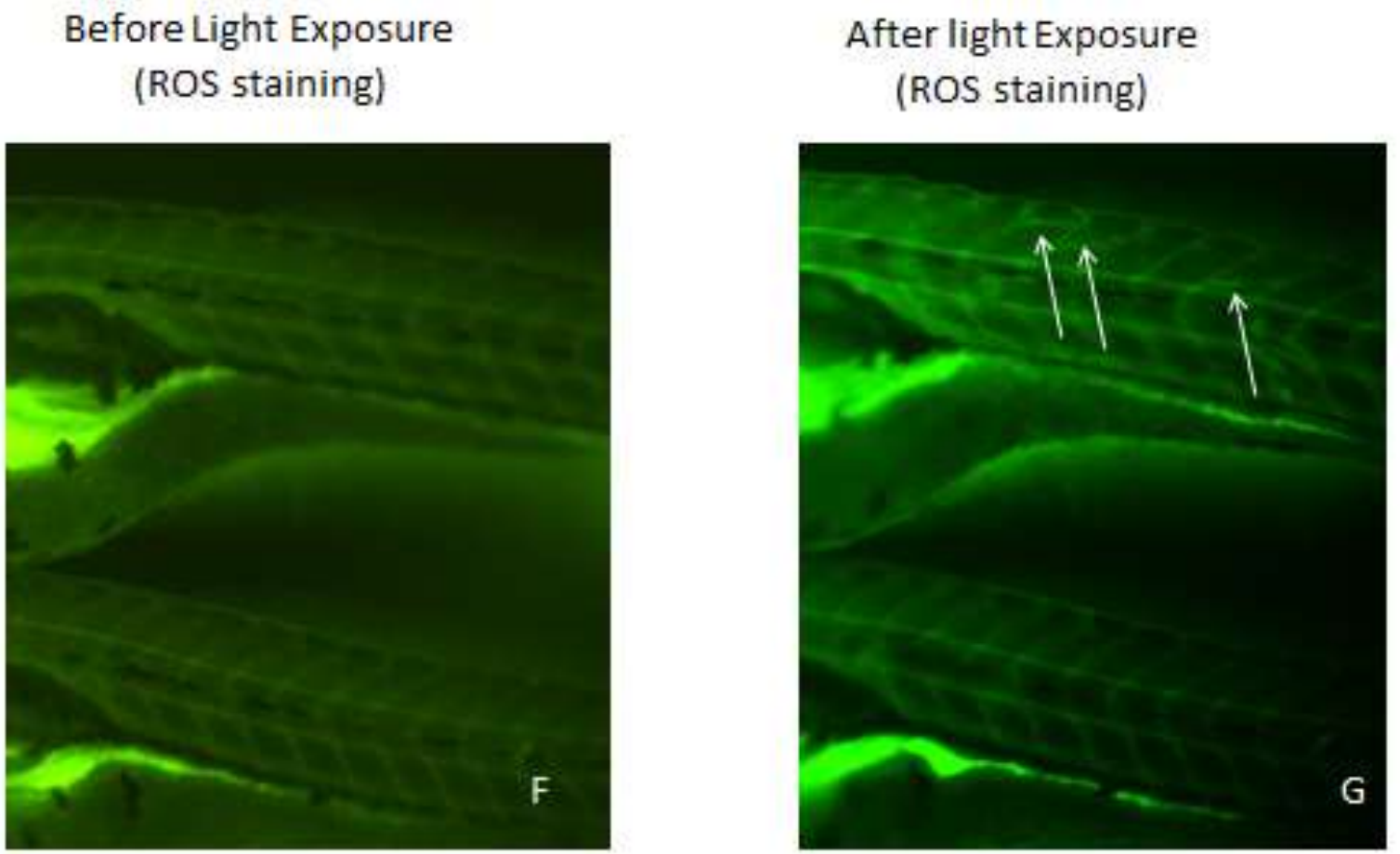

Figure 4.17b- Light illumination of the mKillerRed and mCherry at the same time and same setting, which positive CM-H2DCFDA was staining. (F and G) higher magnification of the CM-H2DCFDA staining before (D) and after (E) light illumination of the embryo. White arrow head is the CM-H2DCFDA staining positive muscle cells in KillerRed zebrafish.

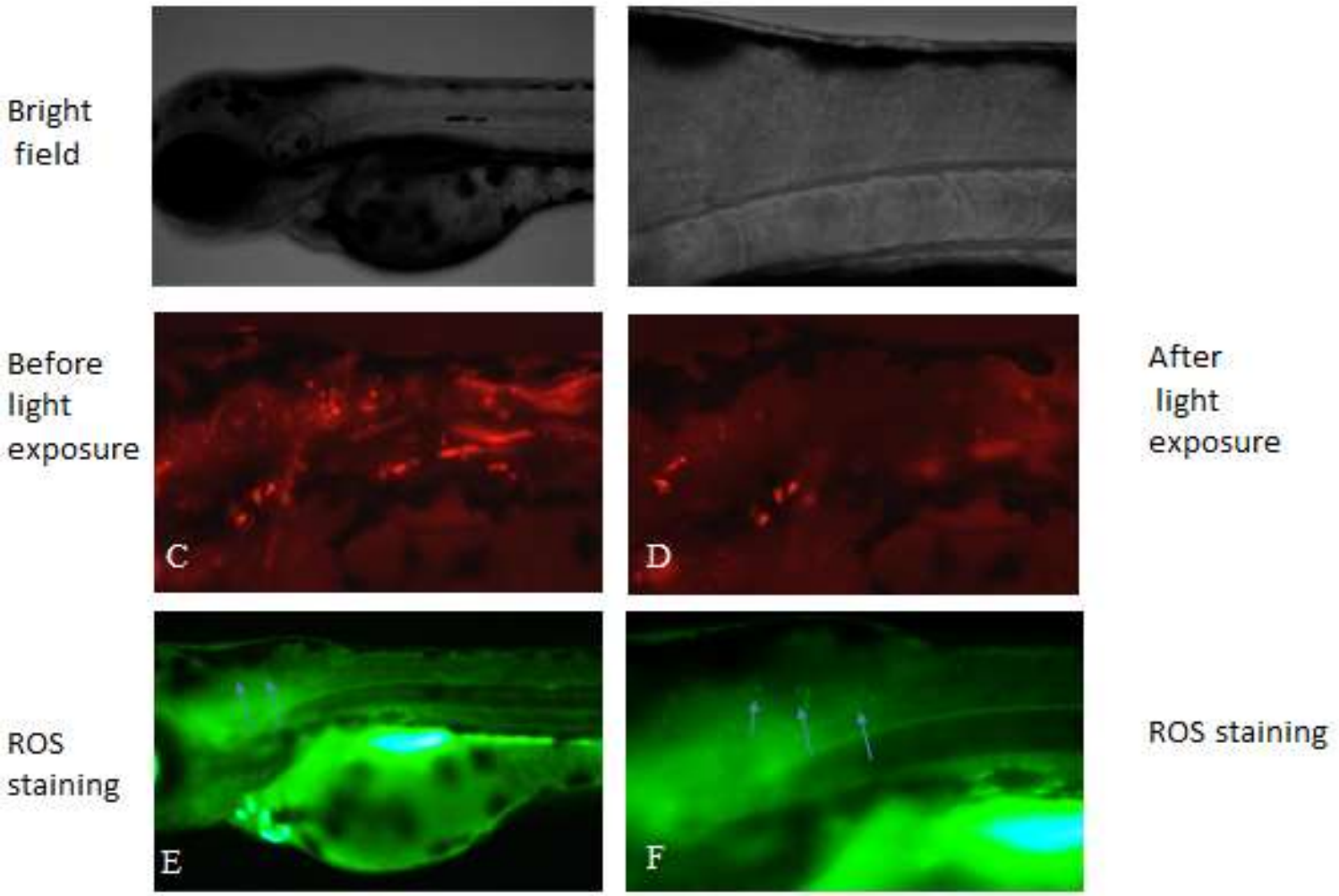

Figure 4.18a - Light illumination of the mKillerRed neuron followed by photobleaching and CM-H2DCFDA positive cells. (A and B) bright field image; (A) of whole embryo and (B higher magnification (C) Fluorescent image of before light illumination. (D) Fluorescent image after photobleaching (E and F) fluorescent image of CM-H2DCFDA staining after light illumination. Blue arrow head ROS detected by CM-H2DCFDA in the motor neuron

Figure 4.18b- Light illumination of the mKillerRed neuron followed by photobleaching and CM-H2DCFDA positive cells, (A) mKillerRed neuron, white arrow head mKillerRed neuron. (B) Embryo stain with CM-H2DCFDA after photobleaching , blue arrow head ROS detected by CM-H2DCFDA in the motor neuron zebrafish.

4.7 Apoptosis of the cells by KillerRed.

After validating that KillerRed produced ROS, we then compared the extent of KillerRed photobleaching and the degree of cells damaged in the zebrafish embryos. KillerRed phototoxicity is directly related to the amount of KillerRed protein concentration (Bulina et al., 2006a). Research has shown that injection of higher concentration of DNA and mRNA into the embryo would result in higher integration frequency into the DNA of that embryo (Sumiyama et al., 2010), and integration of foreign DNA into the host DNA will result in uniform and more predicated expression of protein. Therefore, in order to increase the integration frequency of membrane-KillerRed in the embryos, we injected a higher dose of DNA plasmid, 2ng of 200µg/ml of DNA, into the one cell stage of zebrafish embryos.

The duration of the illumination was usually done until twice the duration for the complete photobleaching of the KillerRed takes. When we illuminated with the green light, KillerRed-positive cells were effectively photobleached within 5 to 10 minutes (figure 4.19 E and F). After photo bleaching for 15 to 20 minutes, double the time taken for complete photobleaching of KillerRed protein, we were able to detect an increase in the amount of apoptosis cells within the light illuminated part of the zebrafish (figure 4.19 G and H). However, even with the high dose of mCherry DNA plasmid injection and illumination with the green light for 25 minutes, we were not able to photobleach the mCherry- positive fish (figure 4.20 E and F). Moreover, we did not notice any increase in the amount of apoptosis cells after illuminating with the light (figure 4.20 G and H). This observation suggested that KillerRed can induce cell death if illuminated with the light.

