Cell Biology
Neuroscience

Zebrafish live imaging reveals a surprisingly small percentage of spinal cord motor neurons die during early development

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eLife Assessment

The authors have developed a biosensor for programmed cell death. They use this biosensor to provide cell death measurements in a specific early development time. The findings **useful** in a specific context; however, the application of this biosensor is **incomplete** as it does not take into account existing literature and is missing controls.

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Abstract

It is widely accepted that large numbers of neurons die during the early development of vertebrates; however, the tracking of this dying process in live animals remains challenging. Here, we generated sensor zebrafish achieving live imaging of motor neuron apoptosis at single- cell resolution. Using these sensor zebrafish, we observed for the first time that in an apoptotic motor neuron, caspase-3 activation occurred quickly within 5-6 min and at the same time between the cell body and axon. Interestingly, we found that only a surprisingly small percentage of spinal cord motor neurons died during zebrafish early development, which is quite different from the generally believed massive motor neuron death occurred in the embryonic stage of chicks, mice, rats, and humans. We also observed that most of the apoptotic bodies of dead motor neurons were not colocalized with macrophages. These sensor zebrafish can serve as powerful tools to study motor neuron apoptosis *in vivo*.

Introduction

Programmed cell death or apoptosis is a dynamic process that occurs in most tissues in live animals. In the nervous system, apoptosis plays an essential role in the regulation of neuronal cell numbers (Hollville, Romero, & Deshmukh, 2019). During the embryonic development of

vertebrates, including chicks, mice, rats, and humans, a large number of neuronal cells die through apoptosis (Fuchs & Steller, 2011). In pathological states, such as Alzheimer's disease, Parkinson's disease, and motor neuron disease, the death of neuronal cells severely impairs the normal function of the nervous system (Butterfield & Halliwell, 2019; Michel, Hirsch, & Hunot, 2016; Moujalled, Strasser, & Liddell, 2021).

When apoptosis occurs, either through intrinsic or extrinsic pathways, it ultimately leads to the cascade activation of caspases and the degradation of cellular structures, which is no exception for neuronal cells (Blanquie et al., 2017; Heck et al., 2008; Hernandez-Baltazar, Mendoza-Garrido, & Martinez-Fong, 2013). Due to the unique and complex morphology of neuronal cells, especially long axons, many important issues related to neuronal cell apoptosis have not been fully addressed, including: (1) How to visualize neuronal cell death in live animals? (2) How fast caspase-3 can be activated within a single neuron during apoptosis? (3) Which part of a neuron (cell body vs. axon) will degrade first? To address these important questions, we need to develop novel tools that can track neuronal cell apoptosis in live animals.

Previously, we have developed a fluorescence resonance energy transfer (FRET)-based biosensor, named sensor C3, for detecting cancer cell apoptosis. Our *in vitro* results showed that sensor C3 could detect the apoptosis of cancer cells both in 2D and 3D cultures by changing its color from green to blue (Anand, Fu, Teoh, & Luo, 2015; Fu, Peh, Ngan, Wei, & Luo, 2018; Hao, Huang, Wu, Peng, & Luo, 2023; K. Li, Wu, Zhou, Tong, & Luo, 2021; Luo, Vivian, Pu, & Chang, 2001; Tian, Ip, Luo, Chang, & Luo, 2007; Wu, Li, Yuan, & Luo, 2021; Yang, Jia, Zhao, & Luo, 2022). Recently, we showed that sensor C3 could also be used to indicate zebrafish skin cell apoptosis (Jia, Song, Huang, Ge, & Luo, 2020).

In the present study, using *Tol2* transposon-based transgenic technology, we generated novel sensor zebrafish and achieved the noninvasive tracking of motor neuron apoptosis at single-cell resolution in live zebrafish. More importantly, using these sensor zebrafish, we were able to obtain novel insights into the spatiotemporal properties and occurring rates of motor neuron death during early development.

Results

Generation of transgenic zebrafish expressing sensor C3 in motor neurons

Sensor C3 is a fusion protein of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), between which is an amino acid linker containing the caspase-3 cleavage site DEVD (**Figure 1A**). In live cells, when sensor C3 is excited with a 458 nm laser, due to the energy transfer between CFP and YFP, the emission light of CFP can excite the YFP to emit green fluorescence, which can be detected at the wavelength of 535 nm. In apoptotic cells, activated caspase-3 proteins cleave sensor C3 at the DEVD site; due to the disruption of the FRET effect, sensor C3 emits blue fluorescence, which can be detected at the wavelength of 480 nm (**Figure 1B**).

In our previous publications, we have conducted many experiments to validate the specificity and sensitivity of sensor C3 for detecting caspase-3 activation and apoptosis. First, we confirmed that there was a strong energy transfer from CFP to YFP in purified sensor C3 proteins when CFP was excited. Second, we showed that the cleavage to sensor C3 is specific to caspase-3 and requires the presence of the cleavage sequence of DEVD in sensor C3 proteins. Third, sensor C3 is highly sensitive to caspase-3 cleavage, as it could detect caspase-3 activation at a nanomolar concentration and in 5 min when sensor C3-labelled HeLa cells underwent UV irradiation- induced apoptosis (Luo et al., 2001).

