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2020년 12월

서울대학교 대학원

뇌과학 협동과정

이 지 아

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이 논문을 이학석사 학위논문으로 제출함
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The eGRASP expression on neurons, astrocytes, and HEK293T cells *in vitro*

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A dissertation submitted to the Graduate Faculty of Seoul National University in partial fulfillment of the requirement for the Degree of Master of Science

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Abstract

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Studies on astrocytes have been focused on unveiling its interplay with neurons to understand the physiological properties of nerve systems, leading to both fundamental interests and clinical applications including the origins of neurodegenerative diseases. In terms of synaptic transmission, major role of astrocytes is forming 'tripartite synapse' among postsynaptic neurons and presynaptic neurons. However, progress in the understanding of interactions among tripartite synapses has been limited to topical observation for lack of effective technologies to visualize synapses. Recently, the dual−eGRASP was developed to label synapses of targeted neurons

in the mammalian brain. The technique made it possible to compare the activity- or region-dependent strength of synapses, potentially providing a tool to elucidate the role of neuronal connectivity in learning and memory. Here, I verified the eGRASP signals to be present in *in vitro* rodent primary neurons and astrocytes, expanding the applications of previously published dual-eGRASP methodology. The eGRASP signals were expressed among various cells including neurons, astrocytes, and HEK293T cells, regardless of transfection methods. Especially, the signals appeared in the form of faces at contacts between cells: dendritic shafts and adjoining astrocytes or HEK293T cells. The results substantiate that the *in vitro* eGRASP provides a promising method to elucidate not only physiologies of nerve systems in controlled environment, but also of any cellular systems with an arbitrary level of external control.

Introduction

Astrocytes are a major class of glial cells which constitute the central nerve system, facilitating proper function of neurons by ensuring concentration homeostasis of ions and neurotransmitters (Nedergaard, Ransom, and Goldman 2003). Astrocytes were named after their star-shaped conformation of their branching processes. The processes are mainly constructed from glial fibrillary acidic protein (GFAP), which is used as a representative marker protein of astrocytes (Hammond et al. 2015).

In synaptic transmission, astrocytes release gliotransmitters to modulate synaptic transmission by regulating neurotransmitters (Schwarz et al. 2017). Astrocytes extend their processes around synapses between presynaptic and postsynaptic neurons to control and interact synapse formation (Stogsdill et al. 2017). 'Tripartite synapse', the term referring to this structures means the synapses are composed not only neurons but astrocytes surrounding neuronal membranes. Dynamic changes of astrocytic processes influence the strength of relevant synapses, implying neurons and astrocytes influence each other (Chung, Allen, and Eroglu 2015).

In vitro studies of astrocytes have been usually performed with

primary astrocytes from rodent embryos. The astrocyte culture system has been utilized to dissect their functions in the normal brains or malfunctions in neurodegenerative diseases (Phatnani and Maniatis 2015). For instance, the *in vitro* assay for the status of astrocytes were performed to classify reactive astrocytes. Astrocytes show the reactivity against infection or trauma of the nerve system. The reactive astrocytes have been modeled as two distinct profiles regarding their inflammatory or ischemic environments. The toxicity assays using the neuron-astrocyte co-culture system could detect discriminate reactive astrocytes (Liddelow and Barres 2017).

Generally, astrocytes *in vitro* have been known to have neuroprotective functions on rodent primary cortical and hippocampal neurons (Liu et al. 2012). A number of *in vitro* culture studies have explained the role of astrocytes to promote synapse formation (Farhy-Tselnicker and Allen 2018). In the absence of astrocytes, cultured neurons form few, immature synapses. Adding astrocytes or astrocyte conditioned media could increase the number of mature and functional synapses by approximately an order of magnitude (Ullian et al. 2001).

Nevertheless, *in vitro* studies of neurons and astrocytes have been

stymied because of the lack of effective culture protocols. The most established method to prepare astrocytes is including fetal bovine serum in media (McCarthy and De Vellis 1980), which is far from *in vivo* states of astrocytes. Astrocytes in the brains are heterogeneously distributed, depending on brain regions or developmental periods of the fetus (Buosi et al. 2018; Sun et al. 2017).

Synaptic transmission has been studied as a unit of information processing in the nerve system (Di Maio and Bouteiller 2019). Each neuron receives synaptic input and transmit the informational spike to the next neuron. Though neurons remain stationary during lifetime except small population in dentate gyrus or subventricular zone in the brain, their synapses are continuously remodeled reflecting accumulated memory of organisms. Although various methods to observe synapses have been developed with progress in neuroscience (Südhof and Malenka 2008), they still have been limited to topical observation utilizing electron microscopy (Farhy-Tselnicker and Allen 2018).

GFP Reconstitution Across Synaptic Partners(GRASP) is a tool using two fragments of GFP tethered to the membrane domain to label synaptic contacts on cells in proximity (Feinberg et al. 2008).

The split-GFP system constitutes the stable and fast-folding 214 residues of GFP (spGFP1-10) and the small 16 residues (spGFP11). The split-GFP glow when they come into close contact, hence they can be applied to labeling synapses between two neurons. The GRASP technique has been recently optimized to mark synaptic connections with clear signal (t-GRASP) (Shearin et al. 2018) or extend into the mammalian brain (mGRASP) (Kim et al. 2012).

However, previous GRASP techniques suffered from several limitations including weak fluorescence signals (Choi et al. 2018). To overcome the obstacles, the enhanced GRASP was developed modifying structures of spGFP1-10 fragment to increase GFP signal intensity. In addition, introducing additional mutations to spGFP1-10 fragment attained to express distinguishable cyan and yellow colors with same spGFP11 fragment. Using the Dual-eGRASP technique, connection from two distinct synaptic population to a single postsynaptic neuron can be visually separated.

The Dual-eGRASP technique has the potential to mark to various cell types in the mammalian brain, yet its application has not reached the tripartite synapses. To confirm the synaptic relationship between astrocytes and neurons, I applied the eGRASP techniques to figure out connections between astrocytes and neurons using mouse

primary hippocampal neurons and astrocytes. To establish robust *in vitro* system of the dual−eGRASP technique, I performed additional experiment using HEK293T cells, a cell line being commonly used for virus transfection (Pear et al. 1993) with calcium phosphate transfection. The eGRASP signals were expressed *in vitro* regardless of cell types and gene delivery methods. This dissertation provides a robust methodology to construct the eGRASP in various cell types *in vitro* environments.

Materials and methods

Construction of eGRASP plasmids

The plasmids used for eGRASP expressions were modified from Choi et al.,2018. To be specific, the pre－eGRASP construct was composed of an IgG Kappa signal peptide, strand 1－10 of split GFP with mutation expressing yellow highlights, nurexin1b stalk, and transmembrane and intracellular domain. It was inserted with along with myristoylated iRFP670 by In－fusion cloning in the AAV vector with the GFAP promoter. The post－eGRASP constructs consisted of an IgG Kappa signal peptide, Abl SH3 domain, neuroligin1 stalk, and transmembrane and intracellular domain. The human synapsin promoter was substituted for the TRE3G promoter in AAV vector with myristolylated mScarlet and the post－eGRASP construct at the MluI－NheI restriction sites.

AAVs production and infection

Adeno-associated viruses serotype 1/2 were used to infect primary neurons and astrocytes. AAV 1/2s were extracted from HEK 293T cells which were transfected with calcium phosphate methods. Plasmids to assemble AAV 1/2s were the gene of interests in AAV2 ITRs, p5E18-RXC1, p5E18, and pAd-ΔF6. 6-8 hours after transfection, the media were exchanged by opti-MEM and cells were cultured four days. Media with AAV1/2s were harvested.