To further validate our finding, we stained the KillerRed-positive and the mcherry – positive zebrafish after illuminating with the light with TUNEL assay. An exposure of the KillerRed-positive embryos to light caused a substantial increase in the number of TUNEL-positive cells only in the light exposed part of the zebrafish (Figure 4.21). On the other hand, no increase in TUNEL–positive cells was found after the exposure of the mcherry-postive embryos to the light (Figure 4.22). This demonstrates that photobleaching of KillerRed causes cell death and without green light illumination KillerRed is not phototoxic.

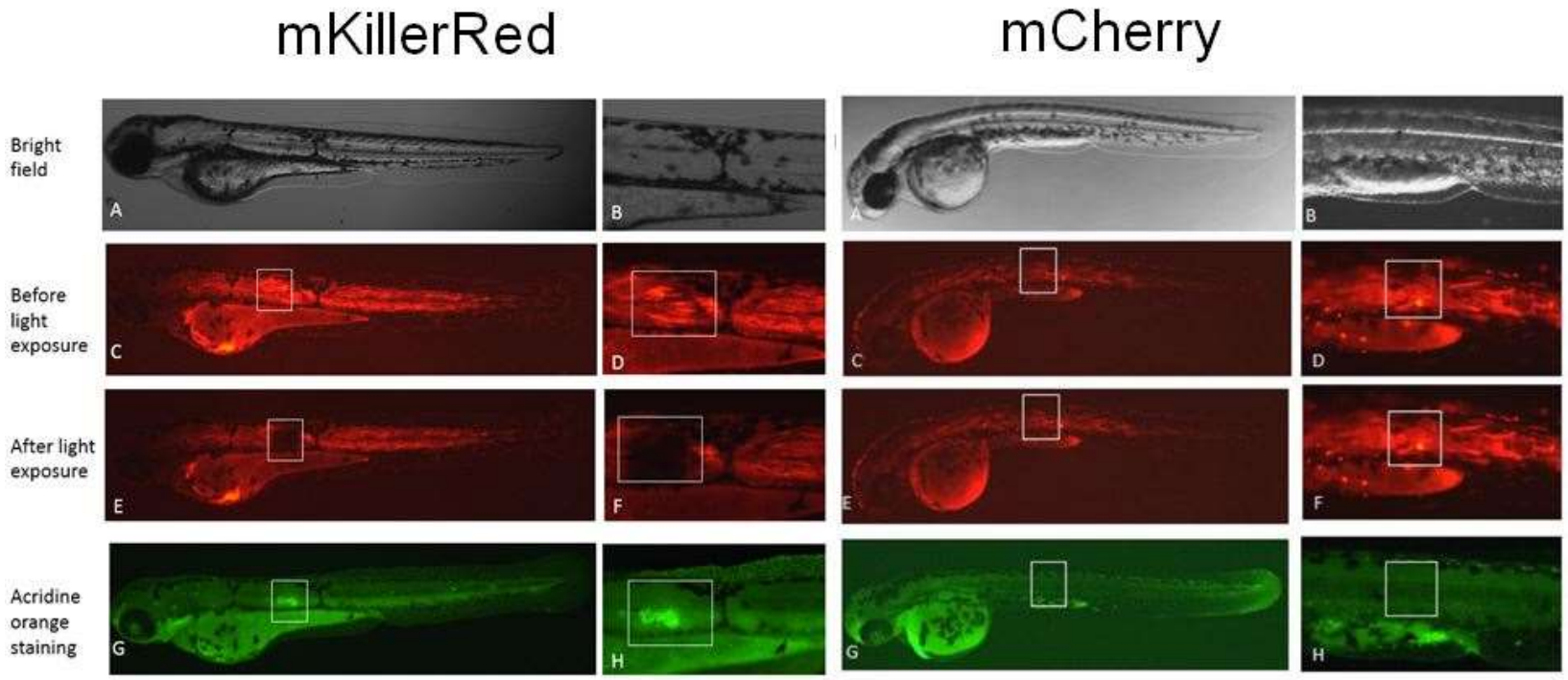

Figure 4.19- Illumination of the KillerRed positive cells caused bleaching of KillerRed followed by increased in apoptosis cells detected by acridine orange. (A and B) bright field image. (C and D) fluorescent image before photobleaching. (E and F) efficient photobleaching of KillerRed cells. (G and H) Embryos stained with acridine orange after photobleaching, white arrow head showing apoptosis. Area under the white square box is light exposed part of the zebrafish.

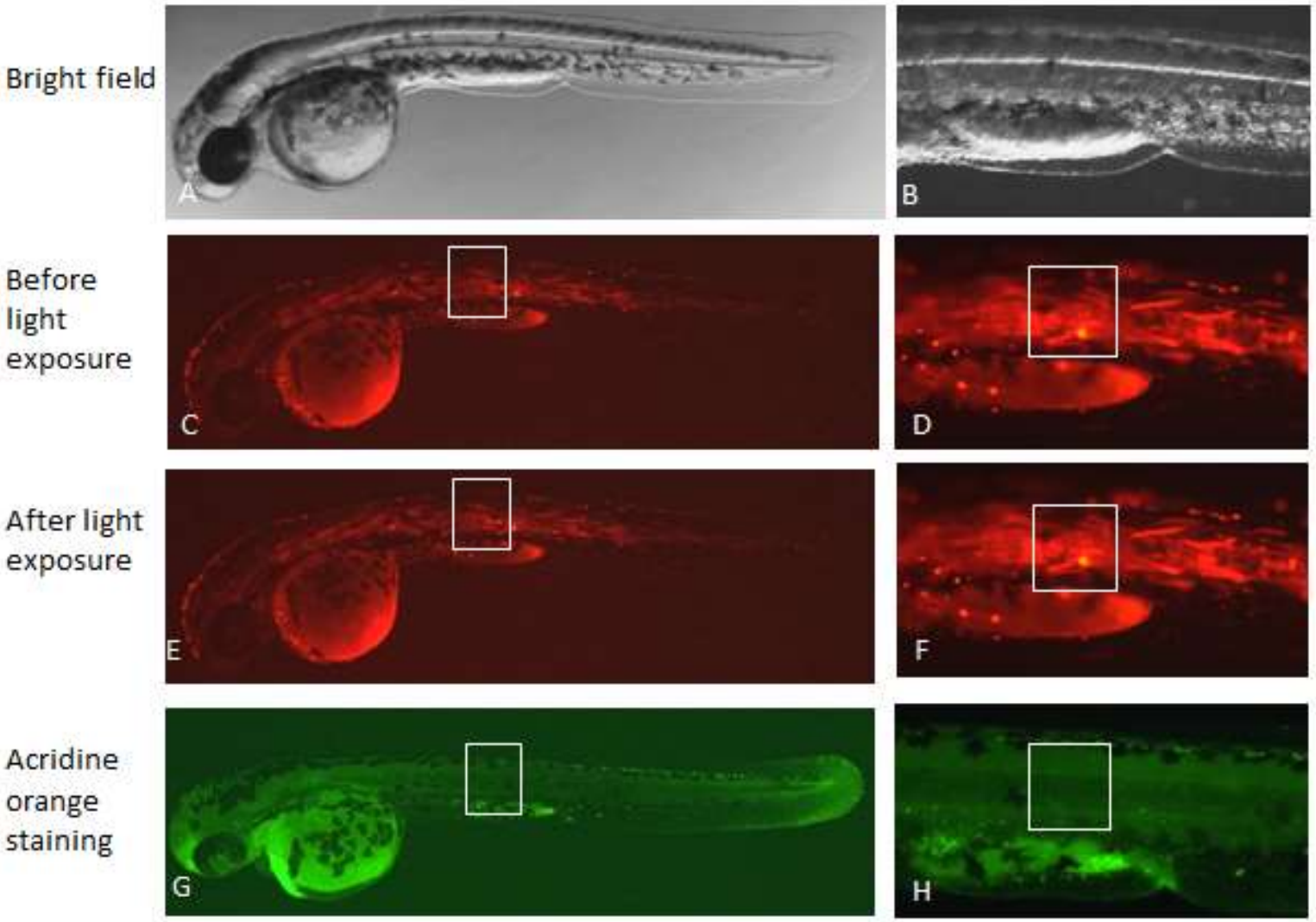

Figure 4.20- Illumination of the mcherry positive cells neither caused bleaching of mCherry nor did acridine orange detected any increased in apoptosis cell. (A and B) bright field image. (C and D) fluorescent image before photobleaching. (E and F) fluorescent after light illumination of mcherry cells. (G and H) Embryos stained with acridine orange after light illumination. Area under the white square box is light exposed part of the zebrafish.

