Manipulating behaviors of targeted single cells in vivo by using IR-LEGO

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Abstract:
Methods for turning on/off gene expression at any desired time and place *in vivo* would be useful for analyzing various biological processes. We have developed a novel microscopic system utilizing an infrared laser, IR-LEGO (infrared-laser evoked gene operator), which is designed to deposit heat locally in living organisms. We have shown that IR-LEGO enables us to induce the heat shock response efficiently in targeted single cells of *C. elegans* worms, thereby driving expression of a transgene under the control of a heat shock promoter. By using IR-LEGO we attempted to rescue several mutant phenotypes of worms at the single-cell level. Diverse cell behaviors including differentiation and migration of target cells can be manipulated by gene induction mediated by IR-LEGO. Our results showed that IR-LEGO can be used to manipulate cell-autonomous as well as cell-nonautonomous behaviors, further confirming that irradiation using IR-LEGO has no harmful effects on the targets. Thus, IR-LEGO serves as valuable tools for manipulating biological processes in living organisms .

1. INTRODUCTION

Causing denaturation of proteins, a rise in temperature is harmful to cells. organisms have developed the cellular defense system against heat shocks through evolution. This system, called the heat shock response, is widely conserved from bacteria to vertebrates[1-3]. Heat stress induces transcription of genes for heat shock proteins, which act as protein chaperones to repair cellular damage caused by protein denaturation. In transgenic organisms, this response can be exploited for manipulating gene expression *in vivo*, using heat shock as a trigger to induce expression of a gene cloned downstream of a heat shock promoter. By irradiating living specimens with a laser beam under the microscope, it is possible to induce heat shock responses in individual cells, thereby inducing a specific gene in the targeted cells. The method has advantages over conventional techniques for ectopic gene expression. It allows induction of gene expression in a specific cell without a specific tissue-specific promoter that directs expression in that particular cell. Only a single strain carrying a heat shock promoter-driven transgene is required for the induction of gene expression in different cell types, Most importantly, it enables the induction of gene expression in single targeted cells at a defined time.

A cell-ablation microscope system with a pulsed 440 nm dye laser has previously been used for inducing gene expression mediated by the heat shock response[4-6]. Although several studies reported the successful gene expression induced with a 440 nm laser or other visible lasers[4-8], and in some cases the induced gene expression mediated relevant phenotypic responses in irradiated cells[5, 7], the method has been seldom used. This is because relatively long irradiation times are required for gene induction and the irradiation often had detrimental effects on cells with the previous method [7, 8].

The mechanisms by which irradiation with 440 nm laser induces heat shock response is poorly understood. **Water, which is a major constituent of cells, is almost transparent to visible light, and is heated with it very inefficiently.** It is known that irradiation with visible light may cause a photochemical reaction in cells[9, 10]. Since the heat shock response can be triggered by various kinds of stresses other than heat, it is possible that the laser-mediated heat shock responses in the previous reports were not always of photothermal origin. In fact, a photochemical effect of laser irradiation on induction of the heat shock response was reported previously [11].

In contrast to visible light, water shows

strong absorption in the infrared (IR) region. Therefore, we presumed that infrared light should heat water in organisms much more efficiently compared with 440 nm light. We have been attempting to apply an infrared laser for heat shock response-mediated gene expression. For this aim, we have developed a novel microscope system called InfraRed Laser Evoked Gene Operator (IR-LEGO). The wavelength of IR (1,480 nm) matches the combination of symmetric and antisymmetric O-H stretching modes of water, and can heat water with ~10^5-fold higher efficiency than the 440 nm laser. Designed specifically for heating water in cells, use of IR-LEGO for laser-mediated heat shock should help avoid photochemical damage.

We have addressed whether the IR-LEGO system is applicable to an *in vivo* study. As an experimental organism, we chose the nematode *Caenorhabditis elegans* (*C. elegans*). *C. elegans,* a model animal, is well suited for examining gene induction by laser irradiation; It has a transparent body. Transgenesis is relatively easy. Heat shock promoters of *C. elegans* are well characterized. Furthermore, all the somatic cells of this organism can be uniquely identified under the differential interference optics [12]. Together with an array of cell specific GFP markers, this characteristics allows unambiguous identification of target of irradiation as well as cells that are induced after irradiation. Examining the irradiation conditions systematically, we have shown that IR-LEGO can induce gene expression efficiently in living organisms, and its effect is less harmful to cells, compared with the system using a 440 nm dye laser [13].

In this paper, we report our attempts to rescue various mutant phenotypes of worms at the single-cell level. Diverse cell behaviors including differentiation and migration of target cells can be manipulated by gene induction mediated by IR-LEGO.