Detailed procedures of affinity chromatography are as follows. The poly-prep chromatography columns were loaded onto 1 mL of heparin-agarose suspension. The virus supernatants were poured into the columns. The columns were washed by 4 mL of 4-150 buffer (150 mM sodium chloride, 10 mM pH 4 citrate buffer) and 12 mL of 4-400 buffer (400 mM sodium chloride, 10 mM pH 4 citrate buffer). The viruses were elulted by 4 mL of 4-1200 buffer (1200 mM sodium chloride, 10 mM pH 4 citrate buffer). The virus particles are concentrated with DPBS in Amicon Ultra-15 centrifugal filter units (Millipore). Titer of viruses were measured by quantitative RT-PCR.

AAVs were stored at -80℃ and thawed the day to infect neurons or astrocytes. Unlike injecting viruses to live animals, a small number

of freezing and thawing had little effect on cultured cells. AAV was frozen and thawed three times at the most.

Cell culture

Primary hippocampal neurons were prepared from mouse E18 embryos. $1.0×10^5$ cells were cultured in 24-well plates with poly-D-lysine treated coverslips. Neurons were in the plating media (10% FBS (Wellgene) in MEM (Gibco)) for 2-3 hours. When neurons attached on coverslips, the media were exchanged to the maintenance media (Neurobasal with additional B-27 (Gibco)) with 0.25-0.5μM Ara-C (Sigma-Aldrich) to exclude unexpected glial cell proliferation. 2.5% FBS added to the maintenance media to prepare for co-culture with astrocytes. Every 2-3 days, media were partially exchanged with the maintenance media without Ara-C.

Primary astrocytes were prepared from mouse E18 embryos. $1.0×10^6$ astrocytes were contained in a cryogenic tube and frozen in stock. Every 1-2 batch, astrocytes were thawed and plated to 24-6 wells with DMEM (Wellgene) and 15% FBS media. To stabilize fresh

astrocytes, astrocyte-plated wells were maintained for 3-5 days till cells attached completely.

The neuron-astrocyte co-culture were performed three days before the sample preparation. Transfected astrocytes were dissociated from wells with trypsin (Wellgene) and centrifuged. 20,000-30,000 astrocytes were transferred to each coverslip with cultured neurons.

HEK293T cells were thawed in 100ϕ plates in DMEM with 10% FBS. They were split every 3 day in 1:10 ratio. The day before transfection, 1.0×10^6 cells were transferred to each well in a 6-well plate.

Cell transfection

Nucleofection was tried to deliver genes to neurons. I referred to the official protocol from Lonza (Lonza BioResearch). 4D-Nucleofector device with X unit (Lonza) and P3 primary cell buffer (Lonza) were utilized. For reactions in 100 μL cuvettes, $1.0-4.0 \times 10^7$ of mouse primary hippocampal neuron were centrifuged

at 0.1 g for 10 minutes. Neurons were resuspended in the reagent. Each plasmid was added at 1.5 μg per cuvette. Cell resuspension was transfer to the cuvette. I applied CU-133 protocol in the device. The plating media were added at voltage shocked cuvettes. 2-3 hours after transfection, I exchange media to the maintenance media when all neurons settled to bottom of the wells.

Lipofectamine 3000 (Invitrogen) was used for astrocyte transfection(Rao, Morales, and Pearse 2015). Opti-MEM (Gibco) was used to make serum-free environment to astrocytes. Plasmids and Lipofectamine 3000 reagents were mixed at 1:2 - 1:3 ratio in Opti-MEM and added on astrocyte cultured 6-wells. Media were exchanged to DMEM with 15% FBS 6 hours after transfection.

Calcium phosphate transfection was applied for HEK293T cells. The media were changed with chloroquine (Sigma-Aldrich). Cells were incubated for 15-20 minutes. I used 3 μg of plasmid per well. 2.5 M calcium chloride solution and plasmid constructs were mixed at 10:1 ratio in 267 μL of water for injection (Gibco). 300 μL of the HEPES buffer solution was added. Precipitates were viciously vortexed spread to cells. 6-8 hours after transfection, the media were replaced with fresh DMEM with FBS 10%. The day after transfection, HEK293T cells with pre and post eGRASP constructs

were detached with trypsin and mixed. They were seeded on poly－D－lysine coverslips for the image processing.

Sample preparation

Before culturing cells, sterilized coverslips were coated with poly－D－lysine for 15－20 minutes. To decrease toxicity of poly－D－lysine, coverslips were washed with DPBS (Wellgene).

Coverslips were fixed with 4% paraformaldehyde(PFA) in PBS for 10 minutes at room temperature. 100% methanol was treated for 20 minutes at －20 ℃ thereafter. Coverslips were washed with DPBS once or twice every step to reduce remnants of reagents. Coverslips were mounted in VECTASHIELD mounting medium (Vector Laboratories) with or without DAPI.

Immunocytochemistry

Astrocytes were seeded at 3×10^4 cells/well in 24-well plates in DMEM and 15% of FBS. After fixing wells, cells were incubated with 5% goat serum blocking solution with 0.5% triton-X 100 for 1.5 h. They were incubated 2 days with anti-GFAP primary antibody (Millipore, MAB3402) in the blocking solution at 4 ℃. Cells were gently washed 2 times in PBS with 0.3% triton-X for 15 minutes at room temperature. Goat anti-mouse Alexa Fluor 555 (Invitrogen, A28180) secondary antibody was used in the blocking solution for 2 hour at room temperature. After washing with in PBS with 0.3% triton-X for 15 minutes, cells were stained with DAPI.

The result were analyzed with ImageJ. To count DAPI stained astrocytes, images were adjusted with threshold. After being counted automatically, manual modification with outlined images was followed. GFAP stained astrocytes were manually counted because filament were amorphous. The number of DAPI stained nucleus in one chunk of stained filaments was considered as plural GFAP-positive astrocytes.

Imaging processing

Samples were imaged by a Leica SP8 confocal laser scanning microscope. Images were acquired with LAS X Life Sciences software. To observe overall coverslips, the 2D mosaic scan was used with a 10x objective lens at 0.75 zoom. Yellow fluorescence was confirmed at each marker fluorescence overlapped region. eGRASP images were captured on 63x objective lens with distilled water immersion on Z-stack. To convert the LAS X file to available image files, snapshot of Imaris Viewer (Bitplane) was utilized.

Olympus IX51 microscope with 10x, 20x objectives under bright-field and fluorescence was used to examine AAV expression and the viability of cells in live states, and immunocytochemistry.

Results

Data obtained in the previous study introducing the dual-eGRASP (Choi et al. 2018) showed that the eGRASP signal appeared at contacts between the pre-eGRASP and the post-eGRASP expressed cells. In this dissertation, GFAP and human synapsin promoter were chosen to avoid co-expression of the pre- and post-eGRASP constructs. mScarlet and iRFP670 fluorescence marker proteins were accompanied to trace the contacts (Figure 1A). Neurons were infected with the post-eGRASP first and co-cultured with the pre-eGRASP-transfected astrocytes to make contacts between neurons and astrocytes (Figure 1B).

The signal appeared at the surfaces of neuron-astrocyte contacts, rather than synaptic points (Figure 2). Co-expression of the pre-eGRASP and the post-eGRASP markers indicated that regions of neurons and astrocytes overlapped. However, overlapped fluorescent markers did not mean exact contacts between nerve cells. The eGRASP signals occasionally appeared when cells touched at the longitudinal axis (Table 1).

