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A novel fluorescent timer based on bicistronic expression strategy in *Caenorhabditis elegans*

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ABSTRACT

Fluorescent timers are useful tools for studying the spatial and temporal cellular or molecular events. Based on the trans-splicing mechanism in *Caenorhabditis elegans*, we constructed a "fluorescent timer" through bicistronic expression of two fluorescent proteins with different maturation times. When used in vivo, this "timer" changes its color over time and therefore can be used to monitor the activity of the targeted promoters in *C. elegans*. Using this "timer", we have successfully traced the time-dependent activity of *myo-3* promoter which drives expression in body wall muscle and vulval muscle. We found that the *myo-3* promoter started to be active about 7 h after egg-laying and sustained its activity in the following hatching process. We have also determined the *myo-3* promoter activity during larval development by this "timer". We anticipate that more new "fluorescent timers" with variable time-resolution could be designed by bicistronic expression of different fluorescent protein pairs.

1. Introduction

Since the discovery of green fluorescent protein (GFP) in jellyfish *Aequorea victoria* [1] and the demonstration of the value of GFP as a luminous genetic tag for various biological phenomenon [2], GFP and its mutants and relatives have been employed and used widely in biological research. These fluorescent tools allow researchers to monitor in time and space of molecular and cellular process in living cells and organisms like gene expression, protein localization, protein–protein interaction, trafficking, cell division and so on. However, fluorescent timers which change colors with time could be especially valuable to the knowledge of temporal and spatial cellular events.

The first fluorescent timer (E5) generated using error-prone polymerase chain reaction was described by Terskikh and colleagues in 2000 [3]. This DsRed [4,5] derived fluorescent protein that changes its color from green to red in a time-dependent manner provides the ability to visualize the promoter activity as well as cell differentiation. More recently, Subach et al. have developed new monomeric fluorescent timers by directed molecular evolution strategy using mCherry, one monomeric variants of DsRed [6]. They created three mCherry-derived monomeric variants with different maturation rates that change colors from blue to red over time, and subsequently used these timers to monitor the intracellular trafficking of lysosomal receptor LYMP-2A.

Here we demonstrated that new fluorescent timers could also be generated by bicistronic expression of well-studied fluorescent proteins with different maturation times. We tested this strategy in a very important model organism *Caenorhabditis elegans* based on the SL2 trans-splicing mechanism [7,8] that permits the bicistronic expression of two transgenes under the control of a single promoter. Using this "timer", we have determined the time-dependent activity of *myo-3* promoter in different cell types, as to partially visualize the process of muscle development of *C. elegans*.

2. Materials and methods

2.1. Molecular biology

The SL2 trans-splicing signal (SL2 for short) that used in this study is a minimal size of *gpd-2* and *gpd-3* intergenic sequence which is about 160 bp long [9]. This sequence was PCR amplified from genomic DNA of worms of *C. elegans* Bristol N2 strain, and fused to the upstream of the gene of interest by overlap extension PCR. Primers used to amplify the SL2 trans-splicing signal sequence are as follows: 5′-GTCTCTTCTCAATAAAGGTTG-3′ and 5′-CATGATGCGTTGAAGCAGTTTC-3′. The 2.4 kb *myo-3* promoter containing vector is pPD96.52 (Fire vector kit, 1995). PCR fragment of GFP was amplified from pPD95.67 (Fire vector kit, 1995). tdimer2 [10] was PCR amplified from pRSETB-tdimer2 which was kindly provided by R.Y. Tsien (University of California at San Diego).

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2.2. Germline transformation

Transgenic animals bearing extrachromosomal arrays were produced by microinjection. Strains used were: wild-type animal *C. elegans* Bristol N2 strain. Transgenes were *Pmyo-3:mCherry::SL2-GFP* (50 ng/μl) and *Pmyo-3::GFP::SL2-tdimer2* (50 ng/μl).