2. MATERIALS AND METHODS

Microscope and related equipments

An inverted microscope (IX-70, Olympus Corporation, Japan) was modified as shown in Figure 1. An IR-laser (Model FRL-DC 1,480 nm, maximum power of 3W, IRE-Polus, Russia) was installed for local heating of specimens. A UApo/340 100x (NA=1.3) objective was used in order to target cells accurately. The focal point of the IR-laser was determined by optical trapping experiments on micro beads. Still images were taken with CCD cameras (Penguin, Pixela, USA).

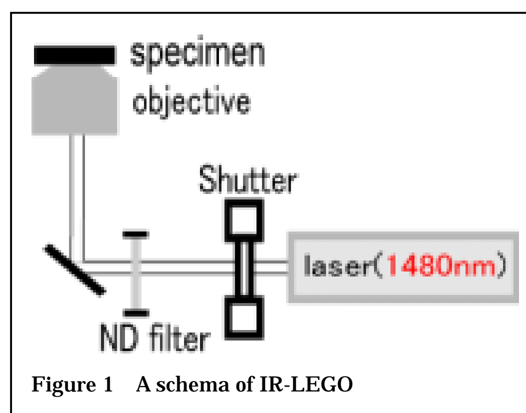

Figure 1 A schema of IR-LEGO

Animals

C. elegans worms were maintained on a nematode growth medium (NGM) agar plate with *E. coli* strain OP50[12, 14], and handled *C. elegans* using a platinum pick. Throughout the experiments, room temperature was kept at 20°**C**.

Plasmids and transgenic strains

cDNA for EGFP (Clontech, USA) to which *KpnI* sites were added by PCR, was inserted into pPD49.78 (a gift from Andrew Fire), a expression vector carrying the *hsp16-2* promoter [15, 16], to generate *phsp16-2::egfp*. This plasmid was used to monitor the gene induction. In worms carrying *phsp16-2::egfp* as transgenes, no expression of the marker *gfp* gene was detected without heat-shock treatment, except that weak GFP expression was only occasionally observed in intestine cells.

For experiments of *unc-6* DTCs, the *unc-6* cDNA with *Kpn*I and *EcoR*V sites was generated by PCR using yk492g9 (a gift from Yuji Kohara) as a template, and was inserted into pPD49.78 to construct *phsp16-2::unc-6. ncEx969[hsp::unc-6]* was generated by injecting *phsp16-2::unc-6, phsp16-2::egfp,* pBluescript KS(-) (Stratagene, USA) (each 0.05mg/ml) and pJS191 encoding *ajm-1::gfp* [17] (0.005mg/ml, a gift from Jeff Hardin), and was then introduced into NW434 *unc-6*(*ev400*) animals. Larval muscle cells at L2 stage were irradiated and the shape of gonads was examined in young adults.

For induction experiments of MIG-24, a

transgene *Ex[phsp16-2::mig-24::3HA]* was generated by co-injecting *phsp16-2::mig-24::3HA* (a gift from Kiyoji Nishiwaki) [18], *phsp16-2::egfp* and *myo-2::mRFP,* a transformation marker expressing mRFP in the pharynx. A chromosomally integrated transgene *Is[phsp16-2::mig-24::3HA]* was generated from *Ex[phsp16-2::mig-24::3HA]* by gamma-ray irradiation [19] , and was introduced into *mig-24(tk68)* hermaphrodites. DTCs were irradiated before the second turn, and the position of gonad tips was scored 10 hr later.

For induction experiments of MAB-5, an integrated transgenic strain CF301 *mab-5(e2088) III; unc-31(e169) IV; him-5(e1490) V; muIs9[hs-mab-5 + C14G10] X* [20] was used. Descendants of V5 and V6 were irradiated unilaterally, and the cell division pattern was examined 10 hr later. The stage of worms at the time of irradiation was deduced retrospectively according to the timing of the cell division after irradiation.

Laser irradiation of C. elegans

Individual worms were mounted on a 6% agar pad of ca. 0.2 mm thickness with 1 mM levamisole (Nacalai, Japan) to anesthetize them, and were then covered with a coverslip (microcover glass, 0.12-0.17 mm in thickness, Matsunami, Japan). We did not use sodium azide, which was used to immobilize worms in a previous study [4], because this treatment by itself activates the *hsp16-2* promoter without irradiation [21]. For irradiation of seam cells, contours of individual seam cells were visualized using AJM-1::GFP for their identification, and cells facing proximally to the objective (100x) were selected as targets. After irradiation, the worms were transferred individually to NGM plates seeded with *E. coli* and allowed to recover before observation. Irradiation of V5/V6 descendants in *mab-5* males was done at an incident laser power of 11 mW for 1 sec. Irradiation of both the body wall muscles and DTCs was done 4 times at 11 mW for 0.25 sec. Under this condition, GFP was induced in 59% (n=200) of the targeted muscle cells and in 53% (n=205) of the targeted DTCs, with no GFP expression in non-targeted cells.

