Developing a CRISPR/cas9 System for Application in Cortical Slice Culture

Senior Thesis

Presented to

The Faculty of the School of Arts and Sciences
Brandeis University
Undergraduate Program in Biology
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In partial fulfillment of the requirements for the degree of Bachelor of Biology

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May 2015

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Abstract

The CRISPR/cas9 system facilitates sited-directed mutagenesis in DNA by using a single gRNA to recruit cas9 endonuclease to the target site. Even though the CRISPR/cas9 system has been widely applied in a variety of cancer and embryonic cell lines, its applications in post-mitotic cells are still limited. Here we first evaluated targeting efficacy of six gRNA targeting the mCitrine gene in cell cultures. 1/3 of gRNAs showed high on-target activity and turned off mCitrine expression. We then delivered lentiviral vectors carrying genes encoding cas9 and gRNAs into cortical slice culture. The delivered CRISPR/cas9 system successfully disrupted the endogenous tdTomato gene expression in post-mitotic cells. Our result suggest that optimized gRNAs requires both low off-target effects and high on-target activity. We also confirmed that the CRISPR/cas9 system is effective in post-mitotic cells and application is possible in cortical slice culture.
Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats, or CRISPR, is a family of short repetitions of DNA sequences present in many bacteria and archaea. CRISPR and its associated proteins (cas) comprise the CRISPR/cas system that functions as an adaptive immune system in prokaryotes. Recently, the CRISPR/cas system has been modified into programmable endonucleases that facilitate sequence-specific DNA editing. Due to its wide application, high efficiency and simplicity in design, the CRISPR/cas system is rising to be a promising tool for genome engineering in basic the sciences, medicine and biotechnology.

Discovering the CRISPR/cas system

In 1987, Ishino and his team first discovered five 29-nucleotide DNA sequences arranged as direct repeats with 32-nucleotide spacers DNA adjacent to the iap gene in E.Coli K12 (Ishino et al., 1987). Over the next ten years, similar DNA repeats were discovered in other prokaryotic genomes. By 2000, CRISPR loci had been discovered in more than 40% of sequenced bacteria and 90% of Achaea, making it the most widely distributed group of genomic repeats in prokaryotes. CRISPR repeats are classically characterized as short palindromic sequence of 24-40 base pair (bp) arranged in clusters with 20-58 unique intervening sequences, known as spacers (Mojica et al., 2000).

In 2002, Jansen and colleagues coined the term CRISPR to describe these loci. They also discovered four core CRISPR associated genes (cas) adjacent to the CRISPR locus and a common leader sequence (Figure-1). The leader sequence is located on one side of the CRISPR locus with conserved orientation regarding the repeats (Jansen et al., 2002). The role of the leader sequence is unknown, but it is suggested that it promotes transcription towards the repeats (Brouns et al., 2008). The palindromic repeats and the leader sequence are homogenous within the same genome, but are dissimilar among different genomes of distant
species. The cas genes are named cas1, cas2, cas3, and cas4 (Jansen et al., 2002). In 2005, additional cas genes, cas 5 (also known as cas9) and cas 6 were identified by Haft and colleagues (Haft et al., 2005). The cas1 gene was found in all 40 prokaryote species with CRISPR loci in the 2002 study, but the presence of the other cas genes varies among different species. The specific order of the cas genes also show variations among different organisms (Jansen, 2002). These variations of cas genes in CRISPR loci form the basis of classification of the CRISPR/cas systems.

![Figure-1](image.png)

**Figure-1**
A simplified example of the CRISPR locus with the cas genes, the leader sequence and the repeat-spacer array. Spacers are shown in colored double helix. The figure shows spacers originate from sequences proceeds the protospacer adjacent motif (PAM) in exogenous DNA source.