2.3. In vivo imaging

The imaging of immobilized worms was performed on Andor Revolution XD laser confocal microscope system (Andor Technology plc, Springvale Business Park, Belfast BT12 7AL, United Kingdom) based on a spinning-disk confocal scanning head CSU-X1 (Yokogawa Electric Corporation, Nakacho 2-chome, Musashino-shi, Tokyo, Japan) under the control of Andor IQ 1.91 software. The microscopic images were obtained on an Olympus IX-71 inverted microscope (Olympus Corporation, Tokyo Opera City Bldg., Nishi-Shinjuku, Shinjuku-ku, Tokyo, Japan) with a Zeiss 40× 1.30 NA oil-immersion objective lens (Carl Zeiss MicroImaging Inc. Königsallee 9–21, Göttingen, Germany), and the 14-bit digital images were acquired by an Andor iXon^{EM} + DV885 K EM CCD camera. Andor LC-401A Laser Combiner with diode-pumped solid state (DPSS) lasers emitting at 491 and 561 nm were used to excite the fluorophores of GFP and tdimer2/mCherry, respectively. The fluorescence was filtered by Semrock bandpass filters with hard ion-beam-sputtered optical coatings (Semrock, Rochester, NY 14624, USA) mounted in a 10 position Filter wheel (Lambda 10–3, Sutter Instrument Company, Novato, CA 94949, USA).

3. Results

3.1. Bicistronic expression of mCherry and GFP in C. elegans

We validated the bicistronic strategy by using the *gpd-2-gpd-3* intercistronic region as the SL2 trans-splicing signal in this study: we constructed a reporter vector containing *mCherry::SL2-GFP* as shown in Fig. 1. Two *Promoter::mCherry::SL2-GFP* constructs were made by insertion of promoter fragments to the reporter vector. With promoters driving expression of target genes in muscles (promoter *myo-3*) and neurons (promoter *rab-3*), we have easily generated independent stable lines with consistent expression pattern of mCherry and GFP. As shown in Fig. 1, under the control of a single promoter *myo-3* or *rab-3*, mCherry and GFP expressed constantly and efficiently in muscle cells or nerve ring (Fig. 1A and B). And there was a good co-localization of mCherry and GFP both in muscle cells and the nerve ring as shown in the merged images. These results show that the SL2 mediates very efficient expression of both mCherry and GFP from a single promoter in muscles and neurons.

3.2. Molecular design of the fluorescent timer

A bicistronic cassette was adopted to design the expression plasmid (Fig. 2). The SL2 was fused to *tdimer2* by overlap extension PCR and the fragment was subcloned into pPD96.52 which contains the *unc-54* 3′ UTR. Then the GFP coding region was placed between a promoter and *SL2-tdimer2* in this construct. After cleavage and trans-splicing, two mRNA cistrons will be produced from the bicistronic primary mRNA: the first encoding GFP and the other one encoding tdimer2 [7,9].

Expression of GFP and tdimer2 from a single pre-mRNA ensures the stable relative expression level of these two fluorescent proteins. And these two proteins used in this construct possess quite different maturation times: $t_{0.5}$ (for maturation) = 2 h for tdimer2 [10], and about 30 min for GFP [11]. So the combinatorial color of these two fluorescent proteins (green and red) expressed from a single bicistronic primary mRNA will change in a time-dependent manner: tissues or cells with green fluorescence indicate very recent activity of the promoter, and yellow or reddish area represents continuous promoter activity. Therefore, it is can be utilized as a fluorescent timer to monitor promoter activity of developing organism.

