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Application of modified fluorophore-assisted light inactivation technique in nervous system cell and explant cultures

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Abstract

Methods of molecular targeting are powerful tools for functional genomics studies. To analyze the biological function of the molecule, acute and temporally restricted inactivation of the targeted molecule is critical. We developed a simple experimental method of protein disruption in living nervous systems. We employed a fluorophore-assisted light inactivation (FALI) technique that uses light irradiation to produce photogenerated singlet oxygen free radical damage that is directed at targeted proteins through chromophore-conjugated antibodies. In order to apply the FALI technique to culture systems over a long period of time, we established a simple and easy-to-use long-term FALI (SELT-FALI) by modifying the original FALI to utilize weak blue light. To address the efficacy of the SELT-FALI technique, we used Neuropilin-2 (Nrp2), a receptor for a repulsive axon guidance molecule of Semaphorin-3F (Sema3F) as a test target molecule. We examined the effect of SELT-FALI of Nrp2 by assessing specific Sema3F-Nrp2 binding in various culture systems. SELT-FALI of Nrp2 resulted in complete inhibition of Sema3F binding to Nrp2 expressed on COS7 cells. This FALI efficacy was dependent on irradiated light power. SELT-FALI of Nrp2 decreased Sema3F-induced growth cone collapse in mouse sympathetic ganglion culture, and also resulted in a significant loss of repulsive response toward Sema3F in a collagen gel 3D culture system. Furthermore, continuous FALI of Nrp2 for 24 hours in developing neural tissue resulted in a significant reduction of Sema3F binding to Nrp2 expressing lateral olfactory tract (LOT) in organotypic culture of mouse telencephalon without any detrimental effects on LOT development. Thus, the FALI technique can be used for protein disruption with temporal resolution. Finally, we applied the SELT-FALI technique for functional screening of key molecules in LOT development, which resulted in the discovery of a novel functional molecule that functions in LOT formation, and this molecule was termed the lateral olfactory tract usher substance (LOTUS). This methodology can be used for functional screening of key molecules in a variety of culture systems.

Keywords: Light-mediated protein disruption, FALI, SELT-FALI, Organotypic culture

1. Introduction

Specific inactivation of a target molecule in an organism or cell is a powerful tool to analyze its molecular function. While genetic knock-out animals are an important tool, it is cost- and time-consuming and it is hard to rule out a possibility that the adult phenotype is caused by a side-effect of gene modification since gene disruption originates at the zygote level. As an alternate tool, RNA interference (RNAi) is a prevailing useful method to reduce levels of a specific protein. The RNAi needs time to become effective, resulting in a time lag between observation and actual expression of the molecule. In molecular function analysis, it is critical to analyze the molecular function at the site of molecule expression and at the time when the molecule is functional. Consequently, spatial and temporal restriction of damage to the targeted protein is necessary for physiological analysis. For this, the technique of spatiotemporally regulated protein ablation is a powerful methodology. Chromophore-assisted light inactivation (CALI) is a representative technique of this methodology, whereby protein ablation is achieved using light irradiation and photosensitizing dye-conjugated antibodies against the target molecule[1-3]. At present, there are many CALI techniques modified from the original CALI technology[1, 4]. For example, fluorophore-assisted light inactivation (FALI) was reported as a similar but more efficient method compared to the original CALI[5]. Subsequently, several CALI techniques were developed with transgenically encoded tags such as an exogenous motif consisting of tetra-cysteine residues bound with membrane-permeable derivatives: FlAsH-CALI[6], ReAsH-CALI[7], or Halo-tag bound by eosin[8]. Another CALI technique modification using genetically encoded fluorescent protein such as enhanced green fluorescent protein (EGFP), yellow fluorescent protein (YFP), KillerRed, miniSOG, and SuperNova was also demonstrated[9].