**CRISPR/cas facilitated adaptive immunity mechanism in prokaryotes**

There are three major groups of CRISPR/cas system, CRISPR/cas I, CRISPR/cas II and CRISPR/cas III. Type II CRISPR/cas systems are among the best characterized CRISPR/cas systems (Ran et al., 2013). Type II CRISPR locus carries the cas9 gene, which encodes a cas9 endonuclease protein. All three CRISPR/cas systems facilitate prokaryotic adaptive immunity through a three-step process. The first step is spacer acquisition. CRISPR spacer DNA shares homology with foreign genetic elements from bacteriophages and conjugated plasmids. In essence, spacers are the genetic traces of past infection by phage and plasmid
Viral challenge triggers genomic incorporation of a new spacer DNA sequence derived from the phage genome through an unknown mechanism. Selection of a spacer precursor, or proto-spacer, depends on recognition of proto-spacer-adjacent motifs (PAMs) (Figure-1). PAMs also direct the orientation of spacers in the repeat arrays. A PAM sequence is usually a few nucleotides long and differs between variants of CRISPR/cas systems. It is found adjacent to, or one position after, one end of the putative spacer (Mojica et al., 2009). Usually a new spacer is inserted towards the 5’ end of CRISPR locus (Deveau et al., 2008). Addition or removal of spacers from a CRISPR locus changes the bacteria’s sensitivity to phage. For example, adding a novel spacer into a CRISPR locus generates resistance against invasion of phages with identical sequences via a mechanism described below. This addition and retention of phage sequences in the bacterial genome is what makes the CRISPR/cas system adaptive (Barrangou et al., 2007).

The second step is expression of genetic elements from the CRISPR locus. The CRISPR locus first gets transcribed into a noncoding RNA called the precursor CRISPR RNA (precrRNA). Type II CRISPR/cas system starts precrRNA processing by transcribing CRISPR locus to produce a noncoding precrRNA. Then, a short sequence on the opposite strand 210-nucleotide upstream of the CRISPR locus get transcribed into the trans-activating crRNA (tracrRNA). TracrRNA is a small, noncoding RNA between 65 to 171 nucleotides long. TracrRNA contains a 24-nucleotide sequence complementary to a repeat region in precrRNA, which allows tracrRNA to bind to the complementary sequence on precrRNA, forming a duplex structure. Upon this RNA pairing, endoribonuclease RNase III proceeds to cleave the duplex into a mature crRNAs:tracrRNA complex. Since none of the cas proteins contains a RNase III motif, it is suggested that an endogenous RNase III is recruited for co-processing. Studies show that cas9 is required for crRNA maturation, as inactivation of cas9 disrupts crRNA maturation process, but its specific function is unknown. One hypothesis is
that cas9 serves as a molecular anchor that prepares the RNA duplex for recognition and cleavage by RNase III (Deltcheva et al., 2011).

After crRNA maturation, the final step is immune interference. Mature crRNA serves as a guide to cascade and cas9, targeting these proteins to a specific site in the phage genome that is complementary to the 20-nucleotide spacer sequence (Brouns et al., 2008). The crystal structure of cas9 reveals bi-lobed structured composed of a nuclease lobe and a target recognition lobe. The nuclease lobe contains two nuclease domains. Each is responsible for cleaving one strand of the target DNA. Cas9 also contains a separate domain for PAM recognition. PAM directs the recognition of the target site (Mojica et al. 2009). It is hypothesized that upon PAM recognition, cas9 protein goes through conformational changes to be activated (Nishimasu et al., 2014). Once target site is located, cas9 or cascade proceeds to cleave both strands of the target DNA, disrupting proper functions of invading phage or plasmid. This endonuclease activity is protosspacer specific and orientation dependent. (Garneau et al., 2010).

**CRISPR/cas9 application in Genome Engineering**

Several groups have modified the CRISPR/cas9 system for genome editing purposes. The most commonly used method involves modifying Type II system to create a chimeric single guide RNA (sgRNA) composed of a fused crRNA and tracrRNA. The sgRNA is capable of directing cas9 endonuclease activity to any site of interest adjacent to a PAM sequence. It inserts a 20-nucleotide sequence complementary to the target site into the crRNA portion of the sgRNA, which give the sgRNA its targeting ability. Once cleaved by cas9, the target locus can be repaired through two pathways, non-homologous end joining (NHEJ) and homology directed repair (HDR). In the absence of a repair template, double strand breaks
are repaired by NHEJ, which is more error-prone and often leads to deletion/insertion mutations. The deletion/insertion mutations cause frameshift that often result in gene inactivation. HDR occurs less frequently than NHEJ, but has higher fidelity given the presence of a DNA template to assist the repair process. HDR can be harnessed to introduce precise modification at the target locus if an exogenous, synthetic repair template is provided (Ran et al., 2013).