Imaging the eGRASP signal, I consistently found clusters around

the astrocyte nucleus (Figure 3). They glowed in the yellow wavelength even at an astrocyte that did not express the pre-eGRASP. These non-specific yellow clusters are easily confused with the eGRASP signals. One possible origin of the clusters could be subcellular components of astrocytes such as metabotropic glutamate receptors (mGluR)(Durand et al. 2011). To distinguish the non-specific clusters from true the eGRASP signals in cultured astrocytes, the immunocytochemistry of possible organelle along with the eGRASP expression is required in future studies.

The strategy to maintain viable neurons

Neuronal maturation requires at least 2 weeks culture periods (Papa et al. 1995). During culture, it was important to maintain a constant condition for the viability of neurons. Therefore, 2.5% FBS was added to the maintenance media from the day primary neurons were seeded. Though serum was not necessary for neurons, changing the media composition in seven days for co-culture with astrocytes damaged neurons. The clear eGRASP signals appeared at

matured neurons with enough number of dendritic spines. Neuronal cultures should be maintained at least for DIV 10, but not exceed DIV 17.

　Neurons were infected by AAVs at DIV 3 and neurons−astrocytes co−culture was followed at DIV 5. The co−culture with astrocytes prevented the degeneration of neurons because astrocytes have neuroprotective functions (Liu et al. 2012). I should leave intervals between infecting AAV to neurons and astrocytes co−culture for fear the virus for neuronal infection would remain and infect additional astrocytes (Howard and Harvey 2017). The strength of mScarlet fluorescence, presenting the post−eGRASP was irrelevant to the day to infect viruses to neurons.

Searching for suitable gene delivery methods for the *in vitro* eGRASP

　To observe the eGRASP expression *in vitro*, I applied the adeno−associated virus (AAV) or Lipofectamine to deliver each plasmid construct (Figure 1B, C). Suitable gene delivery methods for each

cell type were required. Neuronal transfection was especially difficult due to the postmitotic property of neurons (Karra and Dahm 2010). I tried to deliver the post-eGRASP gene to neurons using the AAV 1/2 (Figure 2) and nucleofection (McCall et al. 2012) (Figure 3). The AAV infection method was more appropriate to insert the gene because most nucleofected neurons were dead. Even survived neurons had too weak signal though they survived for 14 days, the period that neurons have mature dendritic spines (Papa et al. 1995).

When using viral vectors to deliver genes, serotypes of virus should be properly determined considering cell types. I chose AAV 2/1, which has been reported to have high efficiency in cultured neuron and astrocytes (Hammond et al. 2017)

To deliver the pre-eGRASP gene to astrocyte, AAV1/2 had higher efficiency than lipofection (Table 1). The efficiency of Lipofection was highly variable according to the ratio of Lipofectamine reagent and DNA. To maintain the viability of astrocytes, P3000 reagent was omitted and the media was exchanged in 6 hours.

Prevention of neuron-astrocyte co-infection

As for long-term (>5 days) primary neuron cultures, glial cells become overrun neurons without additional co-culture procedures(Meberg and Miller 2003). Proliferated glial cells could be infected with the post-eGRASP contained viruses that were supposed for neurons since the human synapsin could be expressed not only in neurons but also in astrocytes for neuroprotective purposes (Wang et al. 2011). This unexpected infection was the problem because the astrocytes could make false-positive eGRASP signals when they had both post-eGRASP and pre-eGRASP constructs (Figure 4). Furthermore, existing astrocytes occupied neighboring regions with neurons hindering new astrocytes containing the pre-eGRASP from contacting neurons to co-express eGRASP signals.

Maintaining the pure neuronal culture, I treated Ara-C to neuronal cultures to inhibit the proliferation of astrocytes. Determining the least functional concentration of Ara-C was critical because excessive Ara-C could damage neurons as well. Besides, pure neuronal cultures were more vulnerable than the natural neuronal culture where existing glia feed and protect neurons. Ara-C treatments after DIV 7 or overdose (1μM) of Ara-C obviously

degenerated neurons. One treatment of 0.25−0.5μM Ara−C at the beginning of culture performed proper function with low toxicity (Figure 5).

Existence of GFAP−negative astrocytes in culture

Despite diverse trials to transfect astrocytes, the pre−eGRASP expression in astrocytes with the GFAP promoter was much sparser than the post−eGRASP expression in neurons (Figure 10). Applying AAVs to infect astrocytes could not increase the expression rate as well (Figure 10A, B). If the GFAP was not expressed at astrocytes of the experiment, the plasmid construct using the GFAP promoter could not be expressed regardless of gene delivery methods. To confirm the GFAP expression in cultured astrocytes, I immunostained astrocytes with the anti−GFAP antibody (Figure 6).

A quantitative analysis based on the DAPI−GFAP signals showed that only one−fourth of astrocytes were GFAP positive. There was no significant morphological differences between GFAP positive or negative astrocytes in bright−field (Figure 6A).

In vitro eGRASP expression among different cell types.

Obtaining the eGRASP signal in HEK293T cells has been utilized only with Nucleofection, a modified form of electroporation (Choi et al. 2018). Electroporation utilizes electrical pulse to perforate holes in cell membranes to insert DNA. While electroporation had high transfection efficiency　(Karra and Dahm 2010), it could raise substantial cell death by high voltage pulses (Potter and Heller 2003). It is difficult to use electroporation as regular procedures because repetitive electroporations were burdensome due to its cost.

The calcium phosphate DNA coprecipitation has been commonly used to transfect in various cell types. It was usually applied to HEK293T transfection to produce various viruses such as AAVs or lentiviruses. I implemented the calcium phosphate transfection method for HEK293T cells to express eGRASP signals (Figure 7A). The eGRASP expressed consistently in repetitive experiments. The signals had different aspects depending on angles where cells contacted with (Figure 8A−C). I found similar false−positive signals

in HEK293T cells to co-expressed astrocytes where I inserted both eGRASP constructs at the same time for the positive control (Figure 8D).

At the next step, I confirm the eGRASP expression in different cell types using calcium phosphate-transfected HEK293T cells (Figure 7B-C). The eGRASP were co-expressed among neurons and HEK293T cells or HEK293T cells and astrocytes. The signal was observed regardless of cell types of gene delivery methods (Figure 9).

Imaging techniques searching for contacts

In contrast to *in vivo* tissue imaging, where cells are compactly organized in tissues, the co-culture of primary neurons and astrocytes was arranged irregularly with low density. Contacts among cells were random as well. The size of synapses was variable according to functional aspects such as neurotransmitter release or postsynaptic receptors. They could be distinguishable when they scanned by electron microscopy(Santuy et al. 2018). Although the

eGRASP can label synapses by fluorescence, labeled synapses could be seen only at a high-magnificent objective lens. It was necessary to scan overall cell arrangements first.

I utilized the mosaic function in the confocal microscope (De Bock 2011) with a low objective lens. After obtaining overall coverslip images, I attempted to search for the yellow eGRASP signals at every contact region in each mosaic scanning in a high objective lens. Even one scanning dramatically reduced time and labor to find the eGRASP signals among numerous cells (Figure 10).