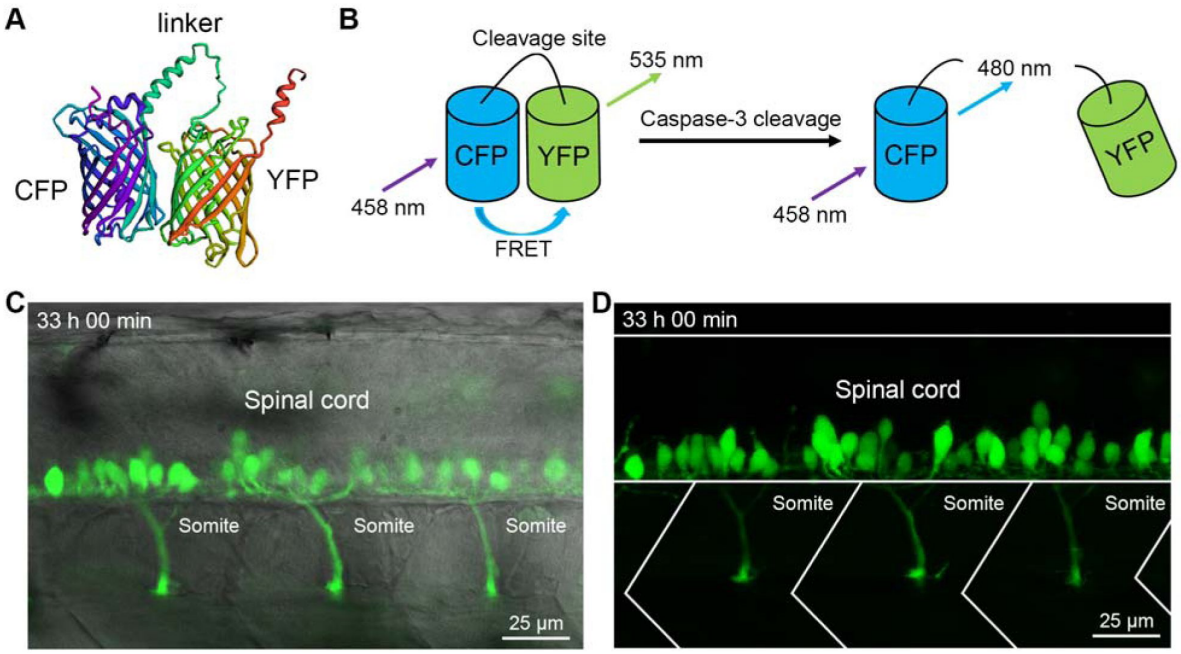

Figure 1.

The live imaging of motor neurons in sensor zebrafish.

(**A**) The structure of sensor C3 was predicted by using Rosetta software. (**B**) The principle of sensor C3 for apoptosis detection. (**C** and **D**) The expression of sensor C3 in the motor neurons of Tg(*mnx1*:sensor C3) zebrafish. The bright field image and fluorescent image were merged in (C) to show the location of motor neurons and their axons. The anatomical structures and developmental time are indicated.

We have confirmed that the apoptosis detected by sensor C3 was consistent with classical apoptotic assays, including chromatin condensation staining, caspase-3 activity assay, DNA fragmentation assay, and morphological changes such as cell shrinkage (Luo et al., 2001; Tian et al., 2007). Furthermore, we showed that sensor C3 could detect the apoptosis of various cancer cells and zebrafish cells by color change no matter whether the apoptosis was induced by UV irradiation, chemical drugs, fluidic shear stress, immune cells-mediated killing, or during normal development (Hao et al., 2023; Jia & Luo, 2021; Jia et al., 2020; K. Li et al., 2021; Luo et al., 2001; Tian et al., 2007; Yang et al., 2022). All aforementioned results show that sensor C3 is a very good tool for detecting apoptosis in live cells and animals.

To detect the death of motor neurons in live animals, we cloned sensor C3 under a motor neuron-specific promoter (Arkhipova et al., 2012; Flanagan-Steet, Fox, Meyer, & Sanes, 2005; Jao, Appel, & Wente, 2012; Rosenberg, Wolman, Franzini-Armstrong, & Granato, 2012; Stil & Drapeau, 2016) and used this construct to generate a transgenic line of sensor zebrafish Tg(*mnx1*:sensor C3) in which the motor neurons specifically expressed sensor C3 proteins. After obtaining the sensor zebrafish, we first examined the morphology of motor neurons in zebrafish. At 33 hours post fertilization (hpf), we observed many green motor neurons inside the spinal cord (**Figure 1C**, D).

Visualizing caspase-3 activation and single motor neuron apoptosis during zebrafish early development

To determine the apoptotic status of motor neurons during the development, we took the images of CFP and YFP separately from the Tg(*mnx1*:sensor C3) zebrafish and merged them into FRET images. The green color will indicate the motor neurons are alive, and the blue color will indicate the neurons are apoptotic. The FRET imaging analysis showed that around 30 hpf during zebrafish early development, the cell bodies of some motor neurons appeared in blue. In contrast, most of the cell bodies of these motor neurons appeared in green. Furthermore, the blue signals were also observed in the axon bundle of the motor neurons (**Figure 2A-D**). **Figure 2C** showed a motor neuron cluster inside of which more than one motor neuron died. Image stacks of the apoptotic motor neuron in **Figure 2D** at different optical section levels better illustrated the blue signals in the axon region (**Figure 2E**). This observation indicates that the majority of the motor neurons are alive, and the minority can undergo apoptosis.

The caspase-3 activation occurred quickly and almost at the same time between the cell body and axon in apoptotic motor neurons

Images in **Figure 2** showed that blue signals appeared in both the cell body and axon of apoptotic motor neurons. To determine which part of a motor neuron degraded first and how fast the degradation process was, we performed time-lapse imaging of caspase-3 activation in live zebrafish. FRET images in **Figure 3A** showed that at 32 h 15 min of zebrafish development, both the cell body (indicated with a white arrowhead) and the axon (indicated with a yellow arrowhead) of the indicated motor neuron were green, suggesting that caspase-3 was not activated. Just 3 min later, both the cell body and the axon started to change color (32 h 18 min). Another 3 min later, both the cell body and the axon became blue (32 h 21 min). At 32 h 27 min, two blue apoptotic bodies could be observed in the cell body region, and multiple apoptotic bodies were also observed in the axon region. In the next 12 min (until 32 h 39 min), this apoptotic motor neuron gradually degraded into more and smaller apoptotic bodies (**Figure 3A**; Video S1).

The time-lapse imaging of another apoptotic motor neuron showed similar results (**Figure 3B**; Video S2). The color of both the cell body and axon of the motor neuron changed from green to blue within 5 min (from 30 h 45 min to 30 h 50 min). This apoptotic motor neuron gradually degraded into apoptotic bodies in the next 15 min (from 30 h 50 min to 31 h 05 min). These images