Further modifications such as mammalian codon-optimization of Cas9 and associated RNAs opens up the possibility of reconstitution of the CRISPR system in mammalian cells (Ran et al., 2013). In 2013, Cong and colleagues first used the CRISPR/cas9 system to successfully induce DNA double-strand cleavage at endogenous genomic loci in both mouse and human cells. They also discovered that RNaseIII is not required for CRISPR/cas9 function in mammalian cells as endogenous mammalian RNaseIII may play a compensatory role (Cong et al., 2013). The CRISPR/cas9 system has also been adapted to generate gene knockouts in many other model organism including rodents, common crops and fruitflies (Sander and Joung, 2014). The CRISPR/cas9 system can be further modified for broader applications in genome engineering. Multiplex genome editing using CRIPSР/cas9 is made possible by encoding several guide sequences into a single CRISPR array. Alternatively, one of the catalytic domains of Cas9 endonuclease can be mutated to turn cas9 into a nickase (ncas9). A ncas9 can be directed to facilitate high fidelity HDR to minimize unwanted mutagenic activity (Cong et al., 2013). If both domains of cas9 are mutated, cas9 loses all nuclease functions and can be directed to regulate transcription by blocking DNA transcription at the target site (Qi et al., 2013).

This rapid development of CRISPR/cas9 related applications is associated with its advantages over other genome engineering technology such as zinc-finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs). All three systems have had
successful applications in various cells models. However, the CRISPR/cas9 system can be easily retargeted by simply altering the 20-nucleotide guide sequence located in the crRNA. In contrast, retargeting TALEN and ZFN requires reconstruction of the nuclease genes, a much more complex process. CRISPR/cas9 system is also more precise as it most commonly cleaves 3 bp 5’ of PAM sequence. TALEN cleavage site is located in the 12-24 bp linker located between the two TALEN monomer-binding sites (Ran et al., 2013). These advantages make CRISPR/cas9 a highly promising new technology in genome engineering.

The major drawback for CRISPR/cas9 mediated genome-engineering technology is its off-target effects. Studies have shown that as many as five mismatches in the off-target sites can be tolerated and many off-target sites are mutagenized with similar or even higher efficiency than the intended target site. One solution to minimize the off-target effects is by using available CRISPR design programs to select gRNAs with fewest off-target sites. The CRISPR Design Tool developed by the Zhang lab from MIT is a widely used program. This online program can take a genomic sequence of interest, screen for suitable target sites and generate one gRNA for each target site. They also predict possible off-target sites and provide a score of targeting specificity for each gRNA based on the number and position of nucleotide mismatches on the off-target sites (Hsu et al., 2013). Although designed to avoid off-targets activities, it does not provide any criteria to evaluate gRNA’s on-target activity. Many reported that only 1/3 of CRISPR gRNAs designed using CRISPR Design Tool and similar programs are in the highest quartile of targeting activity (Doench et al., 2014). Thus we found it necessary to evaluate the efficacy of individual gRNAs in an easy to assay system. We proposed a thesis project that tests the on-target activity of six gRNAs designed to target the mCitrine fluorescence protein gene in mammalian cellular culture. The goal of this experiment is to confirm if only 1/3 of gRNA designed by the CRISPR Design Tool are effective for gene knock out and to learn the differences in gRNA design that affect the
targeting activity. Understanding these questions would help future experiments using CRISPR Design Tool. The experiment results would provide references for determining the optimal number of gRNAs for each gene target and simplify the selection process.

Almost all the cell models tested with CRISPR/cas9 are embryonic and cancer cell lines (Sander and Young, 2014). Very little has been done to evaluate the effectiveness of CRISPR/cas9 in post-mitotic cells, specifically cells in the central nervous system. A recent report by Incontro and colleagues opens up a new field for CRISPR/cas9 system application, which is using CRISPR/cas9 system to target cells in organotypic slice culture. The team produced disrupted expression of two endogenous protein-coding genes in hippocampal slice culture using the CRISPR/cas9 system (Incontro et al., 2014).