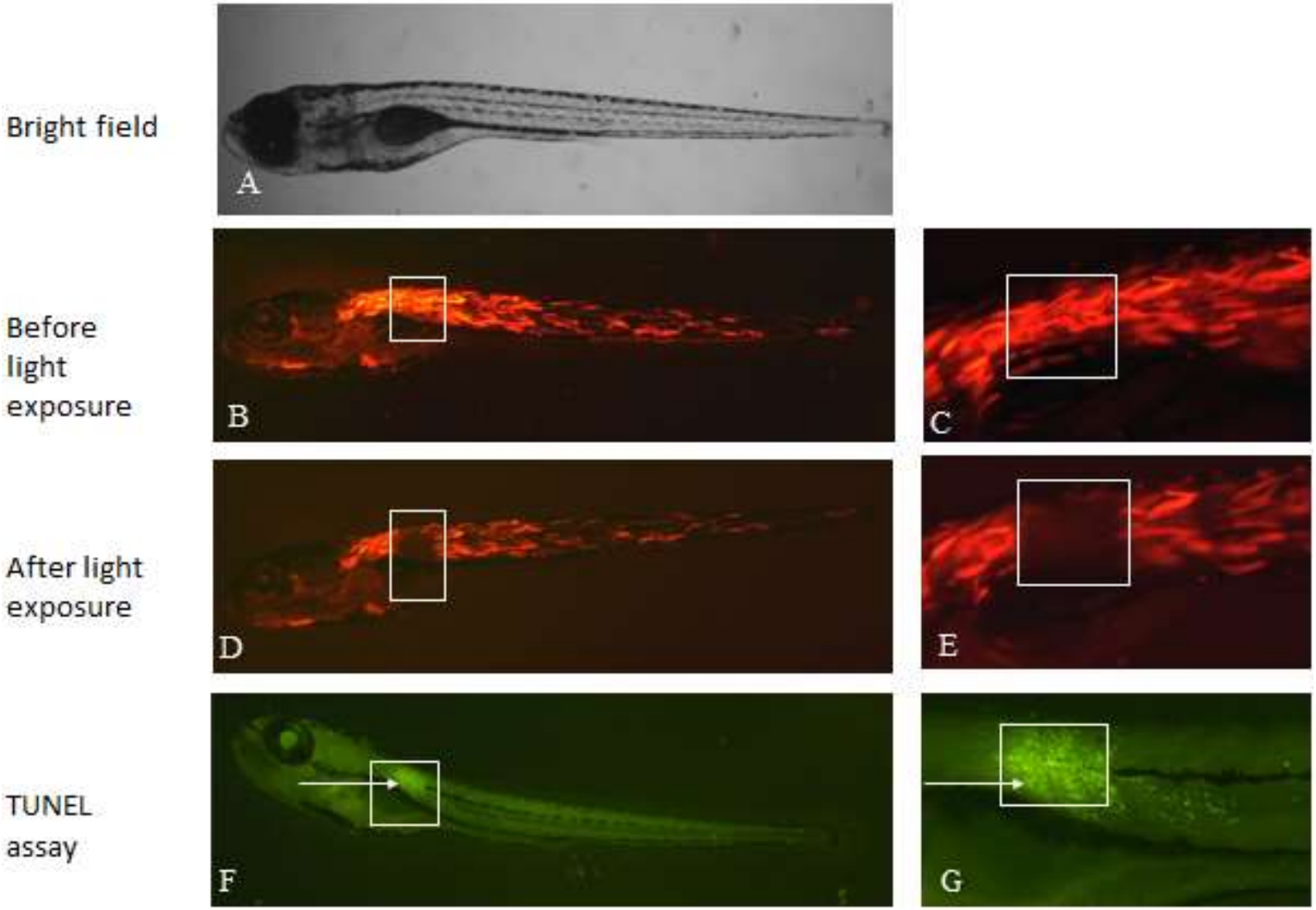

Figure 4.21- Illumination of the KillerRed positive cells caused bleaching of KillerRed followed by increased in apoptosis cells detected by TUNEL assay. (A) Bright field image. (B and C) fluorescent image before photobleaching. (D and E) efficient photobleaching of KillerRed cells. (F and G) Embryos stained with TUNEL assay after photobleaching, white arrow head showing apoptosis cells. Area under the white square box is light exposed part of the zebrafish.

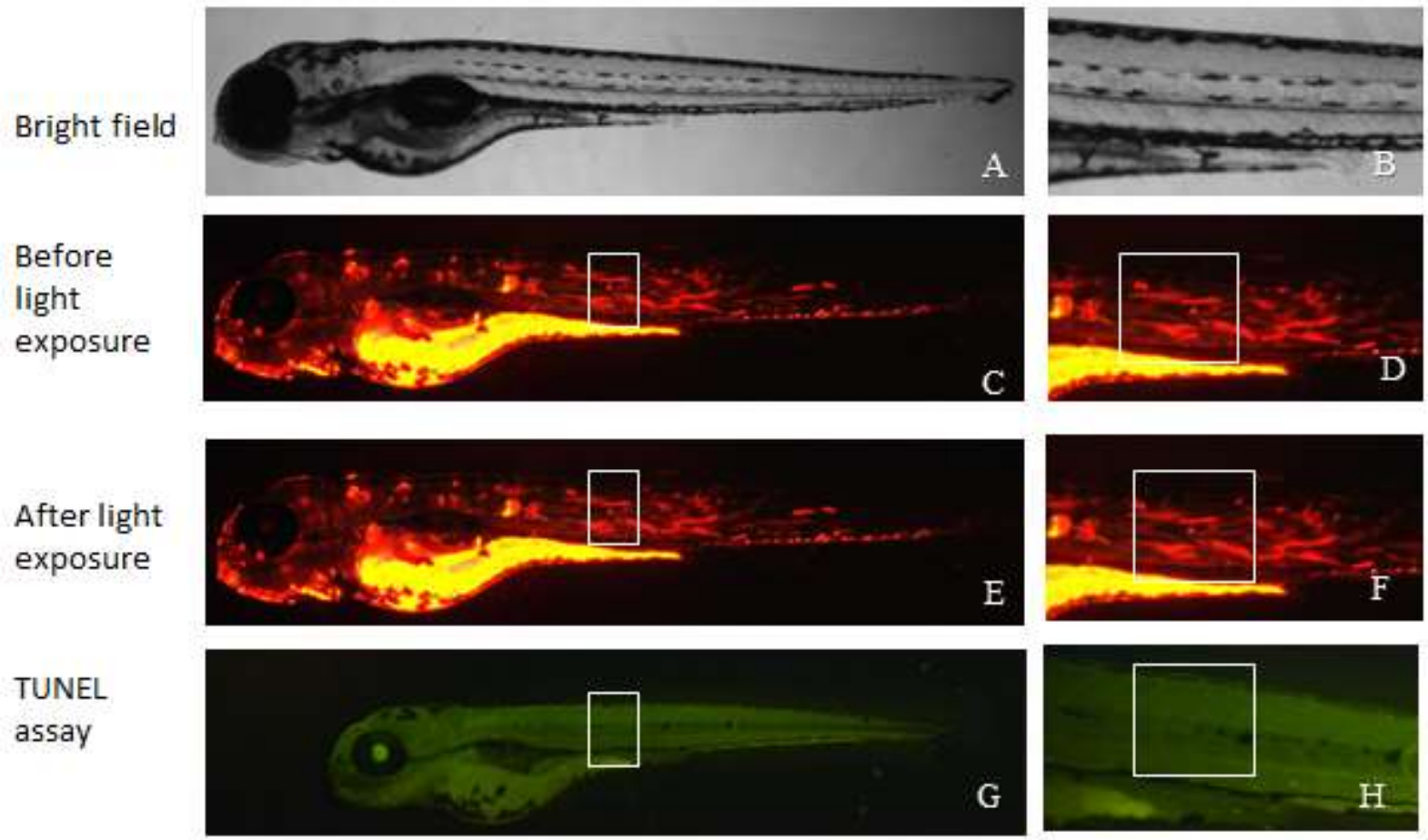

Figure 4.22- Illumination of the mCherry positive cells neither caused bleaching of mCherry nor did TUNEL assay detected any increased in apoptosis cell. (A and B) bright field image. (C and D) fluorescent image before photobleaching. (E and F) fluorescent after light illumination of mCherry cells. (G and H) Embryos stained with TUNEL assay after light illumination. Area under the white square box is light exposed part of the zebrafish.

4.8 Dose dependent effect of ROS

ROS effect cells in a dose dependent manner, where low levels promote cell division, intermediate levels cause growth arrest, and high levels cause apoptosis(Covarrubias et al., 2008). Our result has already shown that the KillerRed can produce ROS within just 5-10 minutes of illumination with the light. Moreover, we have also seen that phototoxicity of KillerRed can cause cell death. We then evaluate to see whether low doses of ROS can kill the cells or not. The amount of ROS produced by the KillerRed is directly proportional to the intensity and duration of light illumination in the KillerRed-positive cells (Teh et al., 2010a). So, if we keep the intensity of the light constant, and change the duration of the light, we can manipulate the ROS produced by KillerRed. As mentioned above, KillerRed can produce ROS within 5 minutes of light illumination. We want to see if ROS produced in a short duration (5minutes) of the light can induce cell death.

For this, we exposed the KillerRed-positive cells with green light for just 5 minutes, which did cause photobleaching of the KillerRed-positive cells (Figure 4.23 E and F; Figure 4.24 E and F). Even after photobleaching, there was no increase uptake of acridine orange in those photobleached areas, which would have occurred if there were dying cells (Figure 4.23 G and H; Figure 4.24 G and H). To evaluate whether it takes a longer time for low doses of ROS to induce apoptosis in the cells, acridine orange staining was done after 3 hours (figure 4.23 G and H) and 6 hours (Figure 4.24 G and H) of light illumination. The result showed that there are no increase uptakes of acridine orange even after 6 hours of light illumination (Figure 4.24 G and H). This observation suggests that KillerRed can be manipulated to oxidative stress the cells without killing them.

