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Genes Stimulation Using Cre / loxp system in *C.elegan*

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ABSTRACT

Background: *C. elegans* is the most fruitful experimental model to advance understanding of the molecular mechanisms of development and nervous system function; it has become such a pin-up animal for scientists. *C. elegans* nervous system is very simple, intersex adults' only 302 neurons, which can be divided into motor neuron, sensory neuron and interneuron. **Objective:** Object of study in our work. Along with logically combine specially promoter to label the single neurons in *C.elegans*, intersection of promoters and site-specific recombinase system (Cre/loxP) to label single neurons. We accomplished promoter library based on Gateway technology, so that promoters and coding regions can be quickly matched and mixed. **Results:** We can label the neuron ASH by intersection of promoter's sra-6p and gpa-11p. We have constructed vectors containing Cre/loxP specific recombination system to explore two specific promoter-driven neuronal expression systems**. Conclusion:** Cre/loxprecombinase is certainly one of the main tools that made many modifications of the genome newly developed possible techniques. In vitro recombination between LoxP sites the excision works efficiently without the expression of any active selection if the Cre is transiently expressed from a strong promote

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INTRODUCTION

Provide basic information for the neurons functions in the neural circuit. And can be applied very rare techniques to mark one single neuron, although the technologies for genes location in live worms are quite numerous. May be the main reason that the expression of genes in *C.elegans* is a wide and promoters which can be determined in certain neurons are limited. Given that, both molecular biological methods (Including Cre/LoxP system) and confocal laser scanning technology has been combined to confirm the promoters that promote expression of fluorescent proteins in certain neurons. Molecular techniques now allow the design of genetic modifications minutes in the *C.elegans*. Not only can define nucleotide changes be engineered in the genome of the *C.elegans*, but can be designed to target the genetic switches or expression of any gene ablation (which molecular basic information is available) to any type of tissue at any given time. It has been more than 15 years now that the Cre /LoxP system has been used as a means to artificially control gene expression. Over the years, this system may allowed researchers create a variety of plants and animals genetically modified with genes that are of their choice being externally regulated (Latchman, 2002).

Cre-loxp technology was presented in the 1980's (Sternberg *et al.*, 1981; Sauer & Henderson, 1988). Cre (causes recombination) recombinaseencoded by P1 coliphageis a 38 kDa protein that recognizes and mediates site-specific recombination between 34 bp long sequences, called loxP (locus of X over in PI) (Sternberg & Hamilton, 1981;Ghosh& Van Duyne 2002). Each the Crerecombinase and loxP sequences are derives from the bacteria P1 bacteriophage, which this system uses recombination in its life cycle to maintain Phage genome and plasmid copy unit in the lysogenic state (Austin *et al*., 1981; Hochman*et al.*, 1983). As a member of the integrate superfamily of site-specific recombinases, does not require recombinase Cre any host factors or accessory proteins help to mediate recombination specific loxP.This fact, in addition to the best operating temperature 37°C (Buchholz *et al*., 1996). LoxP site consists of two 13-bp inverted repeats that flank an 8-bp asymmetric core sequence (Hamilton & Abremski, 1984; Hoess, &Abremski, 1984; Hoess*et al.*, 1982). Cre–loxPmediatedrecombination between

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two loxP repeated direct excise all DNA Sequence located within the two sites as a covalently closed circle.

It has been used recombination Cre-mediated successfully for many applications, tissue (Sauer, B.,1998; Feil *et al.,* 2009) and model organisms including, for example, thezebrafish(Dong & Stuart, 2004), Drosophila (Siegal & Hartl, 1996), Xenopus (Werdien *et al.,* 2001) and Plants (Gilbertson, 2003) .The direction and location of the loxP sites determine whether the Cre recombination induces a deletion, inversion, insertionsand translocations as an outcome of the catalytic power of these recombinases (Nagy, 2000). It was also clarified recombination mechanism through the crystal structure analysis of the Cre and Cre / Loxp interface (Guo *et al.,* 1997).