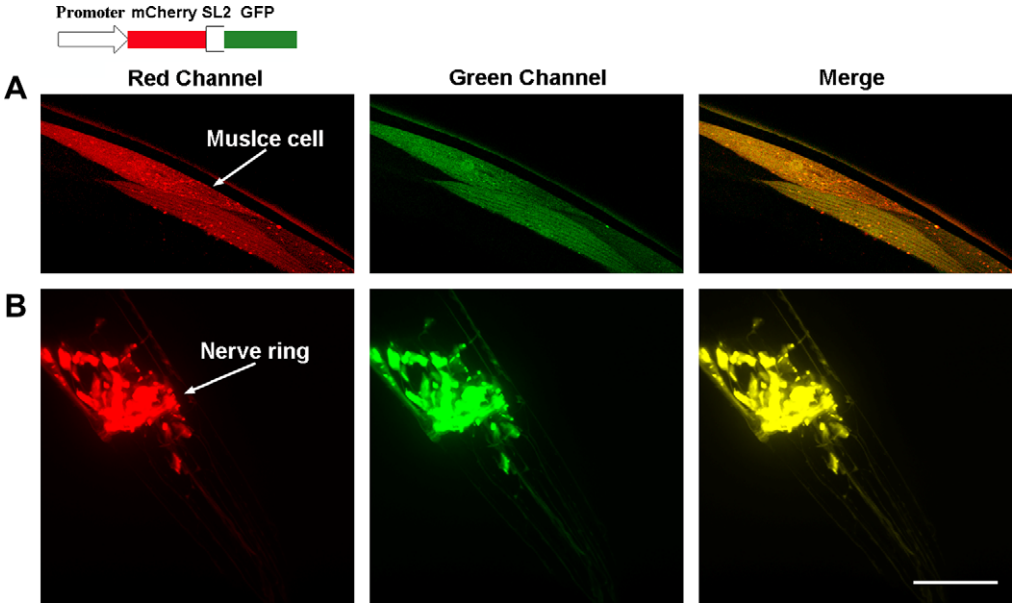

Fig. 1. Bicistronic expression of mCherry and GFP in worm muscles and neurons. The top of the figure is a schematic diagram of the bicistronic expression construct: under the control of the single promoter, mCherry (a monomeric red fluorescent protein) and GFP (including several introns) were closely spaced by the intergenic region (termed SL2) of *gpd-2* and *gpd-3*. Under the control of *myo-3* or *rab-3* promoter, the reporter mCherry and GFP were expressed from a single primary messenger RNA in body wall muscle cells (A) and the nerve ring (B). Projections with maximum intensity of Z-series stacks were obtained and processed by Image J software (National Institutes of Health, Public Domain). The merged images on the right panels are the overlay color of red and green fluorescence imaged by a real time dual-color imaging. Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

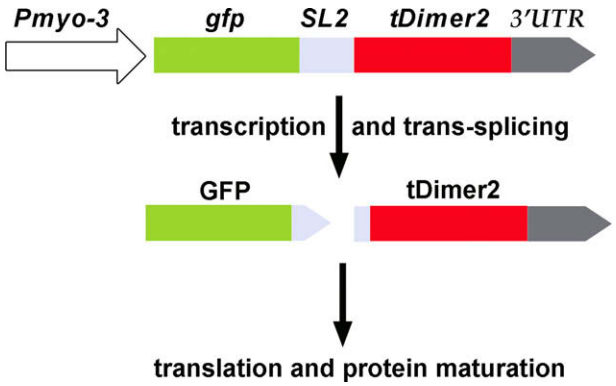

Fig. 2. Schematic diagram of the fluorescent timer. Under the control of a promoter, GFP and tdimer2 (a red fluorescent protein) followed by the *unc-54* 3′ untranslated region (3′ UTR) are linked by SL2. Cleavage and trans-splicing of this biscistronic primary messenger RNA results in the production of two mRNAs: *gfp* and *tdimer2*. And the maturation times of GFP and tdimer2 are quite different. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. To monitor the myo-3 promoter activity in the embryos by the fluorescent timer

To utilize this fluorescent timer as a tool for studying promoter activity, lines carrying *Pmyo-3::GFP::SL2-tdimer2* transgene as extrachromosomal array were established. In our experiments, eggs with green fluorescence were observed about 7 h after egg-laying (Fig. 3A, green channel), while the red fluorescence was really faint (red channel). The merged image (right panel) shows bright green fluorescence indicating that *myo-3* promoter started to be active in the eggs. Then red fluorescent accumulated over time during the following 3–4 h and eggs changed initial bright green fluorescence to yellow gradually (Fig. 3B and C). This process can be observed simply by using a Zeiss (Carl Zeiss MicroImaging Inc., Göttingen, Germany) SteREO Discovery V8 with M^2 Bio Quad stereo fluorescence attachment (Kramer Scientific Corporation, 91 High St., Amesbury, MA 01913, USA) or Leica MZ16F (Leica Microsystems GmbH, Ernst-Leitz-Strasse 17–37, Wetzlar, Germany) fluorescence stereomicroscope. The sustained green fluorescence and the merged yellow images can be observed during flowing hatching process, indicating the sustained *myo-3* promoter activity of this process. The *myo-3* promoter activity visualized from the fluorescent timer indicates the expression "history" of endogenous protein MHC A (myosin heavy chain A) that is essential for thick filament formation and embryonic elongation.