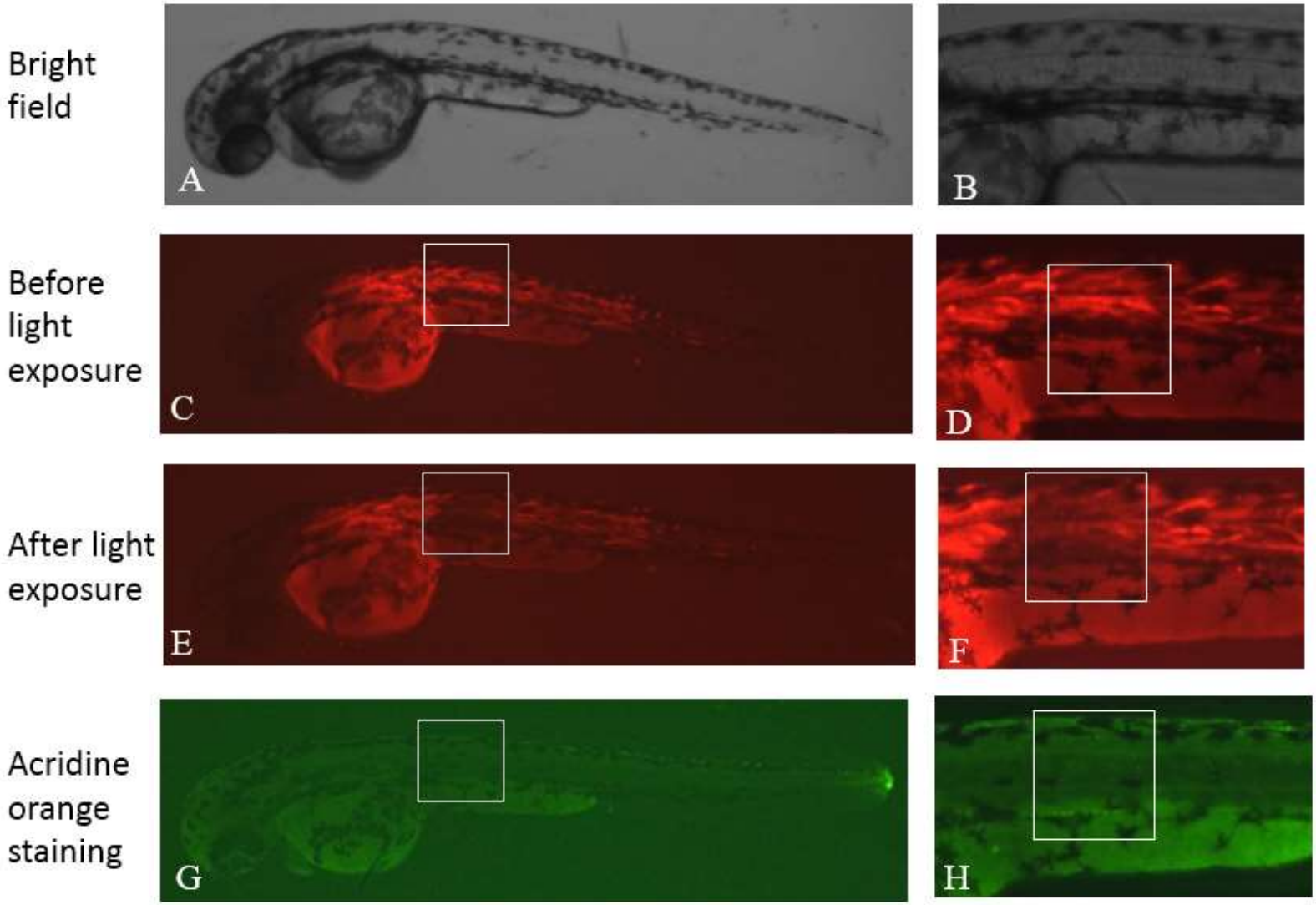

Figure 4.23- Illumination of the KillerRed positive cells just till photobleaching (5 minutes) of KillerRed does not increased in apoptosis cells, acridine orange staining was done 3 hours after exposure (A) Bright field image. (B and C) fluorescent image before photobleaching. (D and E) efficient photobleaching of KillerRed cells. (F and G) Embryos stained with acridine orange assay after photobleaching. Area under the white square box is light exposed part of zebrafish.

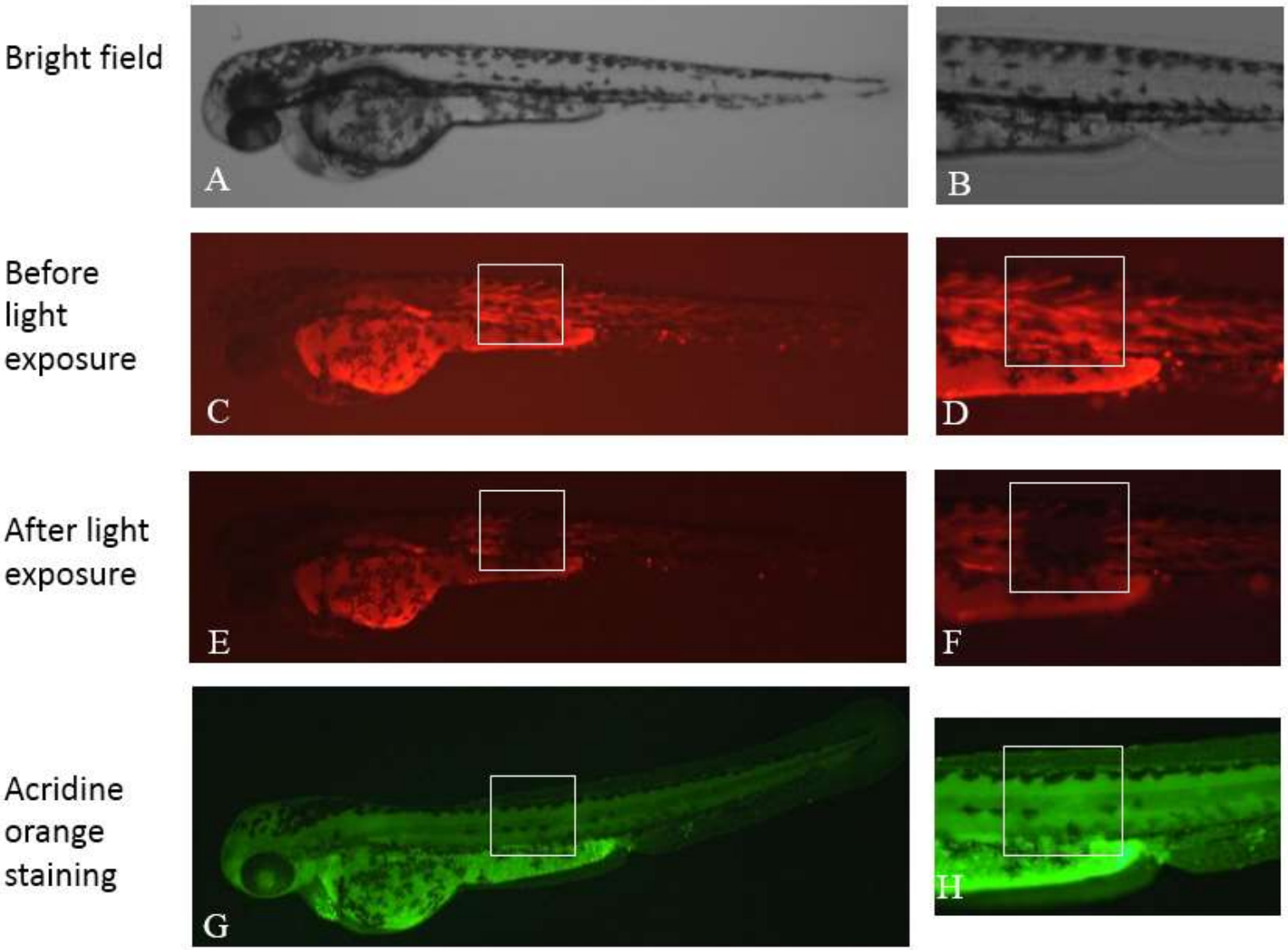

Figure 4.24- Illumination of the KillerRed positive cells just till photobleaching (5 minutes) of KillerRed does not increased in apoptosis cells- acridine orange staining was done 6 hours after exposure. (A) Bright field image. (B and C) fluorescent image before photobleaching. (D and E) efficient photobleaching of KillerRed cells. (F and G) Embryos stained with acridine orange assay after photobleaching. Area under the white square box is light exposed part of zebrafish.

4.7 Generation of transgenic lines

We are currently breeding F0 generation of Hb9-memKillerRed, ubiq-memKillerRed and ubiq-mcherry-CAAX with the wild type fish to identify positive F1 founder for these lines. We have so far identified a positive founder (F1) for HB9-mcherry-CAAX (figure 4.25).

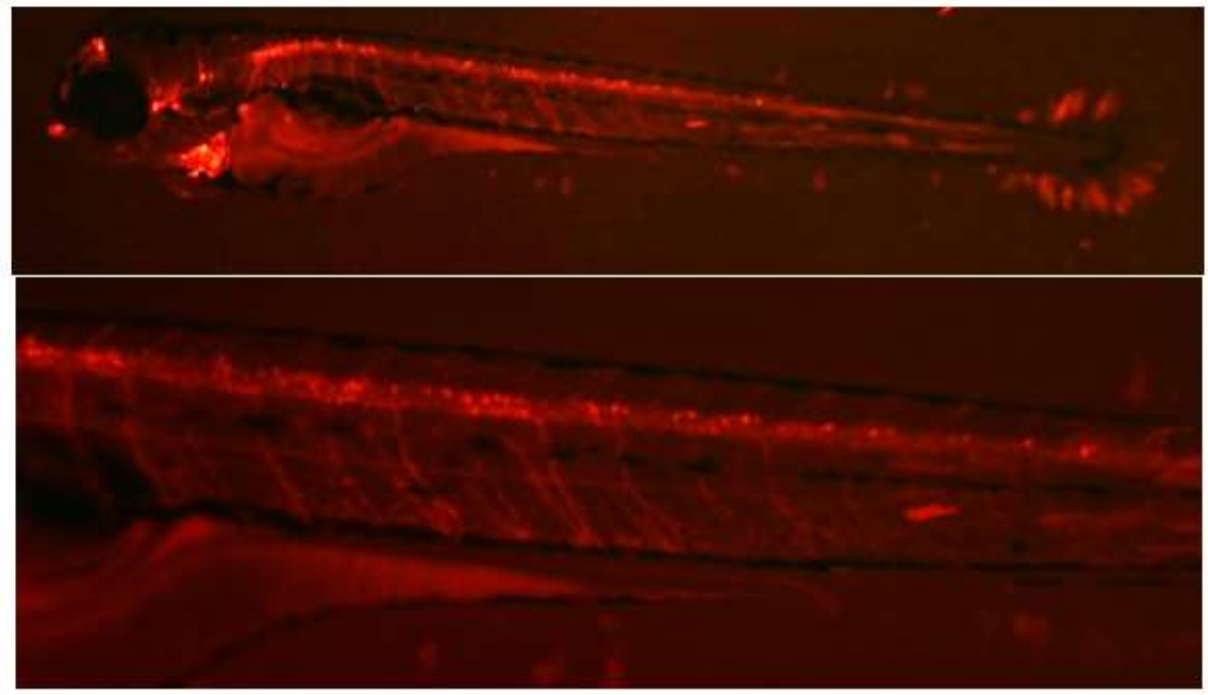

Figure 4.25- Positive founder (F1) for HB9-mcherry-CAAX.