Traditionally, protein function studies use the conditional knockout technology, but the production of conditional knockout animals requires considerable time and expense (Tsien et al., 1996). Being able to use CRISPR in post-mitotic cells has great significance as it allows generation of gene knockout model organisms in a much more efficient and convenient way. A second part of the project was designed to confirm the feasibility of CRISPR/cas9 mediated genome editing in post-mitotic cells of cortical slice culture. We delivered CRISPR/cas9 vectors targeting an endogenous tdTomato fluorescence protein gene into mouse cortical slice culture and collected images of the fluorescence protein expression to monitor CRISPR/cas9 mediated gene editing activity. The goal for this part of the experiment is to expand the use of CRISPR/cas9 in the research fields by developing CRISPR/cas9 tools for application in post-mitotic cells in cortical slice culture.

**Material and Methods**
**Guide sequence identification and selection**

Guide sequences were identified using the CRISPR Design Tool (Zhang lab, MIT). This tool scans the DNA sequence for CRISPR gRNAs that are 20 nucleotides long followed by a NGG PAM sequence required for Cas9 recognition. The tool also predicts possible off-target matches. Candidate gRNAs are ranked by the accuracy of on-target activity based on the combined scores of single off-target hits. The single off-targets hits are calculated using the following algorithm,

\[
\prod_{e \in \mathcal{M}} (1 - W[e]) \times \frac{1}{\left(\frac{19-d}{19} \times 4 + 1\right)} \times \frac{1}{n^2_{mm}}
\]

with “e” representing the number of mismatches between guide and off-target sequence and “m” representing the experimentally determined effects of mismatch position on targeting. A high single off-target hit score indicates high possibility of off-target effects.

The aggregated score combines the individual scores of off-target sites and gives an overall presentation of gRNA targeting accuracy. The calculation is based on the following algorithm. Contrary to the single off-target hit score, a high score shows high on-target specificity.

\[
S_{guide} = \frac{100}{100 + \sum_{i=1}^{n_{mm}} S_{hit}(h_i)}
\]

The DNA sequence of each reporter gene, tdTomato (Figure-2) and mCitrine (Figure-3) was submitted on CRISPR Design Tool’s online submission page (http://crispr.mit.edu/). Target genome was “mouse (mm9)”. The program does not differentiate between introns and exons. In this case, our reporter genes do not include introns. The program can only scan one 250-nucleotide long DNA sequence at once, so the DNA sequences were broken down into several overlapping sections and were scanned separately.
The result page (Figure-4) shows a ranked list of gRNA sequences. The ranking lists, from top down, the highest on-target specificity computed by 100% minus the weighted sum of off-target hit scores. The page displays all off-target sequences for each gRNA and the USCS gene ID if an off-target is located in a coding region.

Guide RNAs were selected with preferences for high on-target specificity score and few off-targets in exons. Because the mCitrine gRNAs were designed for targeting efficacy comparison, gRNAs were purposefully selected to complement sites spread across the DNA sequence. Furthermore, the expression patterns of genes carrying off-target sites were examined using Allen Brain Atlas (Allen Brain Institute) and Mouse Genome Informatics (The Jackson Laboratory). Guide RNAs with off-targets in genes that are known to be critical for neuron survival were eliminated.

Guide RNA sequences used for in this study are listed in Table-1.

Guide RNA oligos targeting tdTomato were annealed and cloned into the pSpCas9 (BB)-2A-GFP (PX458) vector using the published Target Guide Sequence Cloning protocol (Ran et al., 2014). PX 458 was purchased from Addgene (plasmid # 48138), and expresses a sgRNA scaffold from the U6 promoter as well as S. pyogenes cas9 cDNA and GFP separated by a 2a auto-cleavage site (2A).

Similarly, gRNA oligos targeting mCitrine were annealed and cloned into the pL-CRISPR.EFS.tRFP vector purchased from Addgene (plasmid # 57819). This vector is based on the pLKO.005 lentiviral backbone. It bi-cistronically expresses S. pyogenes gRNA scaffold from a U6 promoter and S. pyogenes cas9 from a short EF1a (EFS) promoter with a red fluorescent protein marker separated by a 2a auto-cleavage site.

The newly cloned vectors containing gRNA targeting sequences were transformed into DH5α bacteria (NEB) and plated on LB Agar plate containing ampicillin following the bacterial
transformation protocol (Invitrogen). Individual ampicillin resistant colonies were selected to grow in LB broth containing ampicillin over night. Plasmid DNA was isolated and purified from the bacteria following manufacturer’s instructions with a mini prep kit (Zymo). Purified DNA was sent for sequencing to confirm gRNA was successfully cloned into the vector.