Figures

A

| hSyn | myr_mScarlet-I | P2A | post-eGRASP |

| GFAP | myr_iRFP670V5 | P2A | pre-eGRASP |

B

mouse primary hippocampal neuron → 3 days culture → AAV infection → 2 days culture → lipofected mouse astrocyte transferred → 5 days culture

C

mouse primary hippocampal neuron → 3 days culture → AAV infection → 2 days culture

mouse primary astrocyte → culture maintaining → AAV infection

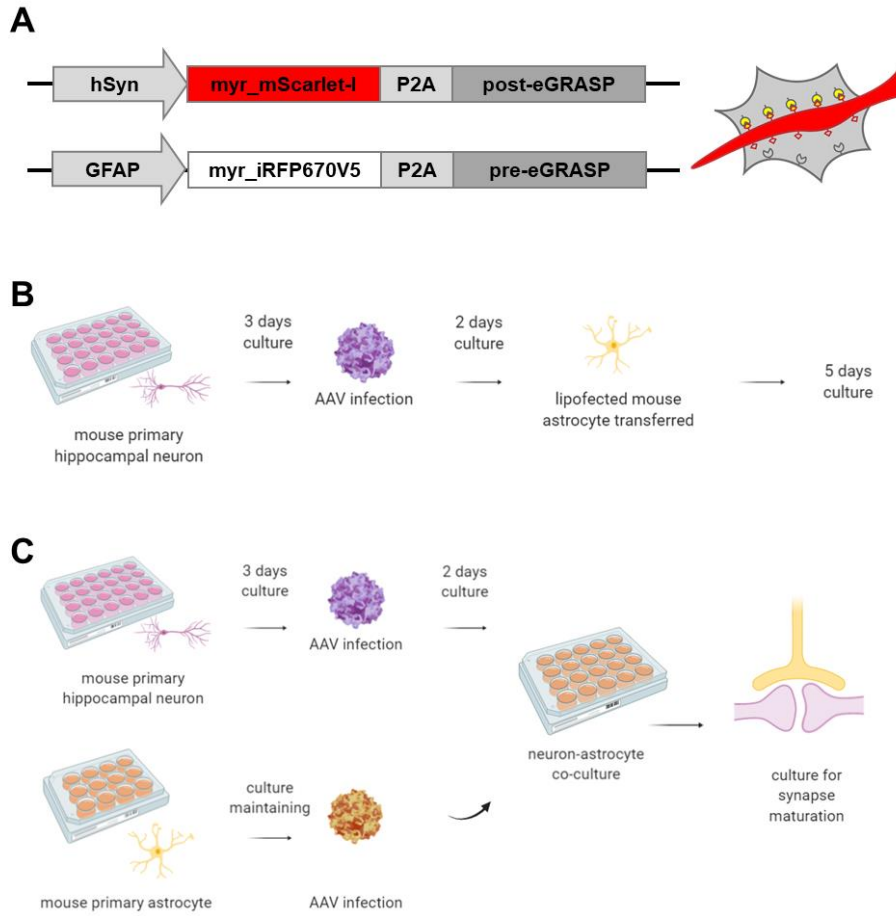neuron-astrocyte co-culture → culture for synapse maturation

Figure 1. Diagrams of *In vitro* expression of eGRASP between astrocytes and neurons.

(A) (Left) Diagram of the eGRASP DNA constructs for astrocytes and neurons. (Right) Illustration of expressed eGRASP within an astrocyte and a neurite. (B) The experimental procedure using AAV infection for neurons and lipofectamine transfection for astrocytes. (C) The experimental procedure using AAV for both neurons and astrocytes
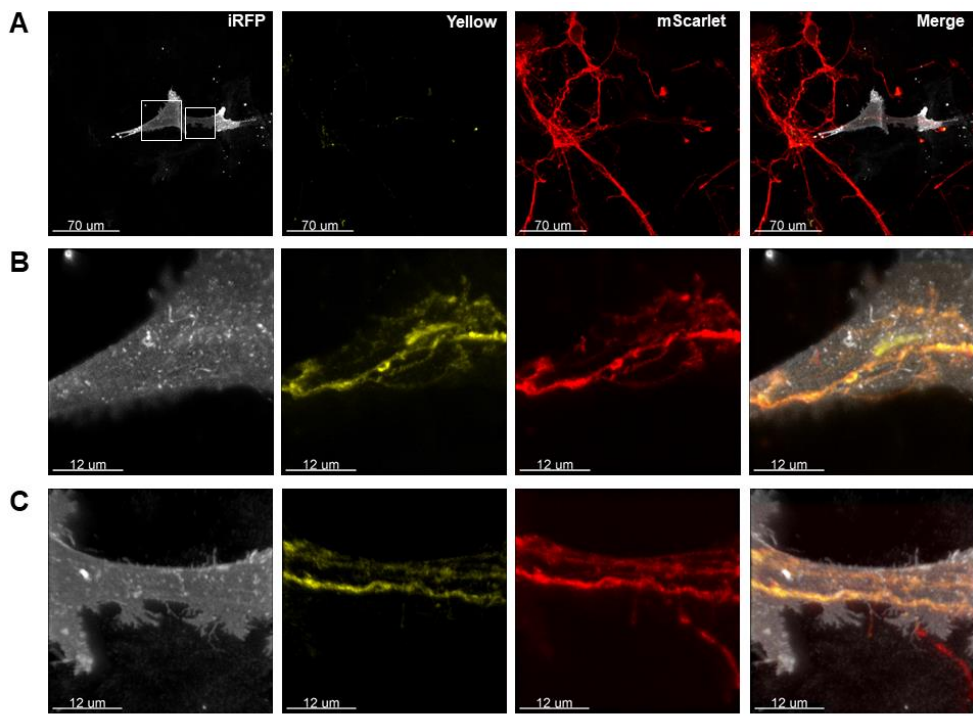
A

iRFP   Yellow   mScarlet   Merge

70 um   70 um   70 um   70 um

B

12 um   12 um   12 um   12 um

C

12 um   12 um   12 um   12 um

Figure 2. Expression of Yellow eGRASPs at contact regions
of between an lipofectamine-transfected astrocyte and a
AAV-infected neuron.

(A) Overlaps between an astrocyte and a neuron. Post-eGRASP
and mScarlet were co-expressed in a mouse primary hippocampal
neuron. Imaged regions were darkened due to photobleaching. Pre-
eGRASP and iRFP were in a mouse astrocyte. (B-C) Expanded
images of (A).

A mouse primary hippocampal neuron → electroporation → Transfer to astrocytes-seeded well → coverslipped well with lipofected mouse astrocyte → 14 days culture

B

150 um

C

iRFP | Yellow | mScarlet | merge

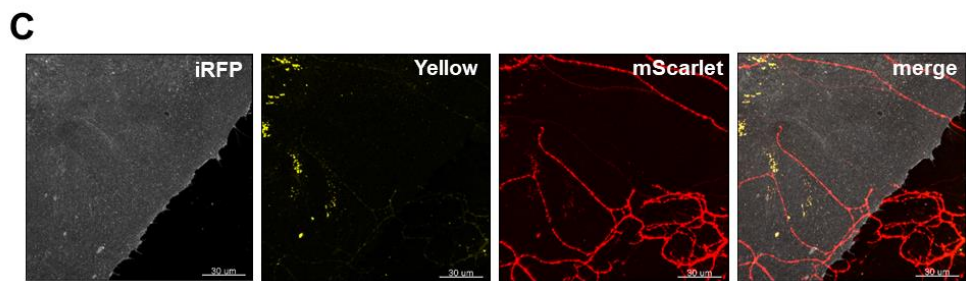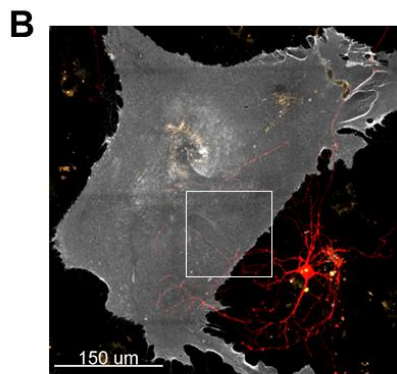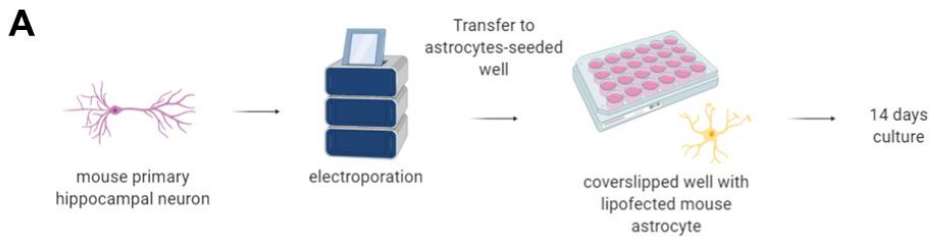30 um | 30 um | 30 um | 30 um

Figure 3. Feeble eGRASP signals between an lipofectamine－transfected astrocyte and nucleofected neurons.