4.8 Alter the function of the Protein

We analysed all our mem-killerRed F0 generations for any structural and behavioural abnormalities. All together we had 27 F0 ubiquitin driven mem-killerRed zebrafish. During the first two and half months of their life, in the majority (20) of the embryos, no structural or behavioural abnormalities were found. In contrast, 4 embryos died of unknown reasons and 3 embryos had some structural abnormalities (figure 4.26 B).

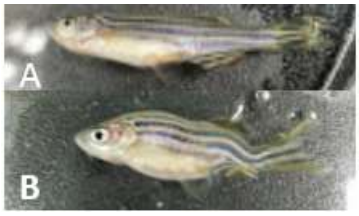

Figure 4.26- Adult zebrafish at 2 months old. (A) Structurally normal zebrafish. (B) Curved spine zebrafish.

Section 4.0: Discussion

4.1 Summary of project

In this study, we investigated the efficacy of KillerRed to produce ROS and cause cell death in a dose dependent manner in live zebrafish. Upon irradiation by green light KillerRed is efficiently photo bleached, which is accompanied by a generation of ROS. ROS effect cells in a dose-dependent manner, where low levels promote cell division, intermediate levels cause growth arrest, and high levels cause apoptosis(Covarrubias et al., 2008). We have shown that KillerRed can be manipulated to control the amount of ROS to be produced, so that desired neurons can be oxidatively stressed without killing them or causing cell death. As, degeneration of the motor neurons is a hallmark of ALS, it is hypothesized that oxidative stress is involved in this process. Therefore, KillerRed provides a novel way to directly induce oxidative stress in the desired motor neuron.

4.2 ROS production of KillerRed

Here, for the first time, we have shown that KillerRed can produce ROS in live zebrafish, including motor neurons. Although studies conducted in cell cultures have shown evidence that KillerRed produces ROS (Bulina et al., 2006b), there is no publication that indicates the production of ROS by KillerRed in the zebrafish (Del Bene et al., 2010a;Lee et al., 2010a;Korzh et al., 2011b;Teh and Korzh, 2014). There is no doubt that KillerRed can produce ROS. However, to study the effect of oxidative stress in the zebrafish, ROS producing properties of the KillerRed in the zebrafish need a more detailed understanding. Now, as we have shown that ROS is produced by the KillerRed in zebrafish, we can unravel a more detailed understanding of the ROS in the live animals cells and neurons.

In our study, after photobleaching KillerRed, usually within 10 minutes, we were able to identify the presence of ROS by using a general ROS detection kit, including in the neurons. Importantly, ROS was detected only in the parts of the embryos that there was photobleaching of KillerRed, and not detected where KillerRed was present but not photobleached. This finding suggests that photobleaching of KillerRed produces ROS and also that KillerRed does not produce ROS before photobleaching. This is an important point because the effect of oxidative stress in one or two neurons in a zebrafish can now be compared to the other neurons that had not been oxidatively stressed in the same animal. As the natural ROS producing capacity and antioxidant defence systems that cope with ROS between two different individuals are not the same, any extra exogenous oxidative stress in different individuals will have different effects. This can obstruct the better understanding of how the same types of cells respond in different levels of ROS. This limitation can be overcome by KillerRed, which allows a better comparison of oxidative stress between the similar cells within the same animal. Similarly, now we can understand what happens to a diseased neuron, such as that of the

ALS mutant zebrafish, with and without the presence of ROS in the same animal at the same time. The well controlled experimental setup achieved with KillerRed will help to reduce the unknown bias between the experiments.

4.3 Inducing apoptosis by KillerRed

ROS affect cells in a dose dependent manner, where low levels promote cell division, intermediate levels cause growth arrest, and high levels cause apoptosis (Covarrubias et al., 2008). Here we tried to explore the effects of different concentrations of ROS in the cells. Our result indicates that a low level of ROS does not kill the cells but with high levels of ROS might be sufficient enough to do so.

KillerRed had been shown to induce apoptosis in various cell cultures and zebrafish. For example, when KillerRed was expressed in cultured Escherichia Coli cells, KillerRed, upon light irradiation killed 96% of point the cells after 10 minutes of irradiation (Bulina et al., 2006a). Applications of KillerRed to kill cells in zebrafish has been demonstrated in different studies (Del Bene et al., 2010a;Lee et al., 2010a;Teh et al., 2010b;Korzh et al., 2011b). In one study, Del Bene and colleagues were also able to induce apoptosis in the neurons of the zebrafish, which they verified using TUNEL and Annexin V assay (Del Bene et al., 2010a). Moreover, Teh and colleagues expressed membrane targeted KillerRed in the hindbrain and the heart of the zebrafish. Then irradiation with an intense green light caused a two-fold increase in apoptosis cell death both in the hindbrain and the heart, as measured by TUNEL assay (Teh et al., 2010b).

However, the ability to manipulate KillerRed to produce ROS in a dose dependent manner, so that it will induce oxidative stress in the cells but not induced apoptosis of those cells have not been well characterized. In one study, the membrane targeted KillerRed was expressed in the habenula, a brain structure involved in avoidance behaviours. After irradiation with light it was found that annexin V was binding to the KillerRed-expressing cells after photo bleaching but there was no uptake of the acridine orange in those cells. The author of this research believes that this suggested the KillerRed-express cells were not killed but were only damaged (Lee et al., 2010a). Although irradiation did not kill those KillerRed express cells, the damaged membranes induced significantly reduced avoidance behaviours in the effected zebrafish (Lee et al., 2010a). To evaluate whether the low dose of ROS will not kill the cells, we explored the effect of light exposure duration to the KillerRed. We found that, exposure of the KillerRed to light just until photobleaching will produce ROS but will not induce apoptosis. However, exposure of the KillerRed to light for a longer duration, double the time it takes to photobleach, will induce apoptosis. It suggests that KillerRed can be manipulated to produce ROS with or without inducing cell death. This very precise ability to manipulate the amount of ROS to be produced within the cells of live zebrafish is of great importance in understanding the role of oxidative stress in various diseases including ALS. Now, we can oxidatively stress the neurons of the ALS transgenic zebrafish with the amount of ROS that will usually not kill the normal viable

neurons. This observation will provide a better understanding if the motor neurons in ALS have increased sensitivity to oxidative stress.

4.4 Factors affecting the ROS production capacity of KillerRed

ROS produced by the KillerRed is directly related to the amount of KillerRed protein concentration (Bulina et al., 2006a), the intensity of the light source and the duration of the light (Bulina et al., 2006b;Waldeck et al., 2012).

We have also noted that KillerRed is more efficient as a photosensitizer after being illuminating with an inverted microscope as compared to that of a dissecting stereomicroscope. This is attributed to the higher intensity of the fluorescent light coming from the inverted microscope at a x40 magnification lens compared to a x4 magnification lens in the dissecting stereomicroscope. The effect of intensity of light on KillerRed had been more meritoriously studied by Teh and colleagues (Teh, 2010). They have shown that photosensitizer of KillerRed is more efficient with the mercury lamp compared to the laser of a confocal microscope (Teh, 2010). These observations illustrate that a low intensity light source will produce a low level of ROS in KillerRed-positive neurons and can be used for a prolonged period of time to produce stable low levels of ROS. This will be helpful to study the continuous chronic effect of oxidative stress in the neurons.

4.5 KillerRed photo toxicity comparison

It has been shown that other fluorescent compounds can emit active electrons producing ROS (Greenbaum et al., 2000;Jiménez-Banzo et al., 2008). In order, to further verify that phototoxicity of the KillerRed is superior to other fluorescent proteins, and more importantly to rule out that the heat and/or laser generated during light illumination is not producing ROS or killing the cells, we compared the phototoxicity of KillerRed to that of the mCherry fluorescent protein. MCherry is a fluorophore with excitation and emission spectrum of 587nm and 610 (Shaner et al., 2004). As, the excitation and emission spectrum of mCherry is very similar to KillerRed excitation and emission spectrum (585 and 610 respectively), mCherry is an ideal negative control. Furthermore, to label the cell membrane by mCherry as done by membrane targeted KillerRed; the CAAX domain was incorporated into the mCherry (Halpern et al., 2008). Even after, 30 minutes of light irradiation we were not able to photobleach the mCherry. Furthermore, we were neither able to detect the presence of any ROS nor apoptosis within the light irradiated part of the mCherry-positive cells in the zebrafish.

Previous research had found the phototoxic effects of KillerRed to be a thousand fold greater than that of other fluorescent proteins (Bulina et al., 2006b). This enhanced phototoxicity of KillerRed has been verified by comparing phototoxicity of KillerRed with green fluorescent protein and yellow fluorescent protein in a cervical cancer cell

culture. After light irradiation, KillerRed induced significantly more cell death than other fluorescent protein (Waldeck et al., 2009). Similarly in another study, when Escherichia Coli expressing different red fluorescent protein, such as KillerRed, mCherry, DsRed, and mRFP was irradiated with light, it was found that there was a significant decrease in surviving bacterial colonies in KillerRed expressing Escherichia Coli (Waldeck et al., 2012). In sum, there is sufficient evidence to suggest that KillerRed is more effective in producing ROS than any other fluorescent protein.