Figure 2

**tdTomato**

```plaintext
atggcttcagagagtgggttcatcgaatgcagctttacggtttagtagaagggatatcaacatcagttcagttggtgtagagcgcgctg
```

Figure 3

**mCitrine**

```plaintext
atggcttcagagagtgggttcatcgaatgcagctttacggtttagtagaagggatatcaacatcagttcagttggtgtagagcgcgctg
```

Figure 2

**tdTomato** coding DNA sequence used for generating gRNAs

Figure 3

**mCitrine** coding DNA sequence used for generating gRNAs
Figure-4
The CRISPR Design Tool (MIT) result page shows the sequence of all potential gRNAs, the off-target sequences, and scores of the on-target accuracy for each gRNA sequence.

Table-1
Using the CRISPR Design Tool (Zhang Lab, MIT), three gRNAs were designed to target the tdTomato gene and six gRNAs were designed to target the mCitrine gene. The gRNAs are 20-nucleotide long and the PAM sequences are underlined. The aggregate score shows the on-target accuracy of each gRNA sequence.

Cell Culture and transfection
The mouse N2A glioblastoma cell line was maintained in Minimum Essential Media (MEM) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C incubation. The Human
Embryonic Kidney (HEK) cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C incubation. Cells from each cell line were seeded onto a 24 well plate for calcium phosphate co-transfection with a mCitrine encoding vector and a CRISPR/cas9-RFP containing vector. For each well, a total of 0.5ug of DNA containing equal molar ratios of each vector was used for transfection.

**Cellular Imaging**

The images of fluorescence expressions in live cells are captured using an Olympus fluorescence microscope connected to the Volocity cellular imaging and analysis software (PerkinElmer).

**Mouse Line**

The mice used for creating slice culture carry a Cre inducible tdTomato inserted into the Rsa26 locus. In this mouse line, TdTomato fluorescence protein is expressed in any cell type following introduction of Cre-recombinase.

**Preparing Slice Culture**

Mice at postnatal day (P) 6-9 were anesthetized according to the IACUC protocol, then perfused with 2.5 mL of ice-cold Artificial cerebrospinal fluid (ACSF). Mice were then decapitated to remove the brain. The brain was sliced at 300µm thickness in a compressstome in ice-cold ACSF. Six slices from the somatosensory cortex were collected from each mouse. Two slices were each placed onto a 0.1µm pore membrane in a six-well plate containing 1 mL of slice culture media (SCM) and allowed to recover and develop at 35°C. Slices were
maintained in culture for up to two weeks, with culture media changed at a minimum of every two days.

**Vector Delivery**

The three PX458 CRISPR/cas9 vectors, each carrying one of the tdTomato targeting gRNAs, were delivered together with a vector carrying Cre-recombinase into the slice culture using the biolistic particle delivery system (gene gun).

The pL-CRISPR.EFS.tRFP CRISPR/cas9 vectors were used for lentiviral production as well as transfected into HEK or N2A cells via calcium phosphate.

**Results**

**Knock-out of the plasmid mCitrine gene in N2A and HEK cell culture.**

Even though there are several programs available to design CRISPR gRNAs, existing programs are designed to avoid off-target activities, rather than incorporating criteria to evaluate the gRNA’s targeting efficacy. Using these programs, most people reported varying targeting efficacy in gRNA and about 2/3 of gRNAs do not work for reasons unknown (Gagnon et al., 2014). Therefore we felt the need to design an experiment to evaluate the efficacy of individual gRNAs in an easy to assay system. The experiment compared the targeting efficacy of six gRNAs targeting the mCitrine gene in N2A and HEK cell cultures. The HEK cell line was selected because of its high transfectability by various techniques including lentivirus transfection (Li et al., 2013). We planned to use the pL-CRISPR.EFS.tRFP lentivirus vectors in mouse cortical slice culture for the second part of the experiment. Thus the N2A cell line was selected because it is a mouse neuroblastoma cell line.