We employed FALI and modified some technical conditions to adapt this technique for long-term use in cell and organ culture systems, in order to facilitate functional screen analysis of key molecules. We thereby established a molecular targeting method by modifying the FALI technique, and termed it as simple and easy-to-use long-term FALI (SELT-FALI). FALI is an acute protein-ablation method directed by binding of fluorescein isothiocyanate (FITC)-labeled specific antibodies. Irradiation of light at a wavelength of 490 nm induces generation of singlet oxygen (oxygen radicals) from the fluorophore. The oxygen radicals generated from the FITC dye react chemically with the nearby target antigen recognized by the FITC-labeled antibody and causes functional inactivation of the target molecule (Fig. 1A).

Fig. 1

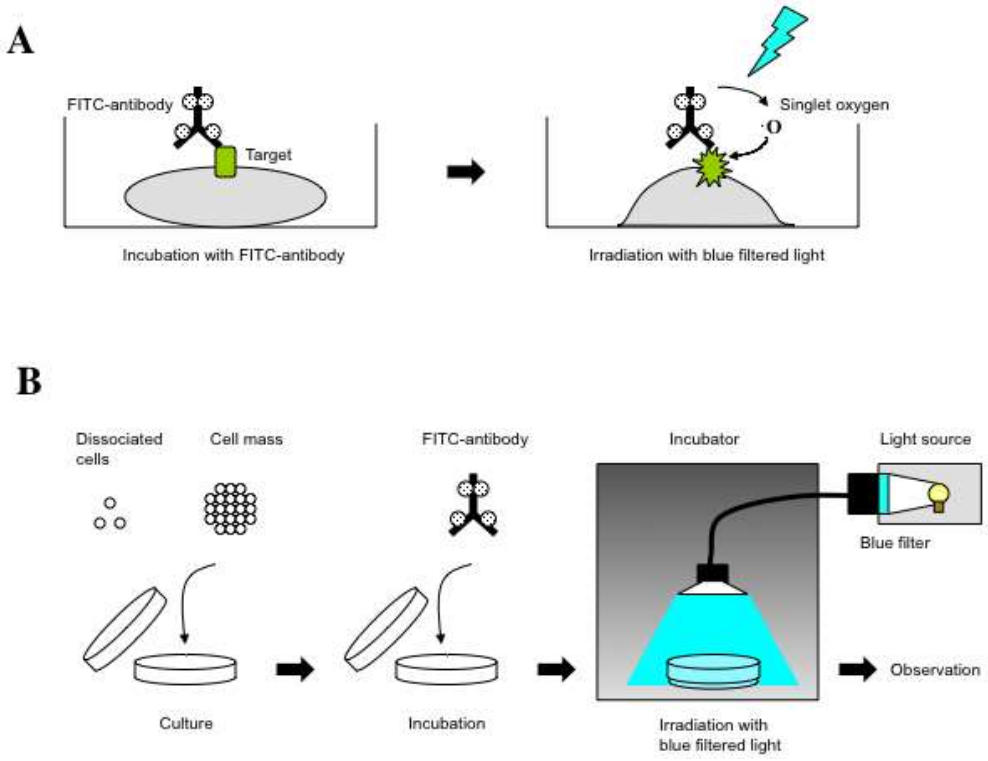

Figure 1. SELT-FALI method. (**A**) Principle of FALI technology. Blue-filtered light irradiation at 490-nm wavelength is used to direct damage of target molecule by photogeneration of singlet oxygen through FITC-labeled antibodies. (**B**) Summary of SELT-FALI in cell or cell mass cultures. The cell culture is incubated with FITC-labeled antibody and illuminated with blue-filtered light for 24 hours in an incubator.

We first examined the effect of SELT-FALI on specific binding affinity between a secreted protein, semaphoring 3F (Sema3F) and its membrane receptor protein, Neuropilin-2 (Nrp2). After confirmation of SELT-FALI efficacy, we then applied the modified FALI technique to cell and organ culture systems.