(A) The experimental procedure to mark the eGRASP using Nucleofection for neurons. (B) A mosaic－scanned image between an astrocyte and neurons. (C) An expanded image of (B). Neurites and astrocytes were overlapped but had few eGRASP signals.
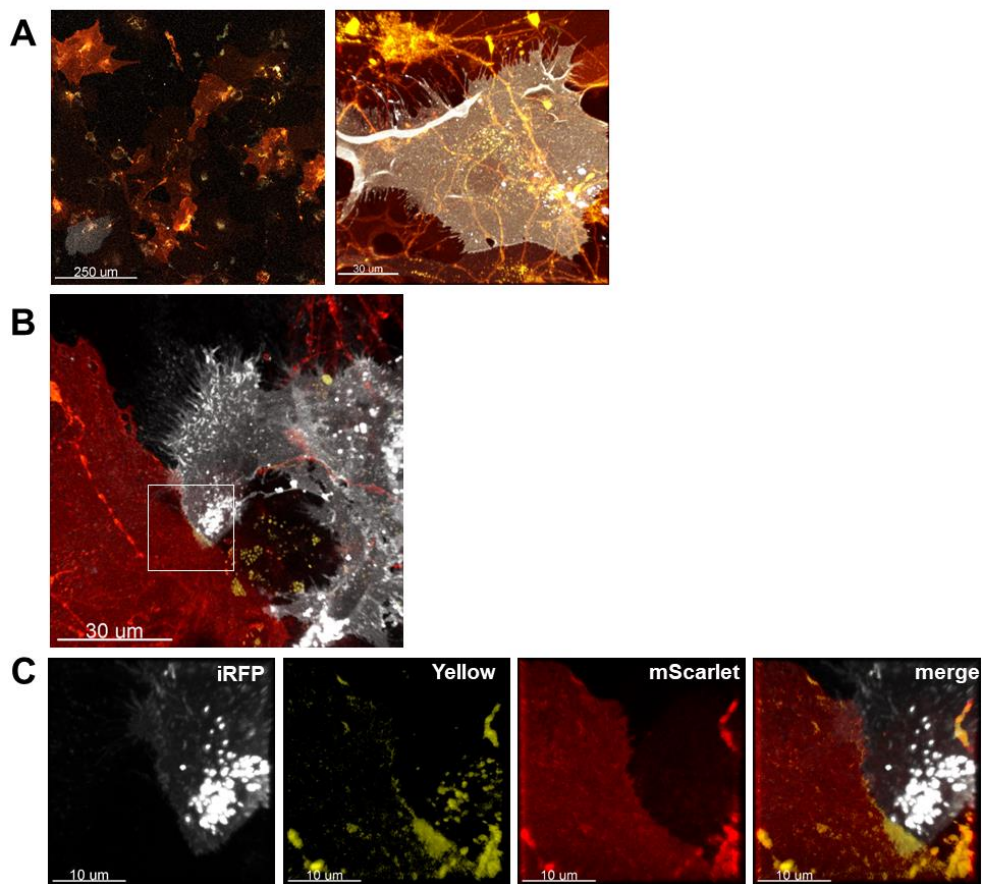
A

250 um

30 um

B

30 um

C

iRFP

10 um

Yellow

10 um

mScarlet

10 um

merge

10 um

3 6

Figure 4. The eGRASP expressions among astrocytes.

(A) Without Ara-C treatment, astrocytes were infected post-eGRASP included AAV for neurons and showed false-positive yellow eGRASP signals. (B) Yellow eGRASP signals were detected within an astrocyte infected post-eGRASP included AAV and the other astrocyte lipofected with pre-eGRASP. (C) Expanded images of (A). eGRASP signals were expressed around contacted regions of both astrocytes.
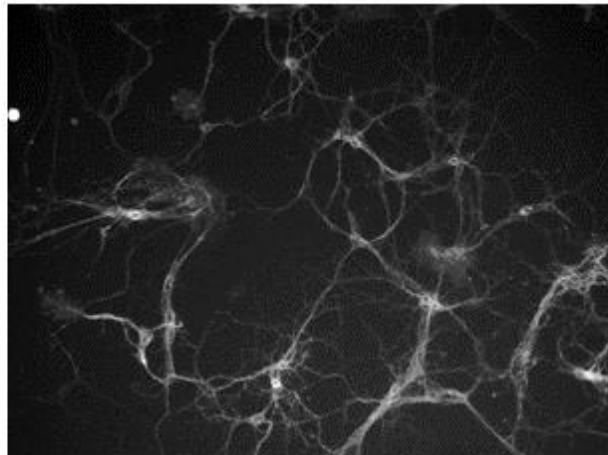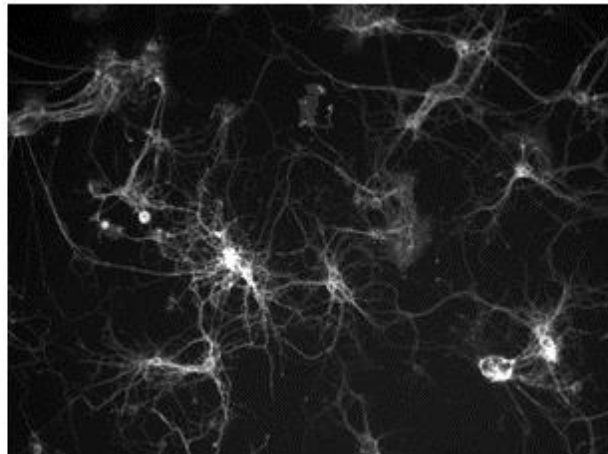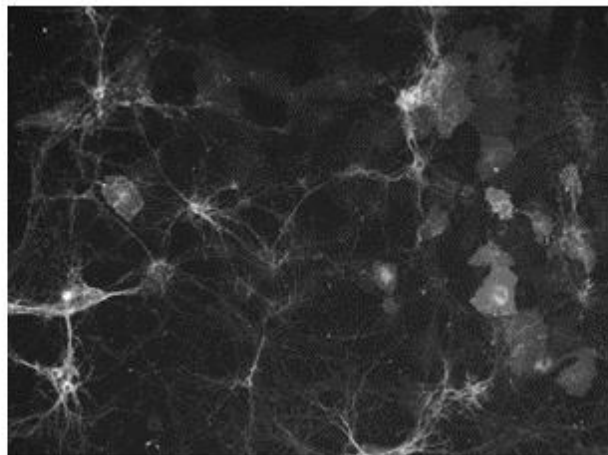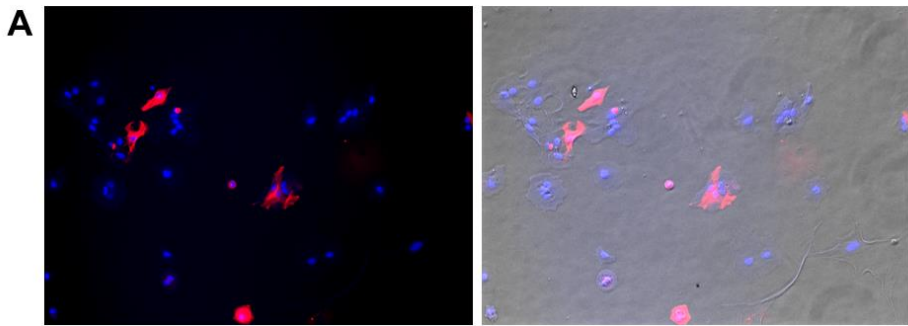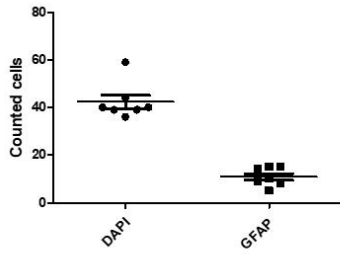
Figure 5. Ara-C treatments for pure expressions of human synapsin promoter in primary neurons at DIV 10.