3. RESULTS

Having established the conditions for gene induction [13], we attempted to manipulate the behavior of *C. elegans* cells *in vivo* using IR-LEGO. First, we examined cell migration during gonadal development [22]. The gonad of adult *C. elegans* hermaphrodites consists of two U-shaped tubular ovotestes, one anterior and one posterior, which are joined together at the mid-ventral side of the body, and has the twofold rotational symmetry in the overall structure. (Figure.2) The gonad derives from the gonadal primordium consisting of four cells named Z1, Z2, Z3 and Z4, which are localized on the ventral side at the middle part of the body of newly hatched L1 larvae. As cells of the gonadal primordium proliferate, the two growing ovotestes elongate in lateral directions, one anteriorly and the other posteriorly during L3. Then, in late L3, the arm of each ovotestis turns 90 degree to elongate dorsally, and after reaching the dorsal side in early L4, they change the direction of elongation again, one posteriorly and the other anteriorly. The elongations of the anterior and the posterior arms of the ovotestes are each led by a migratory cell called the distal tip cell (DTC). Therefore, the final configuration of each ovotestis reflects the trajectory of DTC migration, which provide us with a convenient system to examine migratory behavior of cells.

Netrin is a well-known secreted guidance molecule for growing axons in the vertebrate nervous system. In *C. elegans,* UNC-6, a nematode netrin autholog, is expressed by ventral muscles and other cells on the ventral

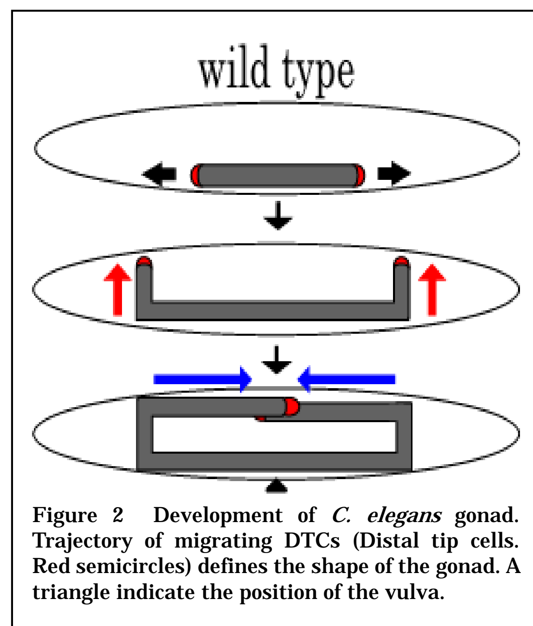

Figure 2 Development of *C. elegans* gonad. Trajectory of migrating DTCs (Distal tip cells. Red semicircles) defines the shape of the gonad. A triangle indicate the position of the vulva.

side of the body [23, 24], and is thought to act as a repellent to steer DTCs dorsally (Figure 3). In *unc-6* mutants, the ventral-to-dorsal (V-D) migration of DTCs is disrupted and the shape of the gonad becomes abnormal; they remain on the ventral side throughout the migration (Figure 3 middle panel). We found that IR-laser-induced expression of UNC-6 in muscle cells in the posterior ventral region just prior to the arrival of posterior DTC to the region completely rescued the V-D migration of posterior DTCs in *unc-6* mutants (Figure 3 bottom panel) **with no apparent effect on anterior DTCs. Conversely, induced expression of UNC-6 in anterior dorsal muscles increased the penetrance of the ventral-to-dorsal migration defects of anterior DTCs. These results proved that UNC-6 exerts local activity to repel migrating DTCs, and that expression of UNC-6 on the ventral side of the body is critical for normal ventral-to-dorsal migration of VPCs.**