2. Simple equipment employed in SELT-FALI

To perform SELT-FALI under culture conditions, we developed equipment that has an excitation filter for FITC in the light source vent (PCS-UMX250, NIPPON P・I CO.,LTD., Tokyo, Japan), that has a metal halide lamp, and guides light into the incubator using a planar light-emitting light guide (PLG-B100X, NIPPON P・I CO., LTD.) (Fig. 1B).

3. Efficacy of SELT-FALI method

Using the above equipment for FALI, we first validated SELT-FALI efficacy by evaluating the molecular interaction between Neuropilin-2 (membrane receptor, Nrp2) and its ligand molecule Semaphorin 3F (Sema3F). We detected the binding of alkaline phosphatase-fused Sema3F (AP-Seme3F) to Nrp2 expressed on COS cells by visualizing the color reaction caused by AP activity. COS7 cells were transfected with Nrp2 and cultured for two days, followed by the addition of FITC-labeled anti-Nrp2 antibody to culture media, and irradiation with blue light at 490 nm for 1hr. This manipulation resulted in remarkable loss of Sema3F binding to Nrp2 (Fig. 2Ae), demonstrating that our methodology of SELT-FALI is effective for inactivating Nrp2 function. The same manipulation with normal (non-labeled) anti-Nrp2 antibody did not result in loss of Sema3F binding (Fig. 2Ac), indicating that this monoclonal antibody for Nrp2 is not a neutralizing antibody. The manipulation with FITC-labeled nonspecific immunoglobulin-G (IgG) also did not result in loss of Sema3F binding (Fig. 2Ad), showing that FITC itself did not affect the binding. These results suggest that SELT-FALI is useful for this biological assay.