(A) Neurons treated Ara-C at DIV 3 and DIV 5 and (B) Neurons only treated at DIV-3. (C) Neurons without Ara-C treatment. Astrocytes proliferated with mScarlet signals with neurons.

A

B

Number of GFAP-pos. astrocytes (N = 4, n = 7)

Ratio of GFAP-pos. astrocytes (N = 4, n = 7)

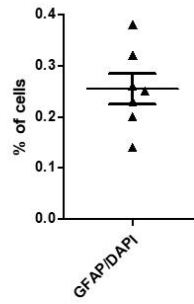Figure 6. GFAP (glial fibrillary acidic protein)-negative
cultured astrocytes from cortices.

(A) (Left) A representative fluorescent microscopic image of
GFAP immunostaining in primary astrocytes cultured for 3 weeks.
(Right) The same image with bright-field. (B) The number and ratio
of GFAP positive astrocytes per all astrocytes based on DAPI. Only
25% of astrocytes were GFAP-positive in culture (n = 7, SD = 0.08)

A HEK293T — CaPO₄ Transfection → transfected HEK293T → Trypsinization and pooling → coverslipped 24 wells → Fixing and Mounting

B HEK293T — CaPO₄ Transfection → transfected HEK293T → Trypsinization and pooling → AAV infected mouse primary hippocampal neuron → Fixing and Mounting

C HEK293T — CaPO₄ Transfection → transfected HEK293T → Trypsinization and pooling → Lipofected primary astrocyte → Fixing and Mounting
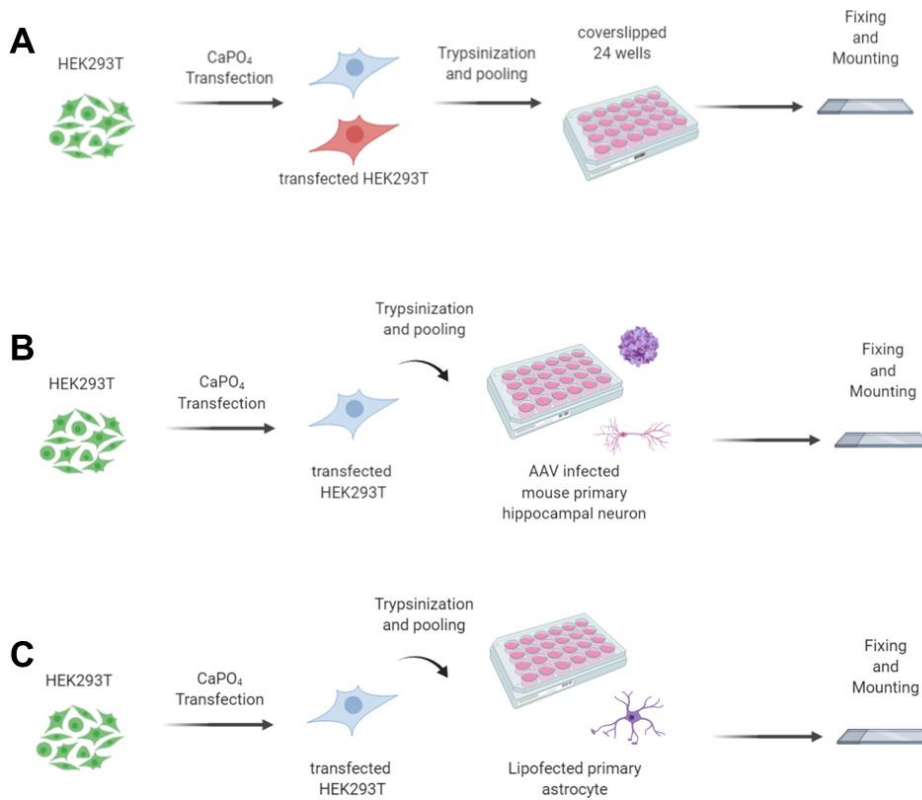
Figure 7. Diagrams of *In vitro* expression of the eGRASP in HEK293T cells.

(A) Schematic diagram of the eGRASP expression procedure within HEK293T cells. (B) Schematic diagram of the eGRASP expression procedure between HEK 293T cells and mouse primary neurons. (C) Schematic diagram of the eGRASP expression procedure between HEK 293T cells and mouse primary astrocytes.
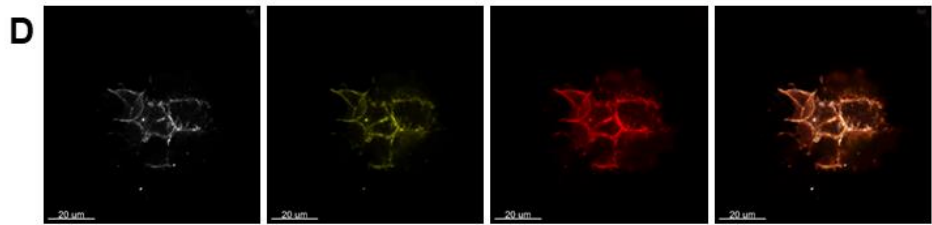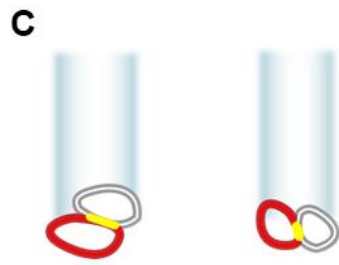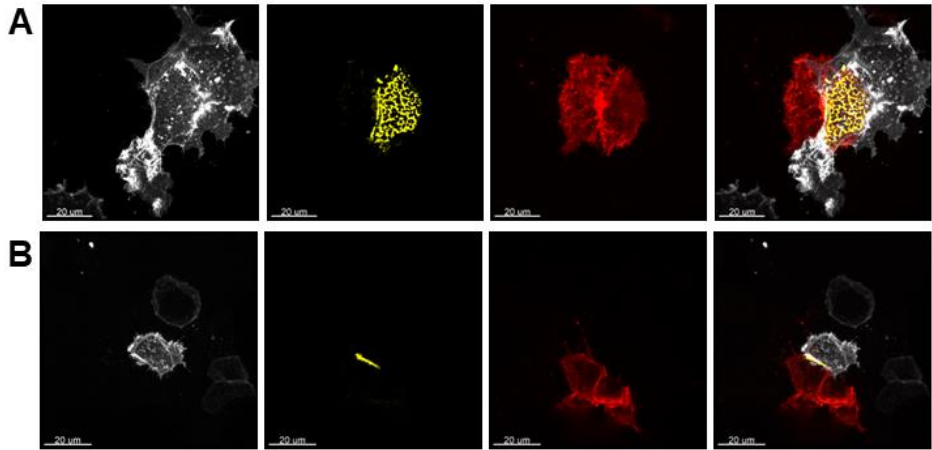
Figure 8. Yellow eGRASP signal expressed in contacted regions of HEK293T cells.