By comparing gRNA’s on-site activity, its specific nucleotide arrangement, and its targeting
site on the DNA sequence, we want to gain a better understanding on the factors affecting the on-target activity of gRNA. Based on previous studies, we estimated that two of the six gRNAs would have high percentile on-site activity that can turn off mCitrine expression. Previous reports also showed that frameshift mutations caused by NHEJ following DNA cleavage are more likely to disrupt expression near the 5’ end. We also hypothesized that gRNAs complementary to sites close to the 5’ end of the DNA sequence have higher gRNA target activity (Doench et al., 2014).

For each cell line, we transfected cells in six individual wells with pL-CRISPR.EFS.tRFP lentivirus vectors carrying gRNAs. Cells in each well received vectors with one of the six gRNAs. The control well was transfected with a pL-CRISPR.EFS.tRFP lentivirus vector without a gRNA. Fluorescence images were taken five days after transfection.

Figure-5 shows the fluorescence images of transfected N2A cells from control well, gRNA#3 well, gRNA#4 well and gRNA#5 well. The mCitrine vector expresses mCitrine green fluorescence protein while pL-CRISPR.EFS.tRFP lentivirus vector expresses Red Fluorescence Protein (RFP). These fluorescence markers provide a simple way to show vector activity in the cells.

As shown in Figure-5, control group cells that express RFP also express mCitrine, indicating transfection success. We introduced the control group to confirm that cells transfected with both vectors remain viable. It was also used to show undisrupted mCitrine expression as the baseline for comparison. Furthermore, this figure shows mCitrine vectors have higher transfection efficiency than RFP, for cells that express mCitrine do not necessarily express RFP, but not vise versa. This observation is important because it suggests diminished mCitrine activity in RFP expressing cells are most likely to be caused by CRISPR/cas9 mediated gene inactivation than failure in mCitrine vector transfection.
Figure-5 also shows fluorescence images of cells transfected with pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNA #3. Cells that express RFP also show similar level of mCitrine expression as the control. In contrast, cells transfected with pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNA #4 (circled by dotted line) express RFP and they show less mCitrine expression compared to the control. The cells that do not express RFP maintain mCitrine expression, which suggests mCitrine was not properly expressed in RFP expressing cells. Similar result was observed in cells transfected with pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNA #5.

Figure-6 shows the fluorescence images transfected HEK cells transfected with same vectors as N2A. As expected, same results as those of N2A were observed in HEK cells.

The results show that vectors carrying guide RNA sequence #4 and guide RNA sequence #5 have high on-site targeting activity that effectively disrupted normal mCitrine expression. Vectors carrying four other guide RNA sequences have low on-site targeting activity and mCitrine was expressed normally.

We then compared on-site targeting activity and the target position on the DNA sequence. Figure-7 shows the relative position of each guide RNA on the mCitrine locus. Guide RNAs #4 and #5 were not the gRNAs located closest to 5’ end. Instead, gRNA #1 and #2 are closer to 5’, but they showed lower on-target efficacy than #4 and #5. This result did not support the hypothesis that gRNAs targeting sites close to 5’ are more active.
The experiment was designed to turn off mCitrine expression using CRISPR/cas9 system. N2A cells were co-transfected with pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNAs and a vector carrying mCitrine. The gRNAs were designed to target mCitrine fluorescence protein. In the control that received an empty lentivirus vector, cells that were transfected express both mCitrine and RFP. The mCitrine vector had higher transfection efficiency than the lentivirus vector, therefore cells that expressed mCitrine do not always express RFP, but not vice versa. Guide RNA #3 shows similar mCitrine expression as the control. In contrast, guide RNA #4 and #5 have selected cells (circled with dotted line) that expressed RFP but no longer express mCitrine as strongly as the control. The results suggested mCitrine expression was turned off by pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNAs #4 and #5.

The experiment was designed to turn off mCitrine expression using CRISPR/cas9 system. HEK cells were co-transfected with pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNAs and a vector carrying mCitrine. The gRNAs were designed to target mCitrine fluorescence protein. In the control that received an empty lentivirus vector, cells that were transfected express both mCitrine and RFP. The mCitrine vector had higher transfection efficiency than the lentivirus vector, therefore cells that expressed mCitrine do not always express RFP, but not vice versa. Guide RNA #3 shows similar mCitrine expression as the control. In contrast, guide RNA #4 and #5 have selected cells that expressed RFP but no longer express mCitrine as strongly as the control. The results suggest mCitrine expression was turned off by pL-CRISPR.EFS.tRFP lentivirus vector carrying gRNAs #4 and #5.
Figure-7 The position of each gRNA on the mCitrine DNA sequence. #4 and #5 were the only ones with high on-target activity. #2 and #3 were located closer to 5’ end than #4 and #5 but they were less active than #4 and #5.