Fig. 2

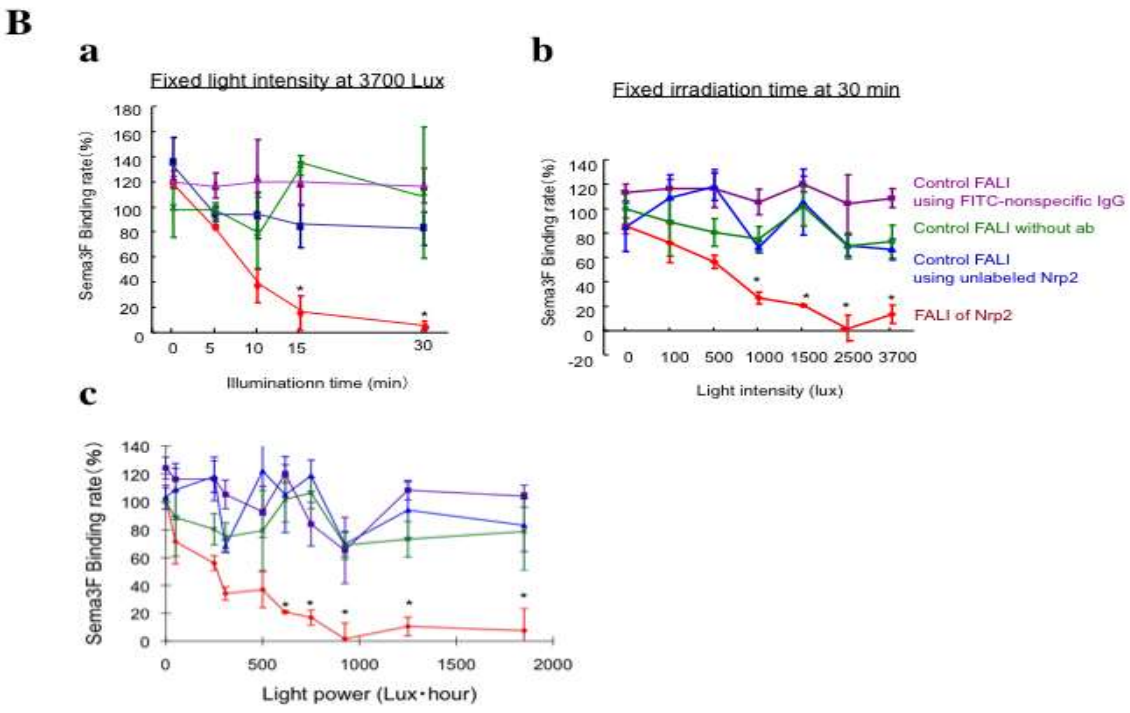

Figure 2. SELT-FALI of Nrp2 in COS7 cells. (**A**) Sema3F (ligand) binding to Nrp2 (its receptor) in COS7 cells. **a**) Summary of SELT-FALI of Nrp2 in AP-fused protein binding assay. **b-e**) FALI of Nrp2 results in complete loss of Sema3F binding in COS7 cells (e) when compared with control FALI without antibody (b), with unlabeled Nrp2 antibody (c) and with FITC-labeled nonspecific IgG (d). Human placental alkaline phosphate (AP)-tagged fusion proteins were generated by a standard PCR cloning technique and a conventional purification method. The binding of the AP-fused Sema3F was visualized with color reaction (see, 14). (**B**) Quantitative analysis of FALI efficacy shown by Sema3F binding affinity under various conditions. **a**) Binding rate (%) in illumination time under fixed light intensity (3700 Lux), **b**) Binding rate (%) in light intensity under fixed irradiation time (30 min), **c**) Binding rate (%) in light power (Lux-hour).

Using this experimental system, we next examined the characteristics of SELT-FALI. A constant intensity of blue light at 3700lux resulted in decreased binding of Sema3F to Nrp2 that was proportional to an increase in irradiation time length (Fig. 2Ba). A constant irradiation time length of blue light resulted in decreased binding of Sema3F to Nrp2 that was proportional to an increase in light intensity (Fig. 2Bb). These results indicate that time and light intensity have additive effects on SELT-FALI. The effect of SELT-FALI was almost proportional to the product of the time length and the light intensity of irradiation and sufficient over 10,000 lux·hour (Fig. 2Bc). Interestingly, the SELT-FALI system can result in various inactivation states through regulation of the duration and intensity of light irradiation. This cannot be achieved by gene manipulation methods and is an advantage of this methodology for functional assays of the molecule.

4. Target specificity of SELT-FALI

The principle of chromophore or fluorophore-assisted light inactivation (CALI / FALI) is that the photogenerated free radical produced from the photosensitizing dye (malachite green in CALI / FITC in FALI) conjugated antibodies by light irradiation is spatially restricted and only damages the antigen proteins[2, 10]. The photogenerated free radical, which is singlet oxygen in FALI, diffuses to a distance of 40Å in the time equal to the half-life of its activity (2). Consequently, we investigated the SELT-FALI effect on other molecules. To address this issue, we used Neuropilin-1 (Nrp1), a paralog of Nrp2 as a test molecule. Nrp1 is a membrane receptor for Sema3A[11]. On COS7 cells co-transfected with Nrp1 and Nrp2, both receptors were expressed at the cell surface membrane (Fig. 3b). FALI of Nrp2 in these cells induced specific reduction of Sema3F binding to Nrp2 but not Sema3A binding to Nrp1 compared with control groups (Fig. 3c). This suggests that

SELT-FALI would have high specificity for the target molecule without affecting other similar molecules.