(A-B) Expressed patterns of eGRASP were vary in different angles between HEK293T cells. (C) Illustration of (A, B). (D) HEK293T cells were co-expressed in both post-eGRASP and pre-eGRASP DNA constructs for positive controls.
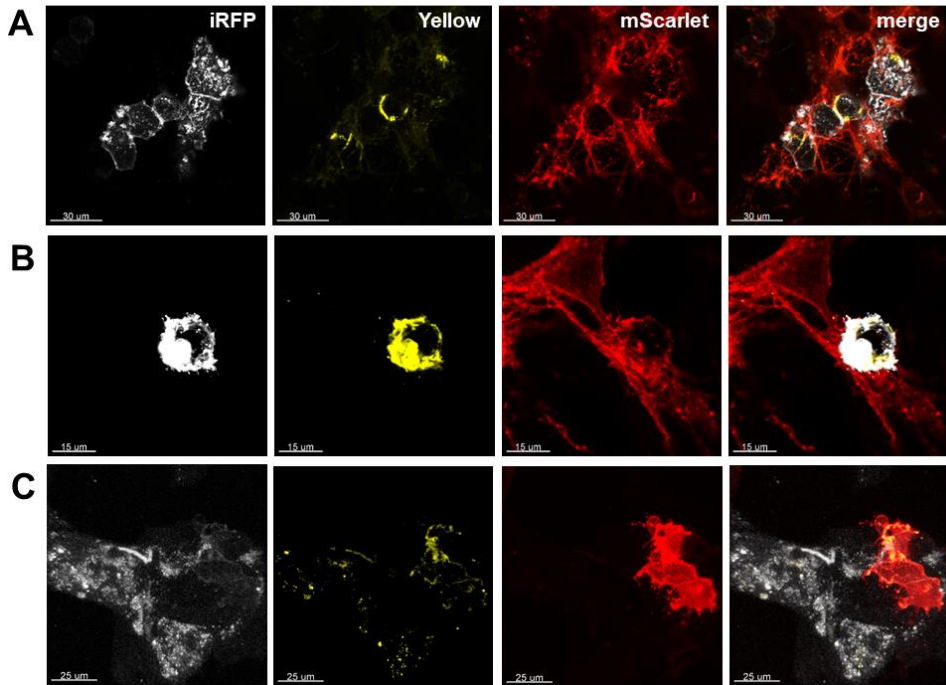
Figure 9. The yellow eGRASP signal expressed in contact
regions of HEK293T cells and neurites.

(A) The eGRASP signals expressed among several HEK293T cells
and neurites. (B) Same signals were among a HEK293T cell and
neurites. (C) The eGRASP signals expressed between HEK293T
cells and an astrocyte.
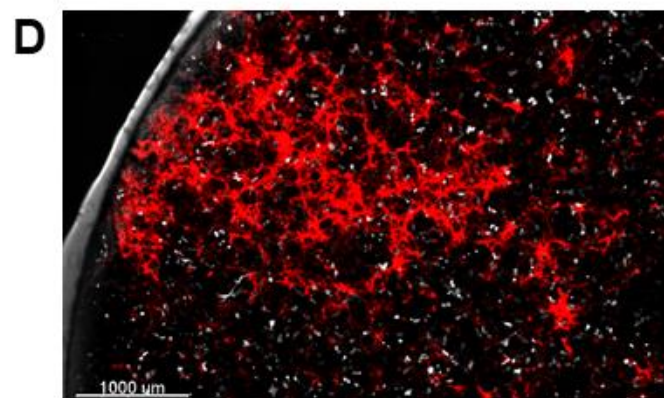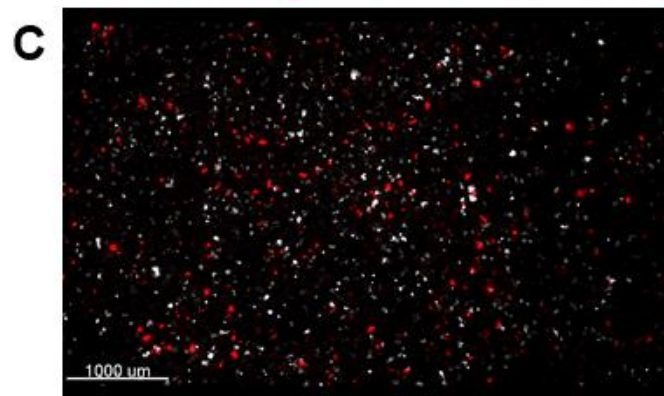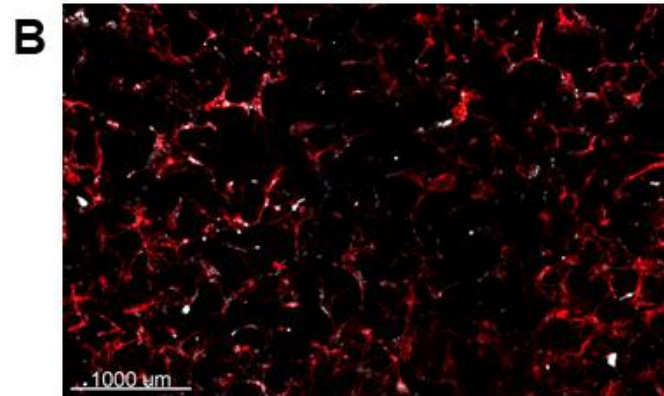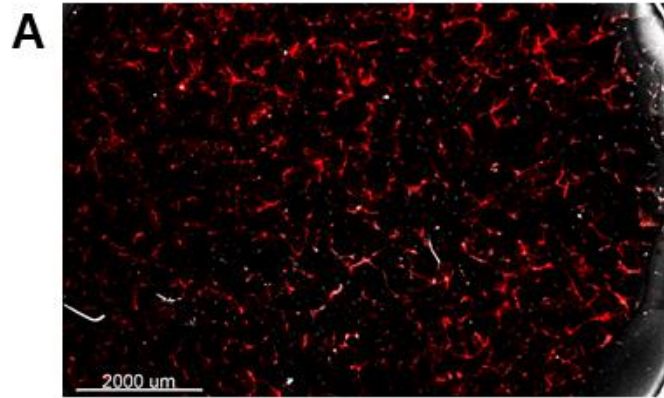
Figure 10. Representative 10x objective mosaic scanned images to find contact among cells.

Yellow wavelength was omitted to reduce photobleaching. (A) The AAV infected post-eGRASP expressed neurons and lipofectamine-transfected the pre-eGRASP astrocytes. (B) Same constitution as (A), but both neurons and astrocytes are infected by AAVs. (C) HEK293T cells were expressed both the post-eGRASP and the pre-eGRASP construct. (D) The post-eGRASP expressed neurons and pre-eGRASP expressed HEK293T cells.

Table 1. Ratio of regions to overall cell counts in Figure 8.

	Expressed constructs		Number of counted cells per image		
	Pre-eGRASP	Post-eGRASP	Pre-eGRASP	Post-eGRASP	Overlapped regions
A	Astrocytes	Neurons	42 (0.05)	836 (0.97)	12 (0.01)
B	Astrocytes	Neurons	48 (0.07)	660 (0.93)	38 (0.05)
C	HEK293T	HEK293T	1003 (0.64)	612 (0.39)	46 (0.03)
D	HEK293T	Neurons	484 (0.26)	1716 (0.91)	316 (0.17)

Discussion

The dual–eGRASP showed that the transition of synapse strengths is the elementary unit of learning and memory, correlating synaptic dynamics to neuronal activities occurring in specific brain regions (Choi et al. 2018; Langille and Brown 2018). *In vitro* application of the eGRASP method can provide a proxy of *in vivo* analysis, enabling various control experiments that is impossible in living animals. However, the *in vitro* eGRASP had different aspects compared to its *in vivo* counterpart.