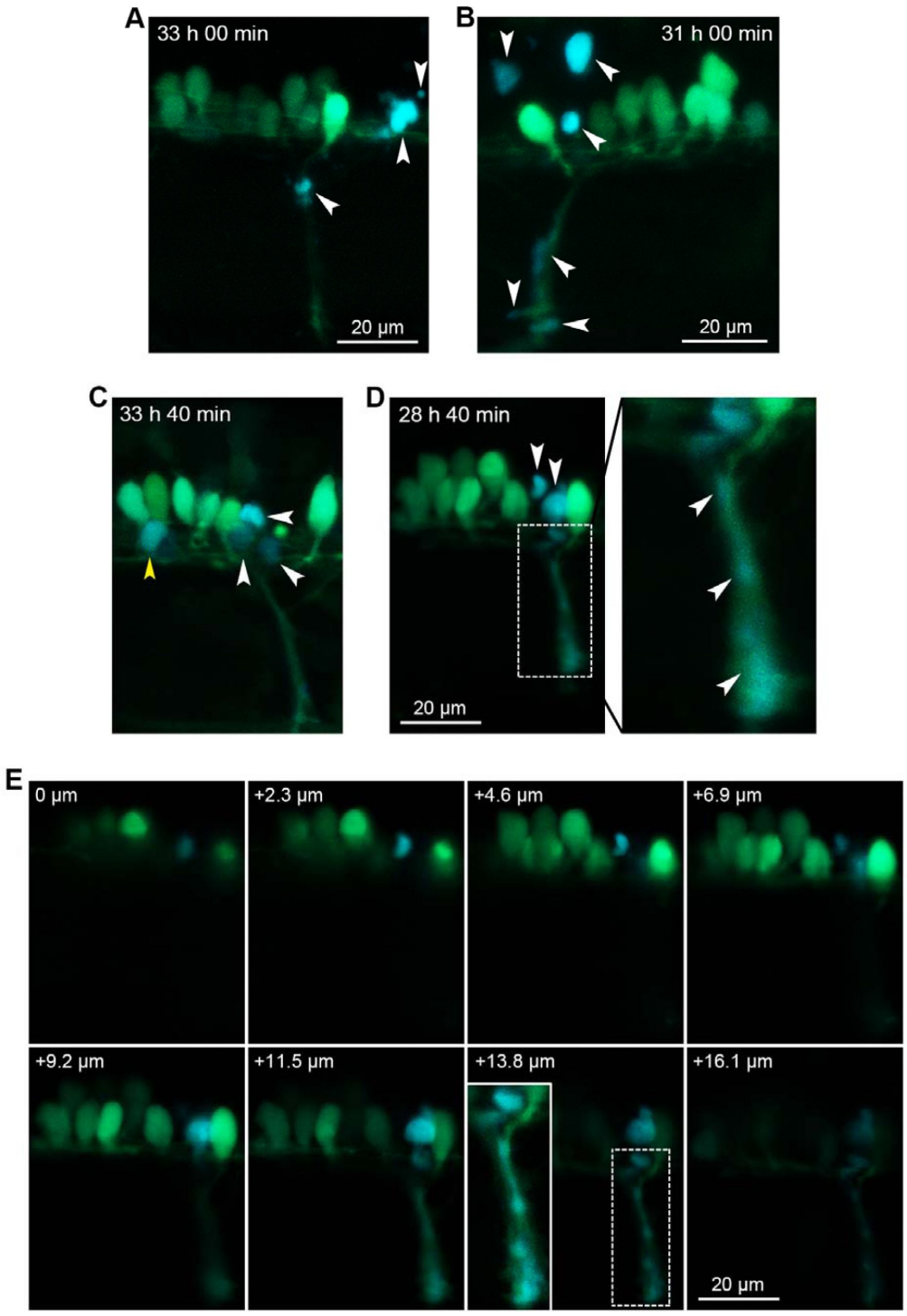

Figure 2.

Visualizing caspase-3 activation and single motor neuron apoptosis during zebrafish early development.

(**A**–**D**) FRET images of four somites showing motor neurons underwent apoptosis during the development of Tg(*mnx1*:sensor C3) zebrafish. Two dead motor neurons in (C) are indicated with yellow and white arrowheads, respectively. The axon in (D) is enlarged for a better illustration. Blue apoptotic signals from cell bodies and axons are indicated with arrowheads. The developmental time is indicated in each image. (**E**) Z-stack imaging showed the apoptosis of the motor neuron in (D). The axon region in the stack of 13.8 μm is enlarged for a better illustration. The depth of each stack is indicated in each image.

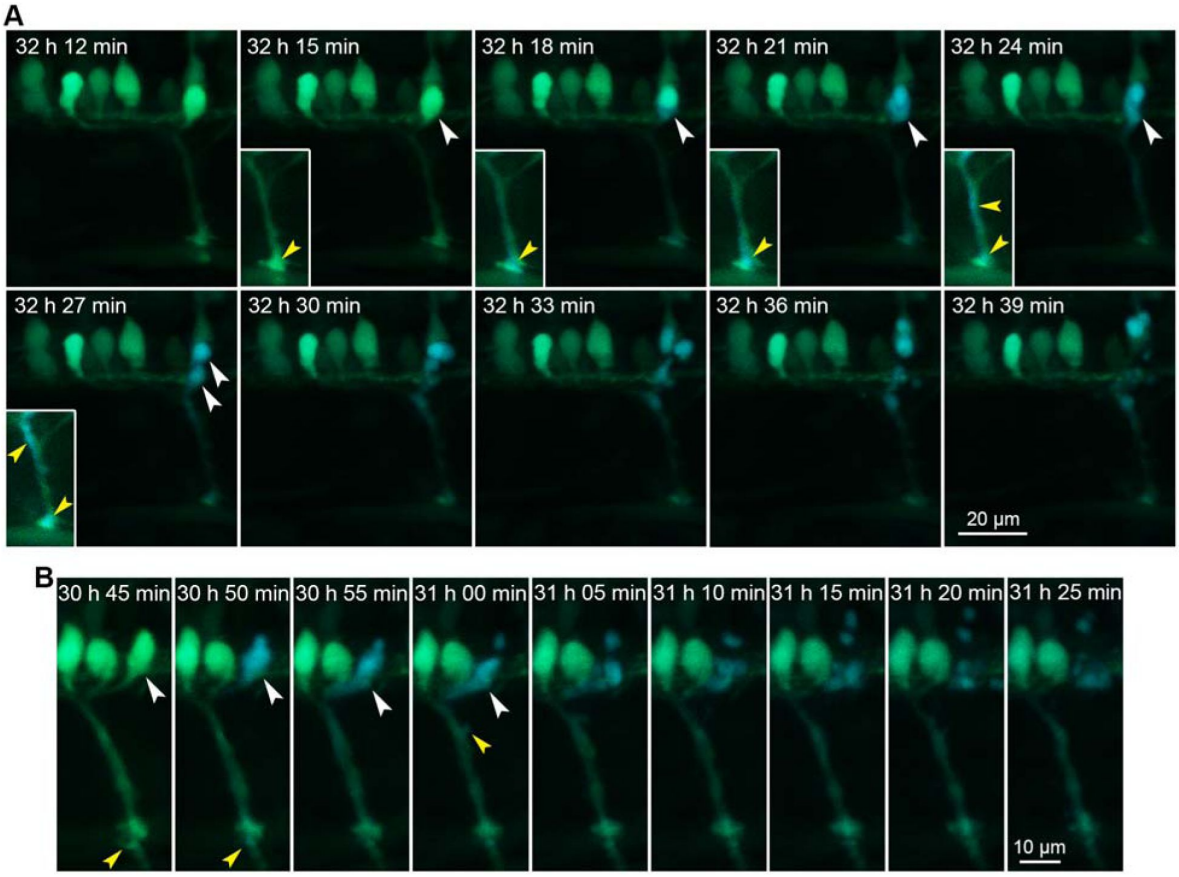

Figure 3.

The caspase-3 activation occurred quickly and almost at the same time between the cell body and axon in apoptotic motor neurons.