4.6 Different effects in different cells

The photobleaching time during the KillerRed activation observed in our study was determined to be slightly longer for motor neurons than muscle cells. This difference in photobleaching time may be the result of the difference in the position of the neuron relative to the muscle in the body wall. This could reduce the intensity of light reaching the nerve compared to the muscle. Or, this might be because different cells handle ROS differently. Moreover, research has found that the ROS generation capacity of KillerRed depends on the cell type. In one study, when KillerRed was expressed in a variety of neurons in Caenorhabditis Elegans, it was found that most of the neurons, such as GABAergic neuron, dopaminergic neuron, and cholinergic neurons, were efficiently killed after KillerRed activation. On the other hand, certain neurons such as amphid neurons and some part of dopaminergic neurons were killed with lower efficiency after KillerRed activation. However, a few neurons within the mechanosensory neuron, such as AVM, PVM and AWB, could not be killed by activating KillerRed (Williams et al., 2013).

Although we have yet to verify the KillerRed capacity to induce cell death in motor neurons and other neurons, the observation done by Williams and colleagues (Williams et al., 2013) is highly inspiring for future projects. They have shown that different types of neurons respond differently to the same amount of ROS. The most interesting aspect is that, motor neurons in Caenorhabditis Elegans were efficiently killed by ROS, but mechanosensory neurons could not be killed by ROS. This finding shows the greater vulnerability of motor neurons to the oxidative stress than to other neurons. In ALS, there is only a degeneration of motor neurons while the function of sensory neurons is preserved in the earlier stage of the disease (Wijesekera and Leigh, 2009; Shaw and Eggett, 2000). This increased sensitivity of motor neurons to the ROS, leaving other cells types unaffected is, not completely understood in ALS. Motor neurons are large, with a large cell body and axon, which has a high metabolic rate and energy demand. The high energy demand of the motor neuron is supplied by mitochondria, but at the same time will produce ROS as a side effect (Shaw and Eggett, 2000). Furthermore, with aging, there is a decrease in mitochondrial efficiency with increased production of ROS (Dröge, 2002). Therefore, the high energy demand and the high ROS level during aging will have deleterious effect in the motor neurons. In contrast, even though Williams and colleagues (Williams et al., 2013) have studied the apoptosis inducing capacity of the KillerRed, the different neuronal responses to different levels of

oxidative stress have not been studied. In our study we have seen that ROS producing capacity of the cells is autonomous. For this reason, the effect of ROS in different types of neurons in the zebrafish can be compared to come up with a better conclusion.

4.7 Alteration of function of the protein by Killerred

KillerRed is a dimer of two monomers (Carpentier et al., 2009;Pletnev et al., 2009). Dimer fluorescent proteins have been found to alter the function of the fused protein of interest (Chudakov et al., 2005). For example, when KillerRed was fused to histone H2A, the KillerRed-H2A-expressing cells were incapable of dividing and were going through spontaneous apoptosis and necrosis even without irradiating with light, due to the effect of the dimer KillerRed in chromatin structure (Waldeck et al., 2009). Tandem-KillerRed, which can act as pseudo-monomer, was developed to overcome this limitation (Fradkov et al., 2002;Bulina et al., 2006b). Tandem-KillerRed is a genetic fusion of two KillerRed coding sequences allowing for intramolecular dimerization and maturation of the protein (Serebrovskaya et al., 2011;Williams et al., 2013).

We found that tandem-KillerRed does not cause any alternation in the function of the protein it binds to, because the majority of the zebrafish that survived for at least two and half months did not develop any structural or behavioural abnormalities. Although a few zebrafish either died or had some structural abnormalities, this can be due to natural phenomenon, toxic effect of the injection, or may be due to the KillerRed. But, given the fact the majority of the fish are normal, the abnormalities found in a few fish is highly unlikely due to KillerRed. The safety of tandem-KillerRed has also been verified by Serebrovskaya and colleagues who fused tandem-KillerRed to histone H2B, and found that those tandem-KillerRed-H2A-expressing cells that were normally dividing in the dark, and stopped proliferation when irradiated with green light (Serebrovskaya et al., 2011).

4.8 Alternative to KillerRed to induce oxidative stress

Recently, fully monomeric version of KillerRed called SuperNova has also been developed by introducing random amino acids mutations into KillerRed(Takemoto et al., 2013). But, the SuperNova generates 10% less superoxide than tandem-KillerRed(Takemoto et al., 2013). In one study, when tandem-KillerRed and monomeric-KillerRed were expressed in the neurons of Caenorhabditis Elegans, it was found that tandem-KillerRed was more effective at disrupting the neurons than monomeric-KillerRed(Williams et al., 2013). Therefore, other alternative forms of KillerRed are not as effective as tandem-KillerRed to produce ROS.

There are various other exogenous insults to producing ROS and inducing oxidative stress in the zebrafish. Chemical reagents that are commonly used in an experimental setting to induce oxidative stress in zebrafish include rotenone, paraquat, hydrogen peroxide, arsenic, and phenethyly iosthiocyanate. Rotenone inhibits the mitochondria

complex I, thus preventing electron transfer down the ETC which causes the build-up of free radicals and induces oxidative stress (Li et al., 2003). Paraquat induces production of a superoxide radical, but the exact mechanism is unclear (Bus et al., 1974). Hydrogen peroxide is also commonly used to oxidatively stress zebrafish.

Despite the use of the various chemical reagents to induce oxidative stress, these chemical agents cannot be controlled to produce the desired amount of ROS within the cells. More importantly, the response of the chemical to produce ROS is highly unpredictable. Our result suggested that only with the high dose (> 2.5mM of hydrogen peroxide for 24 hours), presence of ROS in other parts of the zebrafish beside gills could be detected. Although we did notice an abundance of ROS around the head region with 3.5mM of hydrogen peroxide for 24 hours, we did not notice the presence of ROS in the other organ of the zebrafish. Moreover, toxic effect of 3.5mM of hydrogen peroxide was very high, with only 2 embryos having survived among 90 embryos treated with hydrogen peroxide for 24 hours. This suggests that using chemicals to study the cellular pathways involving ROS- related signalling is specific to desired cells poses a huge challenge. In contrast, the response of KillerRed is specific, predictable and can be adjusted to produce different levels of ROS.

4.9 Application of KillerRed

KillerRed has been used for killing target cells (Del Bene et al., 2010a;Lee et al., 2010a;Kobayashi et al., 2013), the chromophore assisted light inactivation (CALI) of protein (Bulina et al., 2006b;Destaing et al., 2010), studying oxidative stress on various cellular components(Liu et al., 2010) , photodynamic therapy (Serebrovskaya et al., 2009;Shirmanova et al., 2013), and light induced damage of genomic DNA(Serebrovskaya et al., 2011;Waldeck et al., 2011).

We have found that KillerRed can be manipulated to produce the various levels of ROS in the desired cells in the zebrafish. Moreover, our results have also shown that KillerRed can produce ROS in the motor neuron, but we have not yet verified that KillerRed can induce apoptosis in the motor neuron. On the one hand, both Acridine orange and TUNEL assay binds to degraded DNA to verify the apoptosis which is suitable to identify apoptosis in large cells or group of cells (Abrams et al., 1993;Heatwole, 1999). On the other hand, motor neurons are comparatively smaller than other tissues within the zebrafish embryos. Furthermore, there is a naturally occurring apoptosis in the growing embryos, which will also be stained by any apoptosis detecting assay (Cole and Ross, 2001). Thus, there is a high probability of false-positive results with these assays when trying to identify the death of one or two cells. Additionally, both of these assays stain the late sate of the apoptosis (Abrams et al., 1993;Frankfurt et al., 1996), so it will be a time consuming and labour intensive process to predict the exact amount of irradiation provided to the KillerRed and its effects on the cells. Taking these reasons into consideration, it is a challenging task to identify the single neuron that has been killed by KillerRed.

To overcome these limitations, we have planned to use Annexin-V assay (Koopman et al., 1994). Annexin-V assay will identify an earlier stage of apoptosis, stain a large part of dead neurons and can be done in live animal. Annexin V is calcium dependent protein with a high affinity for phosphatidylserine. Phosphatidylserine is a cell membrane protein that is usually present in the inner leaflet of plasma membrane and is completely absent from the outer surface of the plasma membrane in the normal viable cells. During the early stage of apoptosis, phosphatidylserine (PS) is translocated from the inner surface of the cell membrane to the cell surface, which can be recognized by annexin-V. By adding fluorescent reporter to the annexin-V, apoptosis cells can be easily identified in the live zebrafish. Moreover, annexin-V will bind along the cell membrane of the dying neuron due to the exposure of phosphatidylserine. This will make the identity of the dying neuron much easier. Moreover, phosphatidylserine (PS) translocation is considered as the early stage of apoptosis. By using annexin-V as a marker to detect apoptosis, it will be beneficial to find the exact time to expose KillerRed to the light in order to induce apoptosis.

Annexin-V assay detects early stages of apoptosis and will stain large area of a cell, but was limited by not being able to use as an *in vivo* assay. This drawback has been overcome by genetically encoding annexinV with a fluorescent marker (van Ham et al., 2010). For this, annexin V was fused with the mVenus fluorescent protein. Then, a secretion signal peptide was added preceding the annexin V gene fused to mVenus, to effective secret annexin-V fused to mVenus extracellularly. Finally, ubiquitin promoter was used to express annexin –V in all the cells of the zebrafish.