Deletion of tdTomato in mouse cortical slice culture

Reports have shown that CRISPR/cas9 system is a powerful tool for genome engineering for its simplicity in design and capability to mediate site-directed mutagenesis on DNA sequences in endogenous genome in virtually any organism of choices. However, most of these experiments were done using cancer cell and embryonic cell lines. There were few reports about the effectiveness of CRISPR/cas9 in post-mitotic cells, specifically cells in the central nervous system. Therefore, we want to expand the application of CRISPR/cas9 by establishing an effective CRISPR/cas9 system for targeting post-mitotic neurons in organotypic slice culture. Organotypic slice cultures of the central nervous system are simple and convenient systems for studying the neuron and glial cell biology. Application of the CRISPR/cas9 system in organotypic slice culture can be a
powerful tool for future functional genomic studies in the central nervous system. For this experiment, we designed three guide RNAs targeting an endogenous tdTomato gene carried by an established transgenic mouse line from the Nelson Lab at Brandeis University. A previous study by Incontro and colleagues successfully used CRISPR/cas9 to knock out synaptic protein expressing genes in post-mitotic neurons from hippocampal slice culture (Incontro et al., 2014). We thus hypothesized that CRISPR/cas9 system would turn off tdTomato expression in cortical slice culture. We adopted the gene gun strategy Incontro and colleagues used for transfection. We coated gold particles with three pSpCas9 (BB)-2A-GFP vectors, each carrying a different gRNAs targeting tdTomato, and biolistically delivered them into mouse cortical slice culture. The pSpCas9 (BB)-2A-GFP vector carries a GFP marker that is used to confirm plasmid activity.

Fluorescence images were taken between two to seven days after transfection. Figure-8 shows a series of fluorescence images focusing on a single cell (circled with dotted line) in the slice culture. On images taken on two days after vector delivery, the cell expressed both Green Fluorescence Protein (GFP) and tdTomato, suggesting this cell incorporated both pSpCas9 (BB)-2A-GFP vector and the vector carrying Cre-recombinase. The GFP expression remained over past few days, indicating normal expression of genetic elements from the pSpCas9 (BB)-2A-GFP vector including Cas9 and gRNA. During the same period, the tdTomato expression continued to weaken, which suggests tdTomato gene expression was disrupted by the CRISPR/cas9 system introduced.

The same fluorescence images also show a cell adjacent the circled one that did not express GFP, indicating pSpCas9 (BB)-2A-GFP vector was not present. After the cell started turning on RFP expression two days after transfection and RFP expression remained, which shows in the absence of gRNA carrying pSpCas9 (BB)-2A-GFP vector, RFP gene expression was not disrupted. The result suggests that pSpCas9 (BB)-2A-GFP vector carrying gRNAs targeting
tdTomato did disrupt endogenous tdTomato activity. In Conclusion, this experiment shows that CRISPR/cas9 system can be used to target an endogenous gene in cortical slice culture and effectively turn off gene expression.

![Figure-8](image)

**Figure-8**
Three pSpCas9 (BB)-2A-GFP vectors carrying individual gRNAs targeting tdTomato gene and a vector carrying Cre-recombinase were delivered into mouse cortical slice culture using gene gun. The mouse line expressed tdTomato in all cell types in the presence of Cre-recombinase. The pSpCas9 (BB)-2A-GFP vector had a GFP marker that is expressed in the transfected cell in the image to indicate transfection success. The same cell also expressed tdTomato. Over the 6-day-period, GFP expression remained but tdTomato expression was turned off, suggesting that normal tdTomato expression was disrupted by the CRISPR/cas9 system.