Garrison *et.al.,* 2012Showed excision cell-specific Cre / loxP of an ntc-1(+) transgene. Cre was produced from a DVA-specific promoter while target construct include Pntc-1 > ntc-1(+) - SL2-GFPstop >mcherry. This strategy produces simultaneously excision and visualization of circumcision, in this case for both inactivate ntc-1 and the expression of mCherry in DVA. Schmidt *et. al*.,2012availability several technical indicators of intersectional strategy that drive recombinase in one group of neurons and expression of the target in the other group to activate depends on the recombination of gene expression in the field of intersecting expression. The researchers used a combination of promoters with intersecting expression and DNA recombinases to generate conditional expression of channelrhodopsin2 (ChR2) in single neurons (AVA and the ASH, including strains, expressing ASH resulting from (Ezcurra *et al.,*2011).

The main focus of our work is on one of sensory neuron ASH, Cell bodies of neuron type is localized in the nerve ring ganglia in the head (White*et. al.,* 1986).Sensory neurons ASH polymodal in *C. elegans* sense a variety of aversive stimuli and mediate avoid osmotic of high osmotic, chemical stimuli and mechanical (Buchholz*et. al.,* 1990;Flavell*et. al.,* 2013).

These strategies promise to contribute significantly to the increased understanding of the function of individual genes in the development and pathogenesis. Powerful tools, both for design of these genetic switches and speed up the creation of transgenic animals, is Cre DNA recombinase in specific locations of P1 bacteriophage. Rearrange accurate DNA and genetic switches can be generated efficiently straightforward manner using the Cre recombinase. In conjunction with inducible systems for control of the Cre expression and function, and is likely to have a profound impact on evolutionary biology and generate useful animal models of human neuron disease such strategies based on recombination. Here we Description how can a single neuron expression is achieved by using Cre/loxP recombinases.

Methods:

The *C. elegans* strains used in this study is N2 (wide-type) werecultured on nematode growth medium (NGM) with bacterial strain for feeding worms*E. coli.* OP50.The injection builds on the expression plasmid 100ng / µl, marker 30 ng / µl*lin-44p*:: GFP as co-injection marker using the standard techniques.

Design of Plasmids and Entry Clones:

To speed up the assembling of generate and to use of genome reagents generated, we based our generate on the multisite gateway (Invitrogen) .The standard multisite system uses three fragment (promoter, gene of insert, and transcriptional terminator, in name of slots1, slots2,and slots 3, respectively). Were recombined in pDEST R4-R3 Vector II or modified destination using the LR reactions to create the expression clones.

To construct entry clone for slot1 in gateway cloning, was PCR amplified from wild-type N2 genomic *C. elegans* DNA using primer containing attB4 and attB1,purified PCR to recombination with the attP4 and attP1 sites in the slot1 pDONR-P4-P1Rdonor vector.The length of promoter *sra-6p*and *gpa-11p*was 3809 bpand 3300bp respectively.

To generate entry clone for slot2 (linear slot2 *Loxp-stop-Loxp-TagRFP-t*) we used restriction digestion .The linear vector (slot2) 2600bp was PCR amplified using primer containing attL1and attL2 recombination site and then was sequence with primer M13F. *Loxp-stop-Loxp* (997bp)was PCR amplified cloned between Xmal and HinIII.TheTagRFP-t (755bp) was cloned between XbaI and KpnI .The T4 DNA ligase was used for ligation of the *Loxp-stop-Loxp* and *TagRFP-t* into the entry clone (Linear slot2) in the right sequence after the digestion with the conforming restriction enzymes .The slot2 (*Kozak-SV40-Nls-ncre-linker-ER*) 1098bp was PCR amplified using primer containing attB1and attB2recombination site recombined with the attP1and attP2 site in the slot2 vector pDONR221 (Invitrogen) using the Bp reaction to generate entry clone slot2.

To construct *sl2dGFP* or *sl2deTagBFP*entry clone for slot3 fragment with inserted Bp reaction site attB2 and attB3 recombined with attP2 and attP3 site in pDONR P2R-P3 vector using Bp reaction.

After that, we used LR reactions to generate expression plasmids,*Pgpa-11:: Kozak-SV40-Nls-ncre-linker-ER:: sl2deTagBFP* were expression in ASH neurons, we employed a Cre-loxp site-specific recombination system. Through co-injection of the plasmids *Psra-6:: LoxP-stop-LoxP-TagRFP-t:: sl2dGFP*.

