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14. ABSTRACT

Effective *in vitro* models of myelination, dysmyelination, and/or remyelination would substantially speed the development and testing of potential therapies for myelin disorders such as multiple sclerosis. Tissues engineered from human induced pluripotent stem (iPS) may be effective at accurately modeling aspects of human physiology for screening of potential therapies. Highly enriched populations cells of neural lineage were obtained from human iPS cell lines that will serve as sources of microengineered human tissue. Hydrogel micropatterning methods were identified that may suppor the culture of human neurons and remain amenable to optical stimulation and recording. Methods were established for non-invasive monitoring of evoked electrophysiological responses in microengineered tissue constructs with voltage-sensitive dyes. This work represents a unique combination of enabling technologies, including human iPS cells, microfabrication, and optical neural recording. Though the defined tasks were not able to be fully completed, the capabilities and limitations of combining these technologies have been determined and challenges that must be overcome have been identified. The goal of producing a high-throughput, functional assay of human neural activity was demonstrated to be feasible.

15. SUBJECT TERMS
Human induced pluripotent stem cells, hydrogels, 3D culture, electrophysiology, high-throughput assay

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Introduction

Effective *in vitro* models of myelination, dysmyelination, and/or remyelination would substantially speed the development and testing of potential therapies for myelin disorders such as multiple sclerosis. Tissues engineered from human induced pluripotent stem (iPS) may be effective at accurately modeling aspects of human physiology for screening of potential therapies. The most physiologically relevant measure of myelin integrity is compound action potential conduction velocity, which is currently not feasible to measure *in vitro* because of a lack of tissue culture models that mimic myelinated neural tracts. The overall objective of this project is to establish the feasibility of noninvasively assessing myelin integrity with an electrophysiological endpoint in biomimetic, organotypic neural tissue cultures engineered from human iPS-derived cells. The aims of this project are to 1) develop an *in vitro* model of myelinated nerve fiber tracts derived from human iPS cells; and 2) to demonstrate the feasibility of noninvasive, optical stimulation and recording from microengineered tissues. All progress and results discussed in this report are in regard to the revised Statement of Work approved as part of the amended contract effective 4 June 2013.

Body

Since submission of the last report, we have successfully completed Task 1 with some surprising results. Human iPS cells were obtained from NuPotential, Inc. These cells had been derived from human astrocytes and reprogrammed by lentiviral induction of pluripotent genes Oct4, Sox2, Klf4, and c-MYC. Somewhat surprisingly, these astrocyte-derived cells showed a strong epigenetic memory, spontaneously differentiating into neural lineages. When allowed to differentiate in this fashion, iPS cultures produced very high numbers of neurons along with some astrocytes. Nestin expression showed nearly complete enrichment of neuronal lineages, as shown in Fig. 1. The phenotype of each of these cell lines was characterized by RT-PCR for expression of a library of neuronal genes (Table 1), and by immunocytochemistry for expression of proteins. Gene expression was quantified relative to a the housekeeping gene GAPDH and expressed as ΔCT. Though the last few data points are still in process, it is clear that four of the seven human astrocyte cell lines (HA #1, 2, 3, and 7) show very large amounts of neuronal differentiation when using epigenetic memory. We observed differentiation of neural progenitor cells, motor neurons, mature neurons, dopaminergic, glutaminergic, and GABAergic neurons, as well as astrocytes and oligogendrocytes, as shown in Fig. 2. In summary, we have produced highly enriched populations of neurons and glia from iPS cells derived from human astrocytes. These cell lines will serve as an excellent source of human cells from which our model systems may be derived.

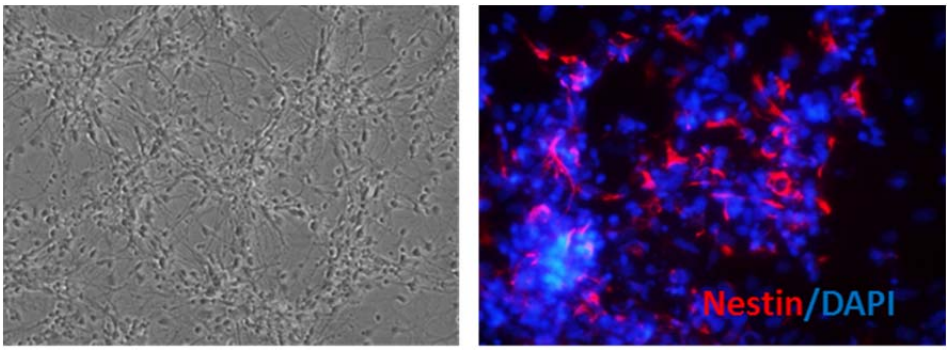

Fig. 1: Spontaneous differentiation toward neuronal lineage of iPS cells derived from human astrocytes. Left: phase contrast image of iPS culture. Right: fluorescence image of mostly nestin-positive cells with DAPI-stained nuclei.

Table 1: RT-PCR quantification of neuronal genes in human astrocyte cell lines, as ΔCT.

Gene	Function	HA#1	HA#2	HA#3	HA#7
ASCL1	neural progenitor marker/mature neuron	7	4	4	9
EMX1	NEURON MARKER (NEURAL TUBE, BRAIN)				
MAPT	BRAIN, PERIPERAL NERVE	6	4	4	5
NEUROD1	Mature Neuron	6	6	2	4
PAX6	brain/retinal progenitor		3	4	
nes	brain development	2	1	2	2
map2	brain				
FABP7	Neuronal stem cell marker	3		3	
FOXG1	Motor neuron	2		3	2
NCAM1	Mature Neuron	5	4		5
NR4A2	Dopaminergic neuron	6			6
SLC1A2	Glutaminergic neuron	4	4	4	4
SLC1A3	Glutaminergic neuron	4	2	4	3
GAD2	GABA neuron	7	5	4	8
SLC32A1	GABA neuron	6	3	4	6
GFAP	Astrocyte marker	6	3	4	8
S100B	Astrocyte marker/mature neuron			4	2
OLIG2	Oligodendrocyte progenitor marker				
NKX2-2	Oligodendrocyte progenitor marker		3		4
CNP	Oligodendrocyte marker				2
MBP	Oligodendrocyte marker		1		4
NAT1	control				
GAPDH	control				

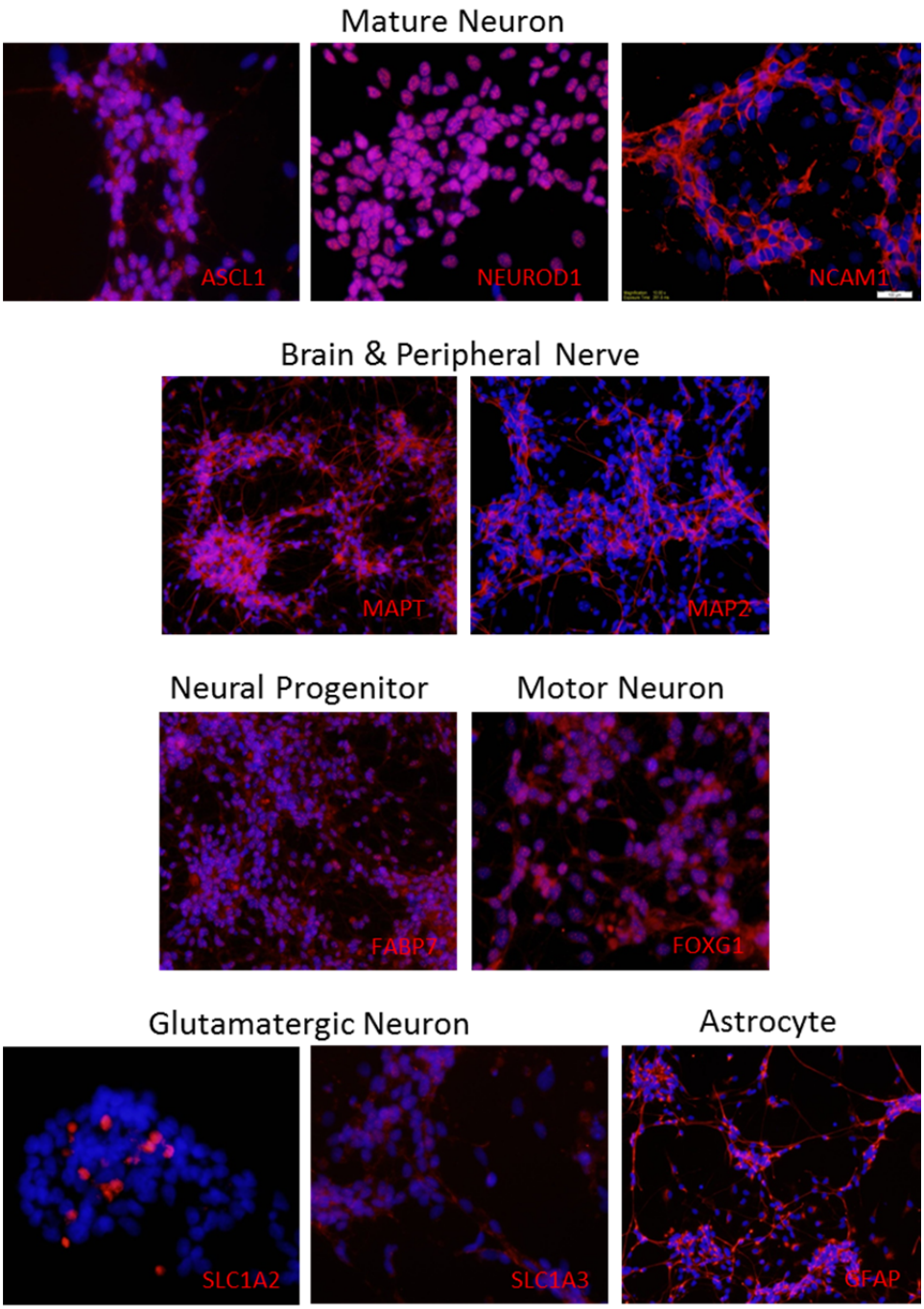

Fig 2: Representative fluorescent images depicting sxpression of various neuronal proteins in cells differentiated from human iPS cells. All images are counter-stained with DAPI nuclei.

In regard to Task 2, we were unfortunately not successful in obtaining robust neurite growth from iPS cells in hydrogels. One setback is that the researcher in the Moore lab who was going to conduct these experiments decided to pursue other options, and so work toward this Task was delayed considerably. We remain optimistic that we could achieve better results if using the most recent cell lines and differentiation protocols to produce cell populations highly enriched with more mature neurons. We also found that the quick method of producing micropatterned hydrogels that we employ may be too crude for the goals of this project, as discussed further in Task 3. We will continue these efforts through other funding sources.

We were able to make substantial progress toward Task 3.1 using recently described voltage-sensitive dyes (Miller, 2012). When applied at a final concentration of 200 nM, we were able to image the 3D rat dorsal root ganglion (DRG) cultures with sufficiently low background as to detect electrically-evoked depolarization events, as shown in Fig. 3. The optical protocols required to perform simultaneous optical stimulation and optical recording in large fields-of-view and at high speeds proved exceedingly difficult. When capturing at frame rates necessary to detect depolarization events, it was necessary to limit the field-of-view (FOV) to image only a fraction of the 3D culture at a time. We were able to optimize speed with FOV sufficiently to obtain recordings in 3D cultures without any optical sectioning, which is a substantial advance. However, we were not able to achieve simultaneous optical stimulation by illumination of channelrhodopsin-2 in specific regions due to the complexity of the optical setup required. We would need to build a novel optical stimulation and recording system specifically for this purpose. Further, we found that the limitations inherent in optimizing speed and FOV may require us to consider an alternative method of producing micropatterned 3D cultures. For these reasons we were not able to complete Task 3.2.

An important finding was that our method of projection lithography of dual hydrogels (Curley & Moore, 2011) is perfectly suited for engineering neural fiber tracts that may be recorded with field electrodes, as discussed in Task 4. However, for optical recording, a higher-precision patterning method, such as cell printing, may be desired. We have formed a collaboration with Dr. Douglas Chrisey, who pioneered laser direct-writing of living cells (Kingsley et al., 2013), in order to continue pursuing this approach.