The biggest difference between *in vitro* and *in vivo* eGRASP is their morphology. In contrast to the expression of *in vivo* dual–eGRASP forming dots at synapses, *in vitro* eGRASP was expressed at the contact regions between cells. Neurexin–1, a constituent of the pre–eGRASP construct, is one of the presynaptic cell–adhesion molecules. However, the same molecules are abundantly located on the surface of astrocytes regardless of synapses (Trotter et al. 2020). Therefore, eGRASP expression among cultured astrocytes and neurons could simply mean contacts for cells, not tripartite synapses.

The difference of the eGRASP expression between *in vitro* and *in vivo* might result from their synaptic maturation and distribution of neurons. On one hand, the neurons in the cultured system is obviously less viable than those in a live brain: primary neurons are more vulnerable compared to neurons in the live tissue. Although the morphology of 2-week cultured neurons is similar to neurons in a live brain of the same stage, excitatory synapses in live brain are distributed in dendritic spines (Harris, Jensen, and Tsao 1992). On the contrary, synapses of cultured neurons are equally formed on dendritic spines and shafts (Boyer, Schikorski, and Stevens 1998).

Another difference might be due to the properties of astrocyte in the co-culture. In general, morphology of astrocytes *in vivo* stands out as dense spongiform with numerous processes. The maturation requires over 4 weeks after birth (Bushong, Martone, and Ellisman 2004), where the tripartite synapse can be readily detected within the spongiform domain of mature astrocytes (Arizono et al. 2020). However, average lifespan of neurons is no longer than three weeks in cultured environment, rendering the full development of the tripartite synapse impossible in *in vitro* systems.

In addition, primary astrocytes have been usually cultured in the serum-containing medium to promote cell proliferation and survival,

yet this operation produces a reactive phenotype of astrocytes contrasted to *in vivo* quiescent states (Prah et al. 2019). In contrast, in the live brain, astrocytes exist in the blood-brain barrier(BBB) composed of dense endothelial cells, preventing the exposure of neurons and astrocytes to the serum components (Abbott, Rönnbäck, and Hansson 2006).

Finally, the lack of the GFAP production would have additionally contributed to the sparse expression of the pre-eGRASP signals at astrocytes. The amount of the GFAP in astrocytes could vastly differ according to the environment (Xu 2018) or culture protocols (Du et al. 2010). In detail, astrocytes expressing GFAP are abundant in hippocampus, but are little detected in cortices (Zhang et al. 2019). Since most astrocytes for culture were extracted from cerebral cortex, it was unsuitable to use GFAP promoter to designate astrocytes. Additional analyses proved that the GFAP expression in this culture system was as low as 25%, suggesting that there is room for four-fold enhancement of signals if promoter for astrocytes were properly chosen. To genetically label cortical astrocytes, there are alternative markers such as S100β or aldehyde dehydrogenase 1 family, member L1 (Akdh1L1). Similar to GFAP, S100β is not expressed at all astrocytes. Instead, ALDh1L1 has recently emerged

as a tool to label astrocytes (Preston, Cervasio, and Laughlin 2019).

These differences raise a necessity of the optimal culture system that imitates live nerve tissues, where a clear the in vitro eGRASP signal can be acquired. Several methods were suggested in recent literature: for example, Poon et al. proposed to add growth factors for synaptogenesis such as TGF−β, TNF−α to promote neuronal health and proliferation (Poon, Choi, and Park 2013). Gordon et al. introduced cell lines from tumors of neurons or astrocytes, resolving the lifespan issue of postmitotic neurons: the clear eGRASP signals are expected from matured neuronal cultures, of which achievement is intractable with primary neurons and astrocytes (Gordon, Amini, and White 2013).

Furthermore, novel culture methods can be developed to improve the eGRASP expression efficacy. Conventional 2D cultures have been criticized for its limited capability to reproduce the complex brain environment (Carter et al. 2017). If the maturation of neurons or astrocytes depends on the microenvironment of the brain, it is critical to provide a culture environment more similar to the live brain. The dual−eGRASP signals in 3D neuronal cultures or organoids would not only enhance the localized contact among neurons and astrocytes, but also lead to novel discoveries on the multi−directional

connectivity between diverse nervous cells.

Utilization of *in vitro* culture system has its own strengths and weaknesses compared to studies using live animals. It is advantageous in that the *in vitro* culture system imitates the complex nerve system with a simple combination of identified cell types in biochemically controlled environments (Giffard and Ouyang 2009). Besides, human nerve system can also be established *in vitro*, derived from the human induced pluripotent stem cells (hiPSCs). Application of the dual-eGRASP to human nervous system in a well-simulated culture environment would provide a glimpse into the complex neurodynamical responses in human brain (D'Aiuto et al. 2018).

Primary cultures of nerve cells have been used to evaluate neuronal responses on particular chemicals (Belle et al. 2018; Liddelow and Barres 2017). Confirming the eGRASP *in vitro* could eventually lead to identify synaptic changes under specific biochemical operations. Early studies using neuronal culture have discovered diverse aspects of synaptic plasticity using chemically induced LTP protocols or repetitive high-frequency presynaptic stimulation (Molnár 2011).

In this dissertation, I expanded the dual-eGRASP techniques

previously studies *in vivo* to the culture system, labeling synapses in more well-controlled environments than experiment using live animals. The *in vitro* eGRASP system is a promising candidate system to observe synaptic dynamics in live states without resorting to the complicated two-photon microscopy. Future studies could utilize the *in vitro* dual-eGRASP to achieve real-time synaptic formation between targeted cell types with progressive microscopic system for live cells (Frigault et al. 2009).

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국 문 초 록

뉴런, 성상세포 및 HEK293T 세포에서의 시험관 내 eGRASP 발현에 대한 연구

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이지아

성상세포와 뉴런 사이의 상호 작용 연구는 신경계의 생리적 특성을 이해하고 신경 퇴행성 질환을 비롯한 임상적 측면에 적용하기 위해 진행되어 왔다. 시냅스 신호 전달의 측면에서 성상세포의 주요 기능은 시냅스 후 뉴런과 시냅스 전 뉴런 사이에서 '삼자 간 시냅스'를 형성하는 것이다. 그러나 시냅스를 효율적으로 시각화하는 기술이 부족하였기에 삼자 간 시냅스에 대한 이해는 국소적인 관찰에 그쳤다. 최근 dual-eGRASP 기술이 개발되어 포유류 뇌에 있는 표적 뉴런의 시냅스 표시가 가능해졌다. 이 기법은 시냅스의 활성 상태나 지역에

따른 강도의 비교를 가능하게 했으며, 학습과 기억에서 뉴런 간 연결이 어떤 기능을 하는지 설명할 도구를 제공했다. 본 학위논문은 dual eGRASP 방법론의 적용을 확대하여 시험관 내에서 일차 뉴런과 성상세포 eGRASP 신호를 검증했다. eGRASP는 형질 전환 방법에 상관없이 뉴런, 성상세포, HEK293T 세포 등 다양한 세포 사이에서 발현됐다. 신호는 수상돌기의 축과 인접한 성상세포·HEK293T 세포 사이의 접촉 사이에서 면의 형태로 나타났다. 이러한 결과는 시험관 내 eGRASP가 통제된 환경의 신경계의 생리학 연구와 더불어 다양한 세포의 생리학을 설명할 유망한 방법을 제공한다는 것을 입증한다.

주요어 : Dual-eGRASP, 삼자 간 시냅스, 일차 뉴런 배양, 뉴런-성상세포 공배양

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