(**A**) Tracking of motor neuron apoptosis during zebrafish development. During tracking, one motor neuron changed from green to blue. The cell body is indicated with white arrowheads. The axon is enlarged for a better illustration and indicated with yellow arrowheads. The developmental time is indicated in each image. (**B**) Tracking of the apoptotic process in another motor neuron during zebrafish development. The cell body is indicated with white arrowheads, and the axon is indicated with yellow arrowheads. The developmental time is indicated in each image.

showed that the activation of caspase-3 can occur within 5-6 min in both the cell body and axon of an apoptotic motor neuron. Thus, we conclude that the caspase-3 activation in the cell body and axon of a dying motor neuron occurred almost at the same time.

Only a small percentage of motor neurons died during zebrafish early development

In the last several decades, it has been widely accepted that during the embryonic development of vertebrates, massive neuronal cells in various regions of the nervous system will die. (Castillo-Ruiz, Hite, Yakout, Rosen, & Forger, 2020; Dekkers, Nikoletopoulou, & Barde, 2013; Kandel, Schwartz, Jessell, Siegelbaum, & Hudspeth, 2012; Lossi & Merighi, 2003; Rubin, 1997).

Regarding motor neuron development, chick embryos have served as the most studied models. Previous studies showed that half of motor neurons in the lateral motor column of the spinal cord died between day 5 to day 10 of incubation during the development of chick embryos (Caldero et al., 1998; Hamburger, 1975; Oppenheim, 1981; Weill, 1991). In addition to the motor neurons in the lateral motor column, a large number of motor neurons in other parts of the spinal cord also die during embryonic development of chicks (Yamamoto & Henderson, 1999). Similar motor neuron death rates were also obtained in mice (E11.5-E15.5), rats (E15-E18), and humans (11-25 weeks of gestation) during early development (Forger & Breedlove, 1987; Harris & McCaig, 1984; Raoul, Henderson, & Pettmann, 1999; Weill, 1991; Yamamoto & Henderson, 1999).

Although the motor neuron death time windows vary in different species, the common feature of these time windows is that they are all the developmental periods when motor neurons contact with muscle cells. The contact between zebrafish motor neurons and muscle cells occurs before 72 hpf (Kaslin & Ganz, 2020; Myers, Eisen, & Westerfield, 1986; Ott, Diekmann, Stuermer, & Bastmeyer, 2001; Panzer et al., 2005; Schmidt, Strähle, & Scholpp, 2013). Most organs of zebrafish form before 72 hpf, and they complete hatching before 72 hpf. Food-seeking and active avoidance behaviors also start at 72 hpf (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995).

To cover the potential time window of motor neuron death, we performed the noninvasive FRET imaging as long as possible: from 24 hpf to 240 hpf. After 240 hpf, the transparency of zebrafish body decreased dramatically, which made optical imaging quite difficult. Then, we counted the number of apoptotic motor neurons in the whole spinal cord from 24 hpf to 240 hpf. The data showed that the apoptosis of motor neurons mainly occurred between 24–48 hpf and peaked between 30–36 hpf (**Figure 4A**, B). Unexpectedly, only a tiny portion (1.6% at 30 hpf and 1.0% at 36 hpf) of the motor neurons underwent apoptosis (**Figure 4C**), which is much lower than the death rates in other animal models and humans.

To validate these results, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays in zebrafish frozen sections. The motor neurons of the sensor zebrafish were identified by immunofluorescence staining using an anti-GFP antibody plus a secondary antibody conjugated with a green dye, and the apoptotic motor neurons were detected by red TUNEL signals (**Figure 4D**). We analyzed 1,045 motor neurons at 30 hpf, 13 of which were TUNEL-positive, with an apoptotic rate of 1.2%. We also analyzed 652 motor neurons at 36 hpf, 5 of which were TUNEL-positive, with an apoptotic rate of 0.8% (**Figure 4E**). These results showed that the low apoptotic rates detected by sensor C3-based FRET imaging could be confirmed by the conventional TUNEL assays.

Most dead motor neurons were not colocalized with macrophages

Thus far, we have shown that sensor zebrafish can be used to detect motor neuron apoptosis. The subsequent question is how the apoptotic bodies of these dead motor neurons are removed.