Laser Scanning Confocal Microscopy Imaging System:

All confocal fluorescence imagines were conducted using Andrew (Andrew plc. Technology, SPRINGVALE Business Park, Belfast, United Kingdom) Revolution XD microscopy system confocal laser based spinning disk head of scanning confocal CSU-X1 (Yokogawa Electric Company,

Musashino Shi, Tokyo, Japan), under the control of Andrew IQ 1.91 software.Was built confocal system on Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan). Was filmed all fluorescent images a ×60 objective lens (numerical aperture =1.45, Olympus) and captured by in Indore iXon^{EM}+DU-897D EMCCD camera. The display images and analyzed using Image J software 1.43b (Wayne Rasband, National Institutes of Health, USA).

RESULTSAND DISCUSSION

The Optogenetic approach in *C. elegans* benefit greatly from generic method that allow the expression of optogenetic tools in single Cell, so that direct whole-field illumination can be used to stimulate just the cell of interest. Such methods, based on the Conditional expression at the intersection of two of the promoters, and be achieved for *C. elegans* for GFP or other proteins, using the Cre recombinases (Macosko*et. al.*, 2009). Site-specific recombinases Cre have been used in many systems to control the expression of genes and the structure (Sauer & Henderson., 1989). These enzymes align Side by side of copies to the target sequence, lead site-specific recombination, and remove the sequence between the targets as a circular DNA molecule. If the sequence of intervention disrupts the expression, and the removal of recombination allows for the modification of the activated.

During the experiment, we found that a single neuron can be labeled more promoters, but each promoter labeled according to the number of neurons in two or more, so we use experiment start driven different fluorescent proteins, by fluorescence imaging method of shooting different fluorescently labeled neurons were then subjected to image processing by the image processing software image launcher, to obtain colocalization of single neurons, but this method can only be used for a single nerve mark element, cannot be in a specific target gene expression of neurons. For example promoter *sra-6P*mark in the neurons ASH, ASI, PVQ, SPD and SPV neurons promoter *gpa-11p* labeled ADLandASH neurons, if using different fluorescent protein promoter and localization by fluorescence method can be very simple mark ADL neurons, but if you want only the ASH neurons in *Caenorhabditiselegans* gene expression experiments TagRFP-t andsl2dGFPcould not continue. To solve this problem we used Cre-Loxp site-specific recombination system. Through literature review to the basic information we need to make the promoter and another entry clone, use multisite gateway technology, the three entry clone by LR reaction to form an expression plasmid .When construction of the plasmid, by T4 DNA ligase methods and multisite gateway technology, this will reduce the build time plasmid so much.

By this idea experiment conducted experiment in addition,thereis not perfectly promoter specific ASH. Thus, we used Cre-Loxp site-specific recombination system to drive specific expression of TagRFP-t in neurons ASH .When expressed worms each of the *gpa-11P::Kazak-Sv40-Nls-ncre-linker-ER::sl2deTagBFP*, and *sra-6P:: LoxP-stop-LoxP-TagRFP-t:: sl2dGFP*constructs, Cre recombinase driven by the *gpa-11P* promoter remove the loxP-flanked transcriptional terminator. This allowed specific expression of TagRFP-t and a green fluorescent protein (GFP) fluorescence marker in ASH neurons that possess a combinatorial intersection between *sra-6 P* and *gpa-11P*promoters .The formulated according to a certain proportion of injection (the expression plasmid 80 ng / µl , marker 30 ng / µl), injected into the nematode body until nematode passaged stable, select sample preparation nematode certain amount, the use of laser scanning confocal microscope image acquisition, the acquired image after image processing software Image launcher processing analysis as shown in (Figure1A and B)after conjunction collected images from the figure we can clearly see that only one pair of neurons, and cilia clearly visible.