Fig. 3

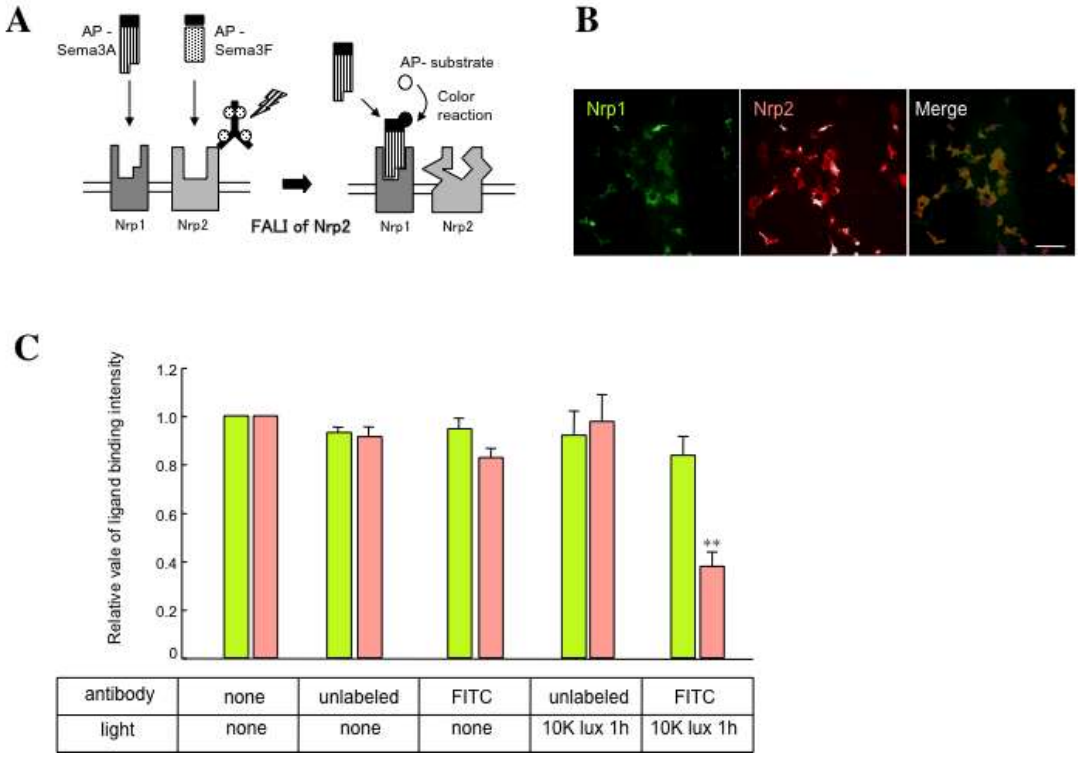

Figure 3. SELT-FALI specificity. (**A**) Schematic drawing of FALI of Nrp2 in COS7 cells expressing Nrp1 and Nrp2. (**B**) Co-expression of Nrp1 (green) and Nrp2 (red) in COS7 cells. Scale bar: 10 μm. (**C**) Quantitative analysis of Sema3A and Sema3F in COS7 cells co-expressing Nrp1 (receptor of Sema3A) and Nrp2 (receptor of Sema3F). FALI of Nrp2 (FITC-labeled antibody plus light irradiation (10K lux, 1 hour)) shows reduction of Sema3F binding, but not Sema3A, indicating that FALI of Nrp2 results in inactivation of Nrp2, but not Nrp1.

5. Application of SELT-FALI to culture systems

We next applied SELT-FALI to primary neurons in culture and investigated whether it would disrupt the physiological function of the target molecule. Growth cone collapse is induced by Sema3F in mouse sympathetic ganglia neurons expressing Nrp2[12]. After observation of neurite extensions by sympathetic neurons, FALI of Nrp2 was transiently applied to the culture using a 2 hour irradiation of 1000 lux blue light. FALI of Nrp2

reduced the growth cone collapse response to Sema3F (Fig. 4Aa), whereas no effect was seen in non-irradiated and irradiation with unlabeled anti-Nrp2 antibody cultures (Fig. 4Ab, 4Ac). These results indicate that transient FALI is feasible for biological assay in primary cultured neurons.

Subsequently, we examined the efficacy of SELT-FALI on long-term physiological function inhibition of mouse sympathetic ganglia neurons co-cultured for two days with an aggregate of COS7 cells expressing repulsive guidance molecule Sema3F. A previously described 3D co-culture system of Sema3F using collagen gel[13] was used in this study. The sympathetic ganglia neurons extended neurites in an opposite direction to the aggregate in response to Sema3F secreted from the aggregate (Fig. 4Ba, 4Bb). SELT-FALI of Nrp2 with 1000 lux blue light for 24 hours enabled neurite extension toward the COS7 cell aggregate secreting Sema3F (Fig. 4Bc), suggesting that SELT-FALI of Nrp2 inhibits the response to Sema3F by inactivation of its receptor Nrp2.