The unique feature of annexin-V transgenic zebrafish is that, whenever any cell undergoes apoptosis, annexinV -mVenus will bind in its outer cell membrane due to exposure of phosphatidylserine. This binding of annexinV-mVenus with phosphatidylserine in the apoptosis cell can be easily visualized using fluorescent microscopy. Moreover, using annexin-V transgenic zebrafish, we will be able to detect apoptosis noninvasively. Furthermore, it is recommended to use more than one method to verify apoptosis (Ulukaya et al., 2011). Therefore, apoptosis cells that have been detected by annexin-V can be used by another *in vivo* or *in vitro* apoptosis detection method, such as TUNEL or Acridine Orange, to further verify the apoptosis of those cells.

Using annexin-V transgenic zebrafish, we are planning to study the effects of the different levels and times of exposure of ROS in the motor neuron of the zebrafish. Currently, we are in the process of the validating the annexin-V transgenic zebrafish line that is in our lab.

4.10 Limitation

4.10.1 Mosaic vs. stable transgenic lines

One of the major limitations of our study is that the experiments were done in the mosaic (F0) generation. This was due to the time restraint of my Master of Research course, 6-8 months of experiment time before writing the thesis. Protein expression of the injected DNA in mosaic generation is highly unpredictable. Moreover, with the same amount and concentration of DNA injected into embryos, the expression pattern of the protein will be different between the embryos. For example, we did not see the expression of KillerRed in the head region of some of the zebrafish, even though the expression of KilllerRed in all the zebrafish was driven by ubiquitin promoter. Ubiquitin promoter should enable the protein assigned to be expressed in every cell of the body including the head. However, this will not change the fact that KillerRed can produce ROS and can kill cells in a dose dependent manner. In contrast, the duration of light required to produce ROS and/or kill the cells might be different between F0 generation and stable transgenic lines.

4.10.2 Apoptosis or Necrosis

KillerRed has been localized to several subcellular compartments, such as cytosol(Bulina et al., 2006a), mitochondrial (Bulina et al., 2006a;Bulina et al., 2006b;Shibuya and Tsujimoto, 2012;Erturk et al., 2014), plasma membrane (Del Bene et al., 2010a;Lee et al., 2010a;Teh et al., 2010b), nuclear lamina (Waldeck et al., 2011;Waldeck et al., 2013), peroxisome (Ivashchenko et al., 2011), lysosomes (Serebrovskaya et al., 2014), and histone (Serebrovskaya et al., 2011;Waldeck et al., 2011;Waldeck et al., 2013). The phototoxic properties of KillerRed localized to various subcellular compartments result in different molecular and cells response. For example, mitochondrially targeted KillerRed induced apoptosis cell death triggered by caspases dependent or independent pathway (Bulina et al., 2006a;Erturk et al., 2014). Whereas, histone and lamina targeted KillerRed can temporarily block cell division if irradiated for a short duration or induce DNA damage if irradiated for a longer duration (Serebrovskaya et al., 2011;Waldeck et al., 2011;Waldeck et al., 2013). Moreover, the lysosome targeted KillerRed induce cell death, either due to necrosis if the exposure of light is of high intensity or due to apoptosis is exposure of light is of lower intensity(Serebrovskaya et al., 2014). Finally, the membrane targeted KillerRed causes snecrosis and reproducible cell death by lipid oxidation (Bulina et al., 2006b;Lee et al., 2010a). Knowing whether KillerRed-activated cells undergo apoptosis or necrosis, would allow for an understanding of the signaling pathways involved.

Our results have shown that membrane targeted KillerRed can kill the cells. But it is hard to interpret whether the cell death was due to apoptosis or necrosis. Research has shown that TUNEL assay cannot discriminate among apoptosis and necrosis (Kraupp et al., 1995). In contrast, although acridine assay stains detect only apoptosis and not necrosis cells (Abrams et al., 1993), acridine orange is toxic to the cells and thus, causes the degeneration of the cells, and also gets autofluorescence (Rodriguez and Driever, 1997). Moreover, ROS are the signaling molecules that can turn on the different types of the death cycle at different levels. For example, at the lowest death promoting level of

ROS, apoptosis is induced; at intermediate level, autophagy occurs; and at very high death promoting level of ROS, ROS can cause necrosis of the cells. Therefore, our results cannot provide any conclusive evidence that the KillerRed induced cell death was either due to apoptosis or necrosis.

4.10.3 Type of ROS

Although there is evidence that superoxides are a major cytotoxic produced by KillerRed (Serebrovskaya et al., 2009;Bernard, 2011; Rosenthal and Ben-Hur, 1995), it does not rule out the fact that other ROS are not produced by KillerRed. More importantly, superoxides are constantly converted to hydrogen peroxide in living organisms. Therefore, which ROS is produced that has a major effect in zebrafish cells has not yet been studied. It would be of great benefit to know exactly which ROS is produced by KillerRed in zebrafish. So that, we might understand the function of the specific ROS within the live cells, as the detailed function of ROS is yet to be fully discovered. However, the time constraints mean that we were not able to verify the specific ROS produced by KillerRed. Instead, we used a general ROS detection kit (CM-H2DCFDA; Life technologies, US) to detect ROS. CM-H2DCFDA is a cell-membrane permeant indicator for reactive oxygen species that is nonfluorescent until it gets oxidised. Upon oxidation it yields fluorescence and also gets trapped inside the cell. Therefore, to know which specific ROS is produced by KillerRed would require further studies (Goody et al., 2012).

4.11 Oxidative stress and ALS

Oxidative stress has been associated with various diseases, such as chronic lung disease, cardiovascular disease, cancer, rheumatoid arthritis, diabetes, and neurodegenerative disease including ALS (Valko, 2007). Oxidative stress refers to the situation where there is imbalance between the production of ROS and the cells inability to detoxify those excess ROS by antioxidants defence (Dröge, 2002). In a normal healthy cell, there is a balance between production of ROS and antioxidant defences. Therefore, oxidative stress can be due to diminished antioxidants, such as a mutation in SOD1 and glutathione peroxidase, or toxic agents that destroy antioxidants, such as xenobiotics, or by reduced intake of dietary antioxidants. On the other hand, oxidative stress can also be due to increased production of ROS, either due to the increase in exogenous reactive oxygen sepsis or the over-activation of endogenous ROS system (Dröge, 2002).

ROS is molecules and ions that have an unpaired electron, thus making them extremely reactive. A common example of ROS includes superoxide anions, singlet oxygen, hydroxyl radicals, and hydrogen peroxide. ROS is either generated exogenously

or produced intracellularly by various sources. Intracellular ROS arises as a by-product of aerobic metabolism. Most of the intracellular ROS is generated from the mitochondrial respiratory chain due to an incomplete reduction of molecular oxygen during oxidative phosphorylation. However, small amounts of intracellular ROS are produced by other cellular enzymes, including xanthine oxidase in the cytoplasm and cytochrome P450 system in the endoplasmic reticulum. Exogenous sources of ROS include ultraviolet light, ionizing radiation, chemotherapeutics, inflammatory cytokines and various environment toxins.

Normally, ROS is constantly generated in the cells. The effect of the ROS depends upon on the cellular systems, and whether the cell is able to handle the ROS. In normal cellular conditions most of the ROS is used for various physiologic functions. For example, superoxide radical is used by phagocytic cells to kill bacteria, are generated as intercellular signal molecules, and used by carotid bodies for detecting the levels of oxygen in the blood. Moreover, hydrogen peroxide is used by the thyroid to make thyroid hormones and also used as an intracellular signal molecule. In contrast, if a high amount ROS can result in detrimental effects such as lipid per-oxidation, protein oxidation and DNA damage, ROS can also kill the cells.

Cells usually cope with excess ROS by either minimizing the formation of ROS or scavenges the excess ROS by an antioxidant defences system. Moreover, antioxidants can also repair the cells that have been oxidatively damaged. Therefore, antioxidant defences prevents cells from any damaging effect of ROS. The antioxidants complex includes both endogenous and diet-derived molecules. Endogenous antioxidants include superoxide dismutase enzymes (SOD), catalase enzymes, glutathione peroxidase, aldehyde dehydrogenases, and sulfiredoxin. SOD is considered as a major component of anti-oxidant response. SOD converts a superoxide, an ROS, into hydrogen peroxide, which is subsequently broken down in to water and oxygen by catalase and peroxidases. Glutathione peroxides remove hydrogen peroxide by using it to oxidize reduced glutathione to oxidized glutathione. On the other hand, α-Tocopherol, derived from the diet such as vitamin E, inhibit lipid peroxidation by peroxyl radical intermediates thus protecting cells from ROS induced damage. Other dietary antioxidants include ascorbate, β-carotene, and flavonoids.

The insight into the role of ROS in the selective killing of the motor neurons in ALS came from the discovery that the mutations in SOD1 as a primary cause of 20% of the familial ALS(Rosen et al., 1993). As already mention above, the SOD1 plays a major role as an anti-oxidant defence. Therefore, the mutation in SOD was proposed to induce degeneration of the neuron, because loss of the SOD function will lead to an increase in the levels of superoxide, which can react with nitric oxide to produce peroxynityite and also can lead to an increase in hydrogen peroxide and hydroxyl radical (Beckman et al., 1993;Deng et al., 1993;Rosen et al., 1993). Some evidence has been found that the loss of the SOD activity results in the apoptotic degeneration of the motor neurons in the spinal cord (Rothstein, 1994). Moreover, a hypothesis that mutant SOD may cause motor neuron disease was further supported by the observation that transfection of an extra normal copy of SOD into SOD1 mutant transgenic mice did not result in signs of

motor neuron degeneration , whereas, transfection with a mutant SOD produced symptoms of ALS (Gurney, 1994). In contrast, not all the research has revealed a clear association of mutant SOD1 with ALS. The ability to develop ALS in the SOD1 knockout mouse (Reaume et al., 1996) and no effect of increased and decreased levels of wild-type SOD1 in mutant SOD1(Bruijn et al., 1998) brings out the fact that mutation in the SOD1 genes is not sufficient to the cause disease. Although a large number of different types of mutations in the SOD gene have so far been identified, it is not clear whether disease is related to the inactivation of the SOD.