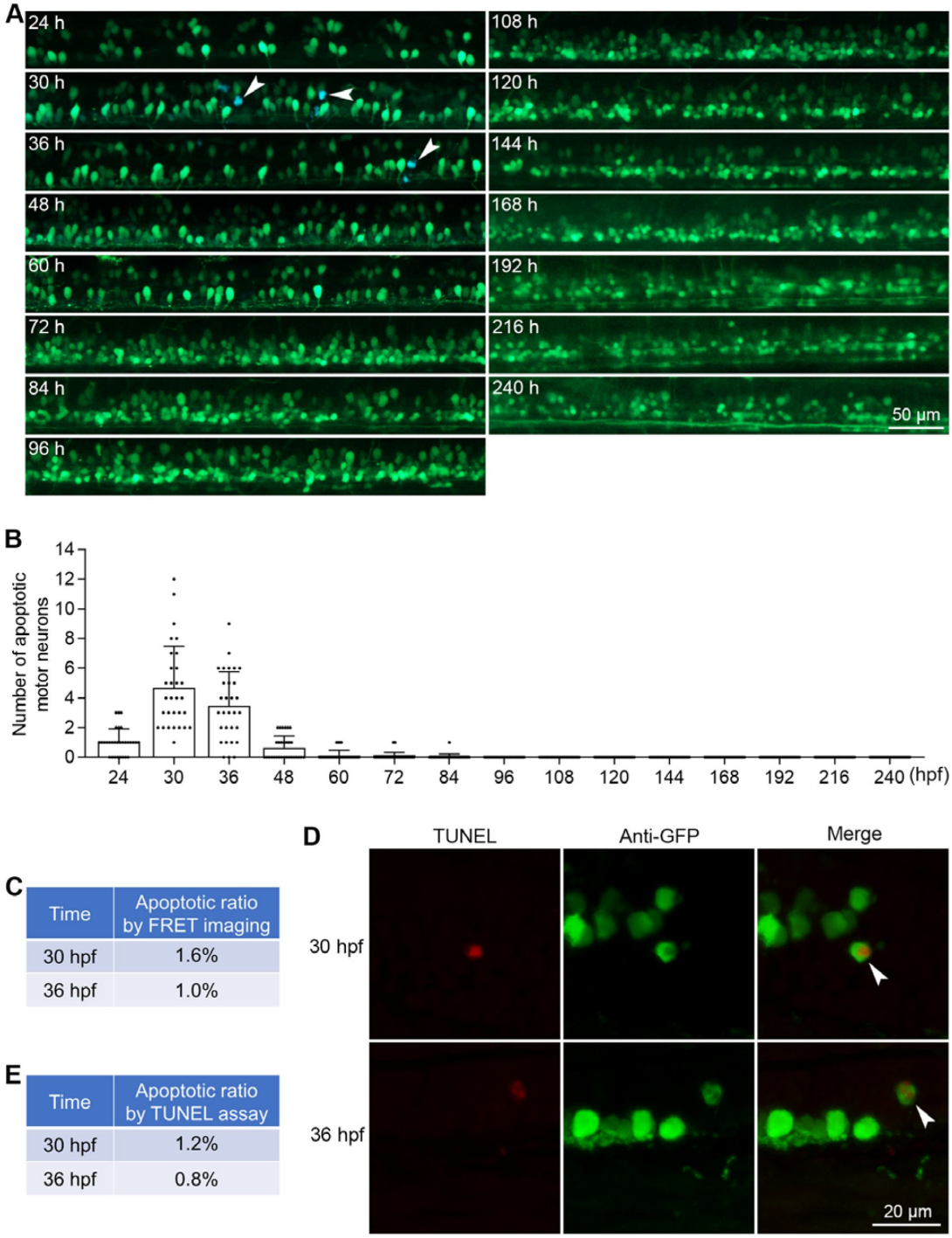

Figure 4.

Only a small percentage of motor neurons died during zebrafish early development.

(**A**) The tracking of motor neuron apoptosis by FRET imaging during zebrafish early development. White arrowheads indicate apoptotic motor neurons. (**B**) The quantified results show the number of apoptotic motor neurons at each time point during zebrafish early development (n = 30 zebrafish for each time point). (**C**) The percentages of apoptotic motor neurons among total motor neurons at 30 hpf and 36 hpf. (**D**) TUNEL assays showing apoptotic motor neurons at 30 hpf and 36 hpf. Immunofluorescence staining with an anti-GFP antibody was used to indicate motor neurons expressing sensor C3 proteins. White arrowheads indicate TUNEL-positive motor neurons. (**E**) The percentages of apoptotic motor neurons detected by TUNEL assays.

Macrophages are the major phagocytes to engulf apoptotic bodies in animals (Nagata & Segawa, 2021); therefore, we focused on macrophages. We fully utilized the advantages of sensor zebrafish, which label motor neurons with green fluorescence, and another transgenic zebrafish line, Tg(*mpeg1*:mCherry), which labels macrophages with a red fluorescent protein (Ellett, Pase, Hayman, Andrianopoulos, & Lieschke, 2011). Live imaging showed that red macrophages distributed everywhere in the zebrafish body at 36 hpf (**Figure 5A**). Interestingly, after examining 164 apoptotic motor neurons from Tg(*mnx1*:sensor C3) zebrafish, we only found one case of macrophage colocalization with a dead motor neuron, in which the macrophage indicated with a yellow arrowhead was colocalized with the apoptotic bodies of the axon part in the somite, which were indicated with a white arrowhead (**Figure 5B**). The colocalization percentage was only 0.6% (**Figure 5C**). These results indicated that the apoptotic bodies of dead motor neurons in the spinal cord might not be cleared by macrophages.

Discussion

By expressing FRET-based sensor C3 in zebrafish, for the first time, we were able to monitor the whole process of caspase-3 activation in single motor neurons and the subsequent breakdown of these dead neurons into apoptotic bodies during early development. We found that caspase-3 was activated within 5-6 min in an individual apoptotic motor neuron, while the caspase-3 in the rest of the neurons in the same bundle was not activated. More importantly, within the same motor neuron, although the cell body is located in the spinal cord and the axon is extended into the muscle tissue, the caspase-3 activation occurred almost at the same time of observation. These data indicate a synchronized activation pattern of caspase-3 in apoptotic motor neurons.

Previous studies showed that some variable primary (VaP) motor neurons died before 36 hpf during zebrafish early development (Eisen, 1992; Eisen, Pike, & Romancier, 1990). Our study examined the overall motor neuron apoptosis regardless of the causes and locations, so we did not limit our observation to VaP motor neurons. The dead motor neurons in panels A, B, and D of **Figure 2** are very likely VaP motor neurons. Besides VaP motor neurons, sensor zebrafish can also capture the death of other types of motor neurons. The reasons are as follows: (1) VaP primary motor neurons die before 36 hpf, but our study found motor neurons died after 36 hpf and even at 84 hpf (**Figure 4B**). (2) The position of the VaP motor neuron is together with that of the caudal primary motor neuron, that is, at the caudal region of the motor neuron cluster.

Although it's rare, we did observe the death of motor neurons in the rostral region of the motor neuron cluster (**Figure 2C**). (3) There is only one or zero VaP motor neuron in each motor neuron cluster. Although our data showed that usually one motor neuron died in each cluster, we did observe that sometimes more than one motor neuron died in the motor neuron cluster (**Figure 2C**).

It is generally accepted that during the development of vertebrates, approximately half of the motor neurons are removed by apoptosis. However, our results showed that motor neurons were not prone to death during zebrafish early development: only a small percentage of motor neurons died of apoptosis during the early development of zebrafish. This large discrepancy may be the unique properties of zebrafish compared with chicks, mice, rats, and humans, which could be further investigated. In our manuscript, we tracked the motor neuron death in live zebrafish until 240 hpf, which was the longest time window we could achieve. But there was still a possibility that zebrafish motor neurons might die after 240 hpf. Another possibility is that the limitations of conventional apoptosis detection methods, such as the TUNEL assay (Loo, 2011), nuclear morphology staining (H. Li et al., 2000), and caspase-3 staining (Wang et al., 2010), may potentially exaggerate the number of apoptotic cells in tissue sections. For example, the specificity of antibodies for apoptotic markers or the quality of other reagents in experiments may not be