Furthermore, we examined the effect of SELT-FALI manipulation on organotypic cultures of mouse telencephalon in which a whole embryonic day 12 mouse telencephalon hemisphere was cultured on a special membrane filter that enabled the formation of an axonal tract derived from the olfactory bulb termed the lateral olfactory tact (LOT). The LOT expresses Nrp2. The organotypic culture was performed as described previously[14]. The telencephalon organotypic culture was performed for 24 hours with SELT-FALI to Nrp2, and the LOT was visualized thereafter using anti-Nrp1 antibody. The LOT was formed normally and was similar to the control without SELT-FALI. This manipulation, however, led to reduced binding of Sema3F to Nrp2 expressed in the LOT (Fig.4C). These findings suggest that SELT-FALI can be applied to the organotypic culture system to analyze the molecular function in the developing brain.

Fig. 4

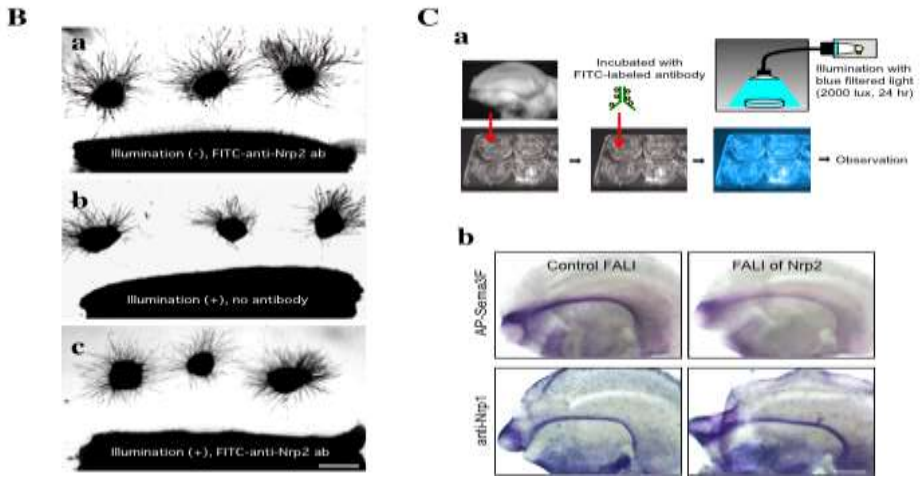

Figure 4. SELT-FALI of Nrp2 in cell and organotypic culture systems. (**A**) SELT-FALI of Nrp2 in cultured neurons. **a**) Control FALI with FITC-labeled antibody and without light irradiation, **b**) Control FALI with unlabeled antibody and with light irradiation, **c**) FALI of Nrp2 with FITC-labeled antibody and light irradiation, Scale bar: 100 μm, **d**) Quantitative analysis of growth cone collapse rate induced by Sema3F in FALI experiments. FALI of Nrp2 with FITC-labeled antibody under Sema3F incubation and light irradiation shown by black filled bar results in reduction of growth cone collapse rate when compared with control FALI experiments. (**B**) SELT-FALI of Nrp2 in 3D collagen cell culture. **a**) Control FALI with FITC-labeled antibody and without light illumination, **b**) Control FALI without antibody and with light illumination, **c**) FALI of Nrp2 with FITC-labeled antibody and light illumination, Scale bar: 500 μm. FALI of Nrp2 results in reduction of the repulsive response of axons extended from sympathetganglia to repulsive guidance molecule Sema3F secreted from aggregates of COS7 cells. (**C**) SELT-FALI of Nrp2 in organotypic culture of mouse brain. **a**) Summary of SELT-FALI in organotypic culture of whole-mount mouse brain. **b**) FALI of Nrp2 results in reduction of AP-Sema3F binding to Nrp2 expressed in LOT (upper panels) and the LOT is visualized by immunohistochemistry of Nrp1, a marker protein of LOT (bottom panels).