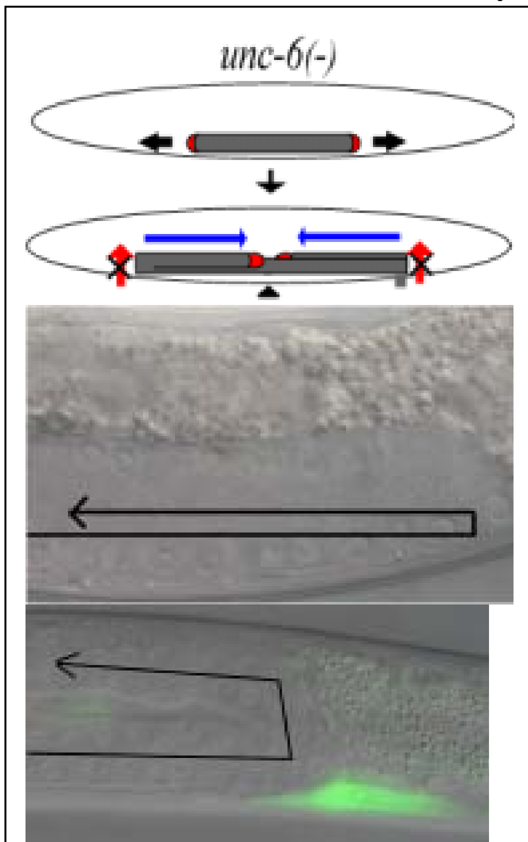

Figure 3 In unc-6 mutants, DTCs remain on the ventral side throughout the migration (Top). Induced expression of UNC-6, which is shown by co-induction of GFP, in a ventral muscle cell rescued the ventral-to-dorsal migration of DTCs (Bottom).

Next we tried to modulate the migratory behavior of DTC by manipulating the gene expression in DTC itself. The MIG-24 bHLH is a transcription factor expressed in DTCs [18]. Among downstream targets of MIG-24 is the GON-1/ADAMTS protease which is known to be essential for DTC migration (Figure 4). As expected, mutations in the *mig-24* gene also cause the arrest of DTC migration. We induced expression of MIG-24 in single DTCs of *mig-24* mutants, and found that induction in early stage of cell migration effectively rescued their migratory defects. Thus, the IR-LEGO-mediated gene induction technique can be used to manipulate cell migration in a cell-autonomous as well as a non-cell-autonomous fashion.

Finally, we examined cell-fate specification by a Hox gene. The *mab-5* is a *C. elegans Antennapedia* homolog and regulates the cell-fate decision of seam cells in the posterior body of males; without *mab-5,*

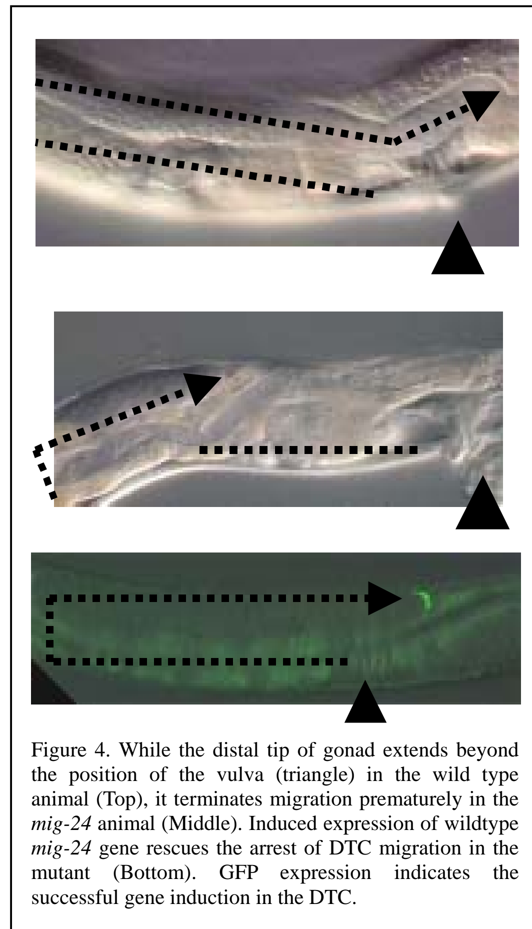

Figure 4. While the distal tip of gonad extends beyond the position of the vulva (triangle) in the wild type animal (Top), it terminates migration prematurely in the *mig-24* animal (Middle). Induced expression of wildtype *mig-24* gene rescues the arrest of DTC migration in the mutant (Bottom). GFP expression indicates the successful gene induction in the DTC.

descendents of the V5 and V6 seam cells in males adopt an aberrant cell-division pattern with complete penetrance; Alae-forming epidermal cells are produced instead of sensory ray cells [20]. We induced expression of MAB-5 in single V5 descendants of *mab-5* mutants at the middle L2 stage, and found that lineal descendants of the irradiated cells completely restored the wild-type cell-division pattern. In contrast to this, induction at the late L2 stage rescued the cell-division defect only poorly. These results proved that IR-LEGO can induce functional gene expression in a finely regulated manner.

4. DISCUSSION

We have shown that gene induction mediated by IR-LEGO successfully rescued the cell migration defect as well as the cell-fate specification defect of single targeted cells in relevant mutant nematodes. Notably, Cell-autonomous rescues of various phenotypes demonstrate that this method can be applied to trigger physiologically relevant responses in multiple cell types without causing detrimental effects. In principle, IR-LEGO could also be adapted to various transparent organisms in which transgenic technology is available. In the future, by combining with other techniques such as site-specific recombination and short hairpin RNA-mediated gene suppression, IR-LEGO will be a more powerful tool for gene manipulation.

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