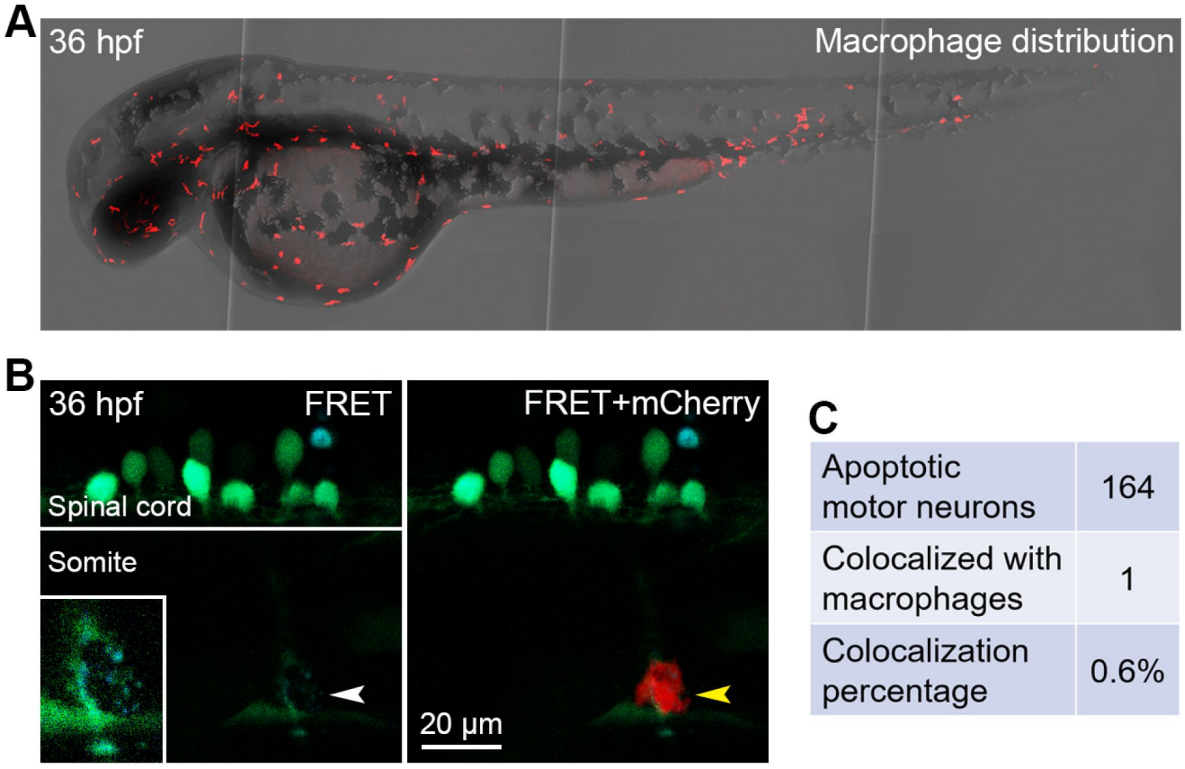

Figure 5.

Most dead motor neurons were not colocalized with macrophages.

(**A**) Live imaging showing the distribution of macrophages in zebrafish body at 36 hpf. (**B**) An example showing the colocalization between apoptotic bodies derived from the axon of a motor neuron and a macrophage in a somite at 36 hpf. The apoptotic bodies are indicated with a white arrowhead and enlarged in the box in the left image. The colocalization is indicated with a yellow arrowhead in the right image. The anatomical structures are indicated. (**C**) The percentage of apoptotic motor neurons colocalized with macrophages. The total number of apoptotic motor neurons observed and the number of apoptotic motor neurons colocalized with macrophages are listed.

good enough, which can result in false positive apoptotic signals. Besides, these staining methods have difficulties in distinguishing neuronal cells and nearby glial cells; thus, dead glial cells may also be counted as dead neuronal cells.

The removal of dead cells by phagocytes can effectively reduce the occurrence of inflammation, which is very important for animals (Arandjelovic & Ravichandran, 2015). Our study showed that macrophages, although serving as professional phagocytes, were not colocalized with the apoptotic motor neurons. Thus, some other cells in the spinal cord may help to engulf the dead cells. For example, a recent zebrafish study reported that neural crest cells can help remove apoptotic cells in the nervous system during development (Zhu et al., 2019). In the future, we will investigate whether red fluorescent protein-labeled neural crest cells could engulf dead motor neurons in our sensor zebrafish.

During the past several decades, the noninvasive tracking of neuronal cell apoptosis under physiological conditions in live animals remains challenging. Since the development of fluorescent proteins, several FRET-based protein biosensors have been designed for the detection of apoptosis in live cells cultured in petri dishes (Ai, Hazelwood, Davidson, & Campbell, 2008; Albeck et al., 2008; Karasawa, Araki, Nagai, Mizuno, & Miyawaki, 2004; Mahajan, Harrison- Shostak, Michaux, & Herman, 1999; Rehm et al., 2002; Tyas, Brophy, Pope, Rivett, & Tavaré, 2000; Xu et al., 1998). However, unlike the good performances of these biosensors *in vitro*, the *in vivo* applications of these biosensors have been unsatisfactory because of their small dynamic ranges and the complex optical environments in live animals (Andrews et al., 2016; Takemoto et al., 2007; Takemoto, Nagai, Miyawaki, & Miura, 2003; Yamaguchi et al., 2011). We compared sensor C3 with other FRET-based apoptotic biosensors and found that sensor C3 can achieve a FRET effect of 4- to 5-fold, while other biosensors usually had FRET effects of less than or near 2-fold. This high FRET effect makes sensor C3 the ideal biosensor for visualizing neuronal cell apoptosis in live animals where other biosensors encounter difficulties.

In summary, our results showed that sensor zebrafish can serve as valuable tools for studying motor neuron apoptosis in live zebrafish, which could improve our understanding of neuronal cell apoptosis during normal development and in neurodegenerative diseases.

Materials and methods

Zebrafish maintenance

Zebrafish (AB strain) were raised at 28 °C in a ZebTEC system. The photoperiod was 14 h of light and 10 h of darkness. Zebrafish were fed with artemia twice a day. The experiments in this study were approved by the Animal Research Ethics Committee of the University of Macau (Protocol ID: UMARE-032-2016).

Generation of sensor zebrafish

The zebrafish genome was extracted using the DNeasy Blood & Tissue Kit (Qiagen, 69504). A zebrafish motor neuron-specific promoter *mnx1* was amplified by polymerase chain reaction (forward primer, ACGCGTCGACGAATTCATTTAAATTAGCCTGGCATC, reverse primer, CCCACCGGTCTGGCCCACCTCACAAACAGATTA). The promoter sequence and sensor C3 gene were cloned into the same plasmid backbone to generate the transgenic vector, named pSK- *mnx1*-C3. *Tol2* mRNA was obtained by *in vitro* transcription using the mMESSAGE mMACHINE SP6 transcription kit (Ambion, AM1340). The transgenic vector containing the sensor C3 gene (final concentration, 50 ng/μL) and *Tol2* mRNA (final concentration, 100 ng/μL) were mixed and injected (2-4 nL) into newly fertilized zebrafish eggs using an MPPI-3 pressure injector. The zebrafish

embryos with green fluorescence indicating sensor C3 expression were selected under a stereomicroscope with a GFP filter. These positive zebrafish were cultured to sexual maturity and crossed with wild-type zebrafish to obtain transgenic sensor zebrafish.

In vivo FRET imaging of neuronal cell apoptosis

Sensor zebrafish embryos or larvae were anesthetized with 0.016% tricaine (Sigma-Aldrich, E10521) solution. Anesthetized zebrafish were embedded in low melting point agarose gel (Promega, V2111) in a confocal dish. The zebrafish were then imaged using a Carl Zeiss LSM 710 or LSM880 confocal laser scanning microscope. For FRET imaging, a 458 nm laser was used to excite sensor C3 proteins, and the emissions were collected simultaneously in two channels: 460-500 nm was collected as the CFP channel, designated as blue, and 520-550 nm was collected as the YFP channel, designated as green. Then, the CFP and YFP images were merged to generate the FRET images. In live neuronal cells, sensor C3 emitted green fluorescence when CFP was excited because of the energy transfer from CFP to YFP, while in apoptotic neuronal cells, activated caspase-3 proteins cleaved sensor C3 at the DEVD site, and the FRET effect was abolished. Thus, sensor C3 emitted blue fluorescence when CFP was excited under the same condition.

Immunofluorescence and TUNEL assay

Tg(*mnx1*:sensor C3) zebrafish were fixed in 4% paraformaldehyde at 4 °C for 12 h. The fixed zebrafish were dehydrated in 30% sucrose at 4 °C for 48 h. The zebrafish were then embedded in Shandon Cryomatrix embedding resin (Thermo Scientific, 6769006), and frozen sections were cut at 25 μm using a Leica CM3050S cryostat. The sections were washed with phosphate- buffered saline (PBS) three times to remove the embedding resin and then were blocked with 1% bovine serum albumin with 0.1% Triton X-100 in PBS for 1 h at room temperature (RT). Then, the sections were coated with anti-GFP primary antibody (Cell Signaling, 2956, 1:400) for 2 h at RT. After that, the sections were washed with PBS three times and then coated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Thermo Scientific, A-11034, 1:200) for 1 h at RT. The sections were washed with PBS three times. The terminal deoxynucleotidyl transferase and Cy3-dUTP labeling solution in the TUNEL kit (Beyotime, C1089) were mixed.

The sections were then incubated with this reaction mixture for 1 h at 37 °C. After the incubation, the sections were washed with PBS three times and then mounted with Mowiol mounting medium (Millipore, 475904). The sections were imaged with a Carl Zeiss LSM880 confocal laser scanning microscope with 488 nm and 561 nm lasers.

In vivo imaging of macrophages

Tg(*mnx1*:sensor C3) zebrafish were crossed with Tg(*mpeg1*:mCherry) zebrafish to get offspring that neuronal cells and macrophages were labeled with green and red, respectively. The zebrafish were then imaged using a Carl Zeiss LSM880 confocal laser scanning microscope. FRET imaging was applied to detect apoptotic neuronal cells. At the same time, a 561 nm laser was used to excite the mCherry proteins for the visualization of macrophages.

Statistical analysis

All of the data are expressed as the means ± SD. Statistical significance was judged by one- or two-way ANOVA using GraphPad Prism 7 software.

Supplementary materials

Supplementary materials can be found online.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary materials.

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Additional information

Author contributions

H.J. and K.Q.L. designed the experiments. H.J. and H.Y. performed the experiments. H.J. and K.Q.L. analyzed the data. H.J. prepared the manuscript. K.Q.L. revised the manuscript. K.Q.L. supervised the study.

Additional files

Video S1

Video S2

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Joint Public Review:

Summary:

Jia and colleagues developed a fluorescence resonance energy transfer (FRET)-based biosensor to study programmed cell death in the zebrafish spinal cord. They applied this tool to study death of zebrafish spinal motor neurons.

Strengths:

Their analysis shows that the tool is a useful biosensor of motor neuron apoptosis in living zebrafish and can reveal which part of the neuron undergoes caspase activation first.

Weaknesses:

As far as it is possible to tell, the authors focus on death of motor neurons innervating axial muscles. Previous work from over 30 years ago revealed that only a small number of these motor neurons die early in development. So this is not new, although following the cells and learning details of their apoptosis is new. Most of the work on motor neuron death in tetrapods was carried out on limb innervating motor neurons. Zebrafish have paired pectoral and pelvic fins, homologs of tetrapod paired limbs. These fins are innervated by distinct sets of motor neurons in zebrafish, as they are in tetrapods. However, the authors have not focused on these particular motor neurons, and thus have not made a fair comparison with tetrapods. In fact, they do not tell us which spinal levels they observed or whether they have been consistent from animal to animal. Pelvic fins emerge much later than pectoral fins in zebrafish, so it is possible that the time frame during which the authors imaged motor neuron death does not include motor neurons innervating pelvic fins.

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Author response:

The following is the authors' response to the previous reviews.

> ***Reviewer 1:***
>
> *(1) The results do not support the conclusions. The main "selling point" as summarized in the title is that the apoptotic rate of zebrafish motorneurons during development is strikingly low (~2%) as compared to the much higher estimate (~50%) by previous studies in other systems. The results used to support the conclusion are that only a small percentage (under 2%) of apoptotic cells were found over a large population at a variety of stages 24-120hpf. This is fundamentally flawed logic, as a short-time window measure of percentage cannot represent the percentage on the long-term. For example, at any year under 1% of human population die, but over 100 years >99% of the starting group will have died. To find the real percentage of motorneurons that died, the motorneurons born at different times must be tracked over long term, or the new motorneuron birth rate must be estimated. Similar argument can be applied to the macrophage results.*

In the revised manuscript (revised Figure 4), we extended the observation time window as long as possible, from 24 hpf to 240 hpf. After 240 hpf, the transparency of zebrafish body decreased dramatically, which made optical imaging quite difficult.

We are confident that this 24-240 hpf time window covers the major time window during which motor neurons undergo programmed cell death during zebrafish early development. We chose the observation time window based on the following two reasons: 1) Previous studies showed that although the time windows of motor neuron death vary in chick (E5-E10), mouse (E11.5-E15.5), rat (E15-E18), and human (11-25 weeks of gestation), the common feature of these time windows is that they are all the developmental periods when motor neurons contact with muscle cells. The contact between zebrafish motor neurons and muscle cells occurs before 72 hpf, which is included in our observation time window. 2) Most organs of zebrafish form before 48-72 hpf, and they complete hatching during 48-72 hpf. Food-seeking and active avoidance behaviors also start at 72 hpf, indicating that motor neurons are fully functional at 72 hpf.

Previous studies in zebrafish have shown that the production of spinal cord motor neurons largely ceases before 48 hpf, and then the motor neurons remain largely constant until adulthood (doi: 10.1016/j.celrep.2015.09.050; 10.1016/j.devcel.2013.04.012; 10.1007/BF00304606; 10.3389/fcell.2021.640414). Our observation time window covers the major motor neuron production process. Therefore, we believe that neurogenesis will not affect our findings and conclusions.

Although we are confident that 240 h tracking is long enough to measure the motor neuron death rate, several sentences have been added in the discussion part, "In our manuscript, we tracked the motor neuron death in live zebrafish until 240 hpf, which was the longest time window we could achieve. But there was still a possibility that zebrafish motor neurons might die after 240 hpf."

We agreed that the "2%" description might not be very accurate. Thus, we have revised our title to "Zebrafish live imaging reveals a surprisingly small percentage of spinal cord motor neurons die during early development."

> *(2) The conclusion regarding timing of axon and cell body caspase activation and apoptosis timing also has clear issues. The ~minutes measurement are too long as*

> *compared to the transport/diffusion timescale between the cell body and the axon, caspase activity could have been activated in the cell body and either caspase or the cleaved sensor move to the axon in several seconds. The authors' results are not high frequency enough to resolve these dynamics. Many statements suggest oversight of literature, for example, in abstract "however, there is still no real-time observation showing this dying process in live animals.".*

Real-time imaging of live animals is quite challenging in the field. Currently, using confocal microscopy, we can only achieve minute-scale tracking. In the future, with more advanced imaging techniques, the sensor fish in the present study may provide us with more detailed information on motor neuron death. We have removed “real-time” from our revised manuscript. We also revised the mentioned sentence in the abstract.

> *(3) Many statements should use more scholarly terms and descriptions from the spinal cord or motorneuron, neuromuscular development fields, such as line 87 "their axons converged into one bundle to extend into individual somite, which serves as a functional unit for the development and contraction of muscle cells"*

We have removed this sentence.

> *(4) The transgenic line is perhaps the most meaningful contribution to the field as the work stands. However, mnx1 promoter is well known for its non-specific activation - while the images do suggest the authors' line is good, motorneuron markers should be used to validate the line. This is especially important for assessing this population later as mnx1 may be turned off in mature neurons. The author's response regarding mnx1 specificity does not mitigate the original concern.*

The mnx1 promoter has been widely used to label motor neurons in transgenic zebrafish. Previous studies have shown that most of the cells labeled in the mnx1 transgenic zebrafish are motor neurons. In this study, we observed that the neuronal cells in our sensor zebrafish formed green cell bodies inside of the spinal cord and extended to the muscle region, which is an important morphological feature of the motor neurons.

Furthermore, a few of those green cell bodies turned into blue apoptotic bodies inside the spinal cord and changed to blue axons in the muscle regions at the same time, which strongly suggests that those apoptotic neurons are not interneurons.

In fact, no matter what method is used, such as using antibodies to stain specific markers to label motor neurons, 100% specificity cannot be achieved. More importantly, although the mnx1 promoter might have labeled some interneurons, this will not affect our major finding that only a small percentage of spinal cord motor neurons die during the early development of zebrafish.

> ***Reviewer 2:***
>
> *(1) Title: The 50% figure of motor neurons dying through apoptosis during early vertebrate development is not precisely accurate. In papers referenced by the authors, there is a wide distribution of percentages of motor neurons that die depending on the species and the spinal cord region. In addition, the authors did not examine limb-innervating motor neurons, which are the ones best studied in motor neuron programmed cell death in other species. Thus, a better title that reflects what they actually show would be something like "A surprisingly small percentage of early developing zebrafish motor neurons die through apoptosis in non-limb innervating regions of the spinal cord."*

In fish, there are no such structures as limbs, although fins may be evolutionarily related to limbs. In our manuscript, we studied the naturally occurring motor neuron death in the whole spinal cord during the early stage of zebrafish development. The death of motor neurons in limb-innervating motor neurons has been extensively studied in chicks and rodents, as it is easy to undergo operations such as amputation. However, previous studies have shown this dramatic motor neuron death occurs not only in limb-innervating motor neurons but also in other spinal cord motor neurons (doi: 10.1006/dbio.1999.9413).

We have revised our title to “Zebrafish live imaging reveals a surprisingly small percentage of spinal cord motor neurons die during early development.”

> *(2) lines 18-19: "embryonic stage of vertebrates" is very broad, since zebrafish are also vertebrates; it would be better to be more specific*
>
> *lines 25-26: The authors should be more specific about which animals have widespread neuronal cell death.*

We have revised our manuscript accordingly.

> *(3) lines 98-99; 110-111; 113; 122-123; 140-141: A cell can undergo apoptosis. But an axon, which is only part of a cell, cannot undergo apoptosis. Especially since the axon doesn't have a separate nucleus, and the definition of apoptosis usually includes nuclear fragmentation. A better subheading would describe the result, which is that caspase activation is seen in both the cell body and the axon.*

We have revised the subheadings and related words in the manuscript accordingly. In the introduction, we also revised the expression of the third aim from “Which part of a neuron (cell body vs. axon) will die first?” to “Which part of a neuron (cell body vs. axon) will degrade first?”.

> *(4) lines 159-160; 178-179: This is an oversimplification of the literature. The authors should spell out which populations of motor neuron have been examined and say something about the similarities and difference in motor neuron death.*

We have revised it accordingly.

> *(5) lines 200; 216: The authors did not observe macrophages engulfing motor neurons. But that does not mean that they cannot. Making the conclusion stated in this subheading would require some kind of experiment, not just observations.*

We did observe few colocalizations of macrophages and dead motor neurons. To more accurately express these data, in the revised manuscript, we used “colocalization” to replace “engulfment.” The subheading has been revised to “Most dead motor neurons were not colocalized with macrophages.” Accordingly, panel C of Figure 5 has also been revised.

> *(6) lines 234-246: The authors seem to have missed the point about VaP motor neuron death, which was two-fold. First, VaP death has been previously described, thus it could serve as a control for the work in this paper, especially since the conditions underlying VaP death and survival have been experimentally tested. Second, they should acknowledge that previous work showed that at least some motor neuron death in zebrafish differs from that described in chick and rodents. This conclusion came from work showing that death of VaP is independent of limitations in muscle innervation area, suggesting it is not coupled to muscle-derived neurotrophic factors.*

> *Figures: The authors should say which level of the spinal cord they examined in each figure.*

We have compared our findings with previous findings in the revised manuscript. The death of VaP motor neurons is not related to neurotrophic factors, but the death of other motor neurons may be related to neurotrophic factors, which needs further study and evidence. Our study examined the overall motor neuron apoptosis regardless of the causes and locations. To avoid misunderstanding, in the revised manuscript, we removed the data and words related to neurotrophic factors.

We also extended the observation time window as long as possible, from 24 hpf to 240 hpf (revised Figure 4). After 240 hpf, the transparency of zebrafish body decreased dramatically, which made the optical imaging quite difficult.

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