6. Further application of SELT-FALI to functional screen

Olfactory information is first received by the olfactory bulb, and the axons of mitral cells emerge from the olfactory bulb and first grow laterally and then elongate caudally at the surface of the telencephalon. This LOT projection is a good system for experimental analysis of key molecules of neural circuit formation, because the LOT can be visualized by immunohistochemistry of Nrp1 in whole-mount brain samples and developmental events

can be observed in an organotypic culture system as shown in Fig. 4. We used this system combined with SELT-FALI to screen for molecules that function in LOT formation[14].

To search for the molecules that function in LOT formation, we first generated monoclonal antibodies that were produced in hamsters using a protein extract homogenate derived from the mouse developing LOT as an immunoantigen. We then selected antibodies that recognized molecules expressed on the membrane surface of the developing LOT by immunohistochemistry. The selected monoclonal antibodies were chemically labeled with FITC dye. FALI with FITC-labeled antibodies was applied to an organotypic culture of developing telencephalon. When FALI results in abnormal phenotypes in LOT formation, the molecules recognized by the FITC-labeled antibody may be involved in LOT formation and may play an important role in LOT formation. Out of over one hundred antibodies, FALI with FITC-labeled H24G11 monoclonal antibody resulted in remarkable defasciculation of the LOT, whereas control FALI without antibody, with unlabeled H24G11 monoclonal antibody, or with FITC-labeled nonspecific immunoglobulin showed no effects. These findings suggest that the antigen molecule recognized by H24G11 monoclonal antibody may contribute to formation of the LOT axon bundle. Using a mouse cDNA expression library of the olfactory bulb, we identified the antigen molecule as cartilage acidic protein-1B also known now as lateral olfactory usher substance (LOTUS)[14]. Thereafter, *lotus*-deficient mice were generated by a conventional gene-targeting method and these mice showed defasciculated LOT. The phenotype in organotypic culture with SELT-FALI of LOTUS was similar to that of LOTUS-knockout mice, indicating that the data obtained by the SELT-FALI methodology is in reasonable agreement with the data of conventional genetic methodology. Therefore, the SELT-FALI method may be a promising technique to achieve loss of function of target molecules in culture systems.

7. Technical considerations

We demonstrated that SELT-FALI is a highly useful method in the temporal disruption of molecular function in culture systems. First, SELT-FALI is easy to use as it utilizes only FITC-labeled antibody and weak blue light. Second, SELT-FALI requires only a short time (~30min) to exhibit disruption of the target molecule when compared with other methods such as RNAi that requires a few days. Third, SELT-FALI has high specificity for the target molecule as it uses a specific antibody when compared with RNAi that might have off-target effects. Fourth, SELT-FALI can provide continuous and long-term inactivation of the

target molecule when compared with the original CALI/FALI methods that enable only transient disruption of the target.

Use of non-neutralizing antibodies can expand the range of its application, because the CALI/FALI technique converts a non-neutralizing antibody to a photoactivated neutralizing antibody, rendering a neutralizing antibody unnecessary. Extended irradiation with a high intensity light results in cytotoxity[15] and a large quantity of singlet oxygen is also toxic to living cells[16]. Therefore, the use of weak light resolves this issue of cytotoxity by light irradiation. In fact, singlet oxygen generated from FITC-labeled nonspecific IgG with weak light does not affect living cells in various culture systems including the telencephalon organotypic culture.

The singlet oxygen diffuses toward various directions within a 40Å distance from the fluorophore so nonspecific oxidation may influence adjacent molecules[2]. However, we demonstrated that SELT-FALI to Nrp2 on a membrane exerts no influence on the function of Nrp1 expressed on the same membrane surface, indicating that SELT-FALI shows highly specific efficacy without nonspecific influence on molecules adjacent to the target molecule (Fig. 3).

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