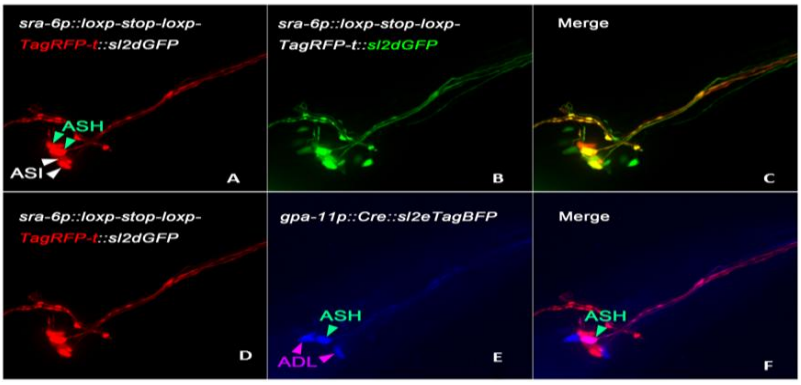

Fig. 1: Laser scanning microscope image of ASH neuron is located by combination of expression plasmid *sra-6P::LoxP-stop-LoxP-TagRFP-t::sl2dGFP* and *gpa-11P::Kazak-Sv40-Nls-ncre-linker-ER::sl2deTagBFP*.As indicating A. RFP, B. GFP, E.BFP, C. Merge of A and B., F. Merge of D and E (Scale bar = 20µm).

Promoter *gpa-11P*labeled neurons in *C. elegans* ADL, ASH and promoter *sra-6P* mark ASH, PVQ, ASI, SPD, the use of these two promoters together mark ASH neurons, reaching only TagRFP-tandsl2dGFPgene expression in the ASH. Because the individual injection two plasmids, *Sra-6P:: LoxP-stop-LoxP-TagRFP-t::sl2dGFP*present and should express green fluorescent protein exhibit neuronal ADL and ASH, but since we added gene *TagRFP-t* 5 'end of the upstream a stop sequence to terminate the promoter behind and fluorescent protein gene. Plasmid *gpa-11P::Kozak-Sv40-Nls-ncre-linker-ER:: sl2deTagBFP* because they carry a fluorescent protein *sl2deTagBFP*capture the image (Figure 1E). Cre has been learned in the introduction of the enzyme that can specifically recognize palindromic sequences loxp-specific, and may be a DNA fragment according to certain direction between two loxP sequences excised.*sra-6 P* promoter-driven gene expression in ASH, PVQ, ASI, SPD, so, and cre enzyme expressed only in ASH and ADL so only when the gene to express TagRFP-t whenloxP sequences and cre enzyme exist, that is, the image rendering green fluorescent moiety. In addition the rate of Cre recombination has been improved by altering the Cre sequence include a better Kozak sequence which both improve translation efficiency, and a (NLS) nuclear localization signal has been added to increase nuclear Cr concentration and thus function(Wilson & Kola,2001; Andrea*et al*., 2002).This result is consistent with the current study, for each of the (Ezcurra, *et.al.*, 2011; Garrison, *et.al.*, 2012; Schmidt, *et. al*., 2012;Flavell*et. al.*, 2013).Utilize promoters in the above test jointly labeled neurons and Cre / LoxP system, combining method can achieve the purpose of gene expression in single neurons.In the above experiments have confirmed that Cre / LoxP system, can be successfully applied to the tagged nematode neurons.

Conclusion:

Cre/lox system is aPriceless tool for molecular biology. Through the creation of tissue specific expression and it allows for isolate individual genes and their functions. Control of genes via Cre / lox system is comparable to the control toy car. You can select the area that will be expressed gene (orientation), and control the level (speed), which gene will be expressed.

Cre DNA recombinase has become a robust tool for the analysis of gene function in the transgenic *C.elegans*recombinational strategies in transgenic *C.elegans* to turn genes on, ablate endogenous genes, and even build a new chromosome-specific and tissue-specific temporally defined manner now to let the level of genetic analysis can hardly be imagined a decade or two ago only. It is likely that further improvements and more sophisticated strategies will be developed using site- specific DNA recombination.

Moreover, the refinement of systems-based inducible gene expression and the development of new systems allow even more accurate control of recombinase expression. The combined use of these molecular tools to be clear of great help in the detection of complex mammalian development and the generation of more sophisticated models of neurological diseases that infect humans.

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