Besides SOD1, studies done on the postmortem tissues from patients with ALS clearly show an evidence of oxidative damage in CNS and cerebrospinal fluid. Protein carbonyl levels have been shown to be elevated in the spinal cord (Shaw et al., 1995) and motor cortex (Ferrante et al., 1997a) from ALS cases compared to the control. Moreover, 3-nitrotyosine levels were found within the spinal cord motor neuron in ALS patients (Ferrante et al., 1997b). Besides the research had found the evidence of the increase in other oxidative stress markers, such as nitric oxide (Abe et al., 1997), lipid oxidation (Shibata et al., 2001), and oxidized DNA (Ferrante et al., 1997a), in ALS patients compared to the control. Studies conducted in the cerebrospinal fluid from ALS patients have also found the evidence of oxidative stress markers in earlier stages of the disease. Such studies have observed an increased in 8-OHdG (Bogdanov et al., 2000), 4-hydroxynonenal (Smith et al., 1998), and ascorbate free radical (Ihara et al., 2005) in the cerebrospinal fluid from ALS patients. Therefore, there is no doubt that oxidative stress is clearly associated with ALS.

The exact triggers that lead to the activation to the different cellular process, including an increase in ROS, in ALS is not known. This had led to confusion as to whether oxidative stress is a primary cause of the disease or whether it a consequence of the disease. However, it is not the underlying cause of oxidative stress that matters the most, but the damaging effect of oxidative stress to the neuron is in need of better understanding. As, ALS is diagnosed after the onset of the disease and except for few familial ALS, who will develop ALS cannot be predicted with certainty. Therefore, further understanding of the association between oxidative stress and ALS is needed.

4.12 Future Directions

As mentioned above, the association between ROS and ALS is in need of a better understanding; therefore, we are planning to further evaluate this relationship. First, we have different types of transgenic zebrafish with ALS mutant genes. More importantly, we have shown that KillerRed can be manipulated to produce a variable level of ROS in the desired neurons. Therefore, we are trying to stimulate signs and symptoms of ALS by oxidatively stressing the neurons of ALS transgenic zebrafish and looking at the cellular response of the oxidative stress in those neurons.

First, we are trying to generate a stable KillerRed transgenic line. Currently, we are breeding F0 generation of Hb9-memKillerRed and ubiq-memKillerRed in hopes of getting our stable KillerRed lines.

Additionally, we are also planning to compare the exogenously oxidatively stressed motor neuron to the motor neurons that have not been exogenously oxidatively stressed in the same zebrafish. We can then evaluate to see if excessive ROS will cause any abnormalities in the growing neuron, such as the change in the length and branching pattern of the neurons. Moreover, the evidence has suggested that axon and neuromuscular junction degeneration eventually lead to the dying of the neuron in ALS (Fischer et al., 2004; Rocha et al., 2013). Likewise, axon and neuromuscular junction degeneration is considered to occur before the onset of any clinical symptoms (Fischer et al., 2004; Rocha et al., 2013). By using KillerRed, any change in the structure and function of motor neuron junction before and after oxidative stress in ALS transgenic can also be evaluated.

Moreover, degeneration of the motor neurons in ALS initially starts form one group of motor neurons, but will spread to other motor neurons as the disease progresses (Barber and Shaw, 2010; Shaw and Eggett, 2000). This might be because certain groups of motor neurons are more sensitive to undergo degeneration. Therefore, die earlier than other motor neurons. Or it might be possible that ALS usually starts from the one group of neurons and with time the disease is spread from diseased neurons to other healthy neurons. To better understand the interaction between the diseased neurons and healthy neurons in ALS, we are planning to induce oxidative stress in certain groups of neurons and analyse if they have any effect in the other healthy neurons within the same zebrafish. The result might reveal whether the pathology of ALS starts ubiquitously or has a focus starting point.

TDP-43 and FUS mislocalization has been observed as one of the pathology features of ALS. Both the TDP-43 and FUS protein, under normal conditions, are mainly localized within the nucleus but abnormal TDP-43 and FUS have been reported to be found in the cytoplasm or intranuclear as an inclusion body in ALS patients (Lagier-Tourenne et al., 2010). The effect on FUS or TDP mislocalization or formation of stress granules/aggregates before and after oxidative stress will shed further light on the cellular effect of ROS in ALS.

Finally, the pathological studies have revealed the abnormalities in the mitochondria in motor neuron from ALS patients (Lin and Beal, 2006), and an increase in ROS during aging has been hyothesized to cause mitochondrial dysfunction (Barber and Shaw, 2010). Genetically-encoded KillerRed has an ability to express the KillerRed in different subcellular locations, including the mitochondria. Mitochondrial function has not been well studied in ALS (Shaw and Eggett, 2000).Therefore, we seek to induce acute and chronic mitochondrial stress, which can encourage an aged phenotype, to address the hypothesis that age related production of ROS can damage mitochondria. Moreover, we may be able to clarify whether mitochondria dysfunction can lead to the development of ALS.

Section 5.0: conclusion

In conclusion, by taking advantage of advanced genetics, we have used the motor neuron promoter to express KillerRed specifically in the motor neuron and ubiquitin promoter to express KillerRed in every cell of the zebrafish. We have successfully demonstrated the efficacy of the KillerRed to induce oxidative stress in the neurons of the zebrafish. Moreover, by activating the KillerRed within the desired cells we were able to selectively kill the desired cells. We have also shown that the amount of oxidative stress induced in the cells by KillerRed can be controlled by manipulating the light, by either reducing the exposure time or the intensity of the light. Therefore, KillerRed is an effective tool to induce oxidative stress in the live zebrafish and will be a novel approach to study the role of ROS in ALS.

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ANIMAL RESEARCH AUTHORITY (ARA)

MACQUARIE UNIVERSITY

AEC Reference No.: 2012/050-8 **Date of Expiry:** 05 December 2014

Full Approval Duration: *06 December 2012 to 05 December 2015 (36 Months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Macquarie Uni, NSW 2109
0431 955 280

Associate Investigator:

Roger Chung	0402 808 958
Marco Morsch	0449 126 528

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above,
Laboratory Manager 9812 3607 **or Animal Welfare Officer** - 9850 7758 / 0439 497 383

Other people participating:

Emily Don	0423 387 488
Claire Winnick	0415 777 021
Andrew Badrock	0421 728 861
Yagiz Alp Aksoy	0420 246 004
Jack Stoddart	0455 493 707
Serene Sze Ling Gwee	0411 514 831
Dasha Syal	0411 147 916
Alison Hogan	0401 857 972
Anuj Dhoj Raut	0421 325 743
Rowan Radford	0403 605 754
Binyang Shi	0425 458 669
Mark Halloran	0403 731 675
Sharron Chow	0413 536 028
Isabel Formella	0421 439 154
Azher Al – Abedi	0420 219 621
Rachael Dunlop	0414 184 452

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Zebrafish models of neural disorders**
Purpose: **5 - Research: Human or Animal Health and Welfare**
Aims: **- To create fish models of inherited motor neural disorders using Zebrafish**
-To understand the processes that occur when motor neuron diseases occur
-To understand the disease and to search for cures for these presently untreatable diseases

Surgical Procedures category: 9 - Production of Genetically Modified Animals
All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
23 Fish	Zebrafish	Adult/Male&Female/-	7200	Own breed & Suppliers
23 Fish	Zebrafish	Larvae/Male&Female/-	15000	Own breed & Suppliers
		TOTAL	22200	

Location of research:

Location	*Full street address*
MND Centre, ASAM, MQ	Building F10A, Research Park Drive, Macquarie University NSW 2109

Amendments approved by the AEC since initial approval:

1. Amendment #1 - Addition of Yagiz Alp aksoy as Phd student (Exec approved 7 November 2013, ratified AEC 5 December 2013).
2. Amendment #2 - Addition of Jack Stoddart as Phd student (Approved 5 December 2013).
3. Amendment #3 - Addition of Serene Sze Ling Gwee as Phd student (Approved 5 December 2013).
4. Amendment #4 - Addition of Roger Chung as Associate Investigator (Approved 5 December 2013).
5. Amendment #5 - Addition of Dasha Syal as Research Officer (Exec approved 31 January 2014, ratified by AEC February 2014).
6. Amendment #6 - Addition of Alison Hogan as MRes student (Exec approved 31 January 2014, ratified by AEC February 2014).
7. Amendment #7 - Addition of Anuj Dhoj Raut as MRes student (Exec approved 31 January 2014, ratified by AEC February 2014).
8. Amendment #8 - Add supplier outside ASAM (as per listed in amendment request) (Approved 17 April 2014).
9. Amendment #9 - Addition of Binyang Shi (Approved 19 June 2014 AEC Meeting).
10. Amendment #10 - Addition of Marco Morsch as Associate Investigator (Approved 19 June 2014 AEC Meeting).
11. Amendment #11 - Addition of Mark Halloran as Research Assistant (Approved 19 June 2014 AEC Meeting).
12. Amendment #12 - Addition of Rowan Radford as Investigator (Approved by AEC 15 May 2014).
13. Amendment # 13 - Addition of Rachael Anne Dunlop as Associate Investigator (Ratified by AEC July 2014).
14. Amendment # 14 - Adition of Azher Al – Abedi as Associate Investigator (Ratified by AEC July 2014).
15. Amendment #15 - Addition of Sharron Chow as research Officer (Ratified by AEC July 2014) .
16. Amendment #16 - Addition of Isabel Formella as research Officer (Ratified by AEC July 2014).

Conditions of Approval:

1. Amendment #10 - Marco Morsch to attend the next Working with Research Animal Workshop (WWRAW) in July 2014.
2. Amendment #16 - Isabel Formella to attend Working with Research Animal Workshop (WWRAW) in July 2014.

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date: 17 July 2014**

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)