**Discussion**

We evaluated the targeting efficacy of individual gRNAs in an easy to assay system. We estimated that two out of six gRNA designed to target mCitrine gene would be effective in turning off mCitrine expression in mammalian cell culture. Because mCitrine is a fluorescence protein, it serves as a good indicator of CRISPR/cas9 targeting efficacy. The result confirmed the hypothesis by showing that only vectors carrying guide RNA sequence #4 and #5 turned off mCitrine expression in the cell culture. The results were also consistent
between both N2A and HEK cell lines. The hypothesis also suggested that gRNAs with high on-target activity were located close to the 5’ end of the locus, but the result showed that the targets position alone could not accurately predict gRNA on-target efficacy. The two gRNAs with high on-target activity were located closer to 5’ end than 3’, but there were two gRNAs #2 and #3 sitting closer to 5’ end but showed low on-target activity. This result suggests, gRNA’s location on the target DNA sequence alone could not accurately predict gRNA on-target activity.

Furthermore, we looked into recent studies investigating factors affecting the on-target efficacy of gRNA sequences. A new study came out in 2014 confirmed several previous groups’ study on factoring determining the gRNA targeting efficacy and incorporated the results to construct an online program that is designed to select highly active gRNA targeting any gene of interest. The study reported that on-target activity of g RNAs are affected by the GC content in each gRNA sequence; guide RNAs with higher GC content tend to be more active. Also, certain positions across the length of gRNA have preferences for certain nucleotides. For instance, in position 20, the nucleotide most adjacent to PAM, guanine was strongly preferred and cytosine was unfavorable. This team used data from nine mouse and human genes to determine sequence feature weights and developed the program predicting guide RNA on-target activity (Doench et al., 2014).

We used the program to evaluate the six gRNA sequences and the on-target activity score was shown in Table-2. Based on this program, gRNA #1, #4, and #5 were the highest scoring gRNAs. Now if we take their target positions on the mCitrine locus into consideration, as previously shown in figure-7, gRNA #1 targets close to 3’ end of the locus and gRNA #4 and #5 target positions close to 5’ end of the locus. Based on previous studies, gRNAs targeting close to 5’ end of the locus tend to be more active (Doench et al., 2014). In conclusion, the result shows that the position where gRNA targets on the DNA sequence alone does not
account for all gRNA targeting efficacy. Instead, further analysis of the experiment result shows that the combination of a variety of factors, including GC content, nucleotide preference at specific positions on gRNA as well as the position of gRNA target, provide a more reliable prediction of gRNA targeting efficacy. This conclusion will help with future gRNA selection by showing the optimal approach to generate gRNA with high on-target activity as well as on-target accuracy. To select gRNAs with high-targeting activity as well as targeting accuracy, programs designed to avoid off-target sites and programs designed to predict on-target activity should be used together. It also shows that the selection should take consideration of the position of gRNA targets because generally gRNAs targeting close to 5’ end of the locus have higher on-target activity.

<table>
<thead>
<tr>
<th>Targeted Gene</th>
<th>Number</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCitrine</td>
<td>1</td>
<td>0.274</td>
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In the second part of the experiment, we wanted to develop a CRISPR/cas9 system that could be applied to post-mitotic cells in the cortical slice culture, a simple and convenient system widely used for neurobiology studies. We hypothesized that based on the success of on recent study using CRISPR/cas9 to target synaptic protein in hippocampal slice culture, CRISPR/cas9 system is effective for mutating an endogenous genes in post-mitotic cells of a cortical slice culture. The result confirms the hypothesis by showing that CRISPR/cas9 turn off tdTomato gene expression in cortical slice culture. The advantages associated with the CRISPR/cas9 system make it a promising tool in site-directed mutagenesis. While it has been proven to be highly effective in a variety of model organisms including mice and humans, its application in post-mitotic cells are still relatively new. Through our experiment, we realized that there was potential to improve the vector delivery efficiency even though the biolistic
particle delivery system is a continent technology with both in vivo and in vitro applications to a variety of organisms and cell types (Xia, Martinez, Daniell & Ebert, 2011). One approach we are considering is lentivirus transfection. Lentivirus transfection is a technology known for its ability to transfect cells in vivo. Therefore, for future direction, we want to use lentivirus transfection for delivery pL-CRISPR.EFS.tRFP lentivirus vectors we tested in the first part of the experiment. Based on our experiment, we think CRISPR/cas9 system holds great potential for applications in cortical slice culture and continuing to optimize the current CRISPR/cas9 system we have will greatly benefit future functional research projects.
Bibliography


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