3.4. To monitor myo-3 promoter activity in body wall muscle and vulval muscle

The *myo-3* gene encodes myosin A protein which is the central component of body wall muscle and vulval muscle filament [12], and expression of reporter gene driven by *myo-3* promoter could be used to identify these muscles. We used *Pmyo-3::GFP::SL2-tdimer2* lines to trace the activity of *myo-3* promoter during larval development. After hatching, there was bright green fluorescence in the body wall muscle of L1 larval, which changed the color to yellow and then sustained in the following larval development stages (data now shown). Intriguingly, in L4 larval the vulval muscles appeared gradually with brighter and brighter green fluorescence which was distinct from surrounding yellow body wall muscles (Fig. 4A), indicating the early development stage of the vulval muscles. As development proceeded, vulval muscles chan-

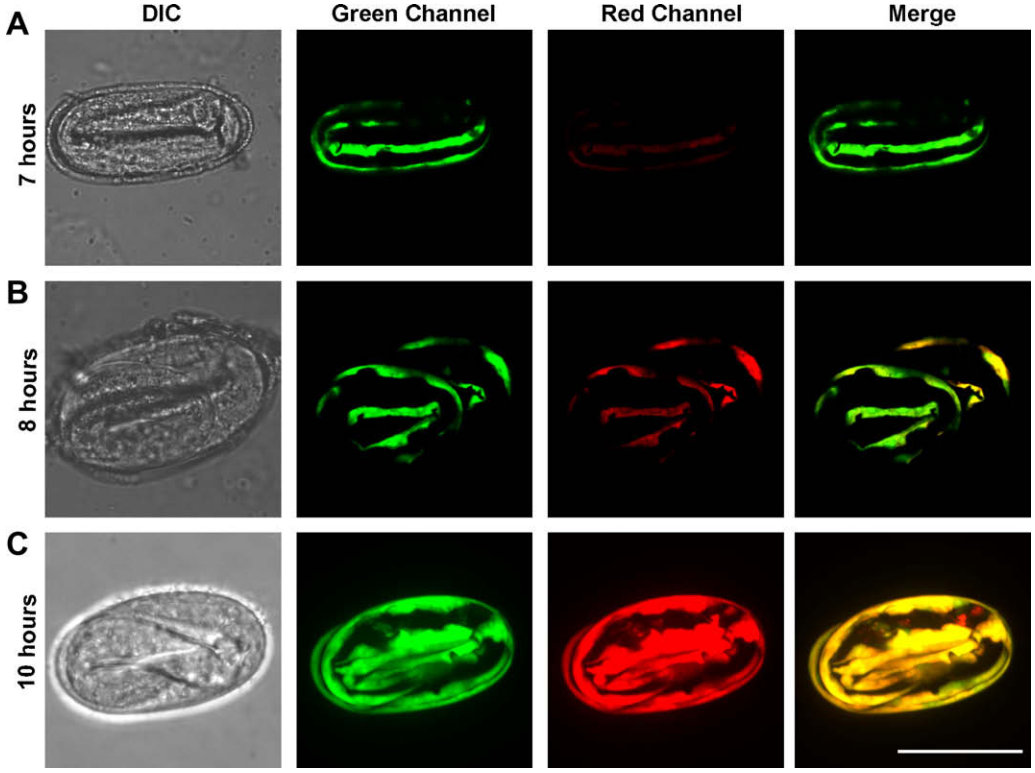

Fig. 3. *myo-3* Promoter-driven expression of fluorescent timer in embryos. (A–C) Representative images of the fluorescent timer under the control of *myo-3* promoter in embryos are shown: the bright field (DIC), GFP filter, TagRFP filter and the overlay (merge). Eggs became green 7.0 ± 0.4 ($p < 0.05$, $n = 5$, mean ± SEM) h after egg being laid outside (A) and changed to yellow gradually in 3.3 ± 0.3 ($p < 0.05$, $n = 5$, mean ± SEM) h (B, C). All the images were acquired under the same conditions. Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

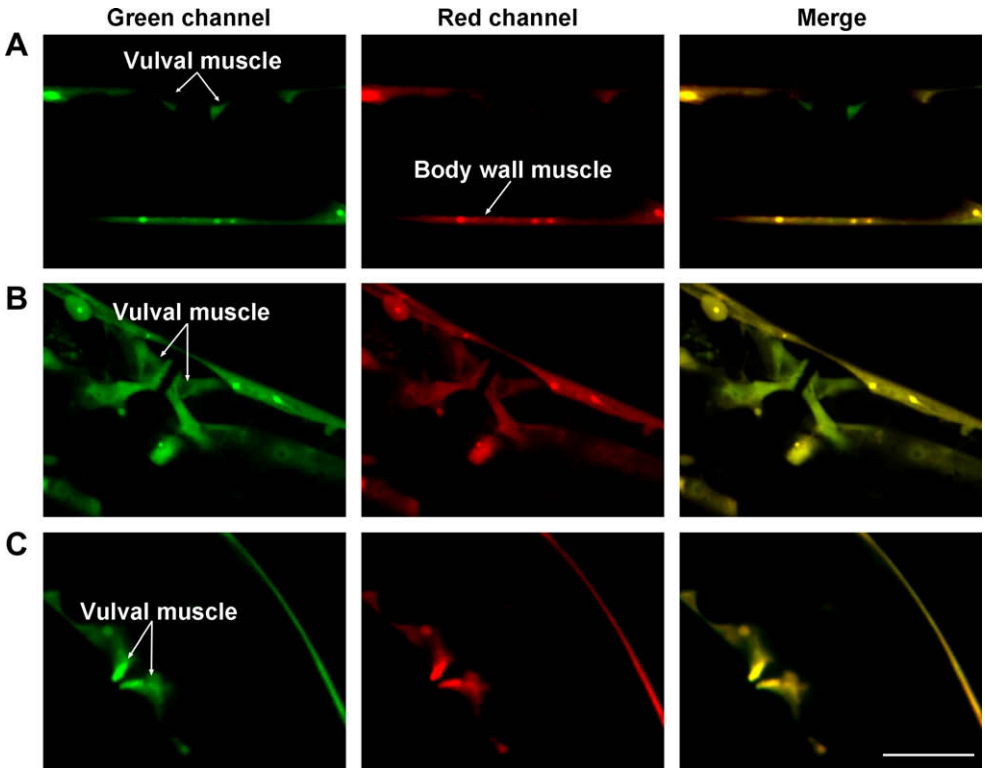

Fig. 4. To trace the *myo-3* promoter activity in the vulva muscles in developing worms. Representative images of the fluorescent timer under the control of *myo-3* promoter in larval and adult animals are shown. The vulval muscles exhibited different colors at different larval stages: green (A, Middle L4), yellow with greenish (B, Late L4), and completely yellow (C, Young adult). And the body wall muscles sustained yellow through L4 larval period and young adult animals. Projections with maximum intensity of Z-series stacks were obtained and processed by Image J software (National Institutes of Health, Public Domain). Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ged colors with increment of red/green fluorescent ratio (Fig. 4B), and then became completely yellow in young adult animals (Fig. 4C). When animals were growing old, the body wall muscle and vulval muscle exhibited a little reddish color (data not shown). In our experiment, the color hue of the vulval muscles can also be distinguished easily by eye using a Zeiss or Leica fluorescence stereomicroscope.

4. Discussion

We developed a new fluorescent timer based on the bicistronic expression of two fluorescent proteins with different maturation times. By utilizing this fluorescent timer, we have successfully reported on the temporal activity of *myo-3* promoter in developing embryos. *myo-3* encodes the minor isoform of MHC (myosin heavy chain) that is essential for thick filament formation, and for viability, movement and embryonic elongation. Monitoring the *myo-3* promoter activity could provide us some useful information of the MHC involved in hatching process. We have also traced the *myo-3* promoter activity in body wall muscle and the vulva muscle in developing animals. The *myo-3* promoter sustained its activity throughout the larval stages. And *myo-3* promoter starts to be active in vulval muscle at the L4 larval development stage of the egg-laying apparatus. The time-dependent activity of *myo-3* promoter visualized from the fluorescent timer provided the expression "history" of the endogenous protein MHC A (myosin heavy chain A).

Fluorescent timers are invaluable tools for understanding the molecular or cellular events and processes. However, there are a limited number of applicable fluorescent timers till now [3,6]. We designed a fluorescent timer through bicistronic expression of GFP and tdimer2 in *C. elegans*. When expressed under the control of a single promoter, these two fluorescent proteins with different maturation times exhibited a combinatorial color that changed over time. We can anticipate that more fluorescent timers with different time-resolution can be generated by bicistronic expression of two or more already well-studied fluorescent proteins based on maturation times and their emission characteristics. Fluorescent timers with slower or faster color-conversion could be obtained by this strategy, and these fluorescent timers would enable more accurate study of the temporal expression profile of a promoter. Fusion proteins (fluorescent timer-targeted protein) could be obtained to study transient gene expression. And this new type of fluorescent timer could also be utilized in mammalian system by using internal ribosome entry sites (IRES) instead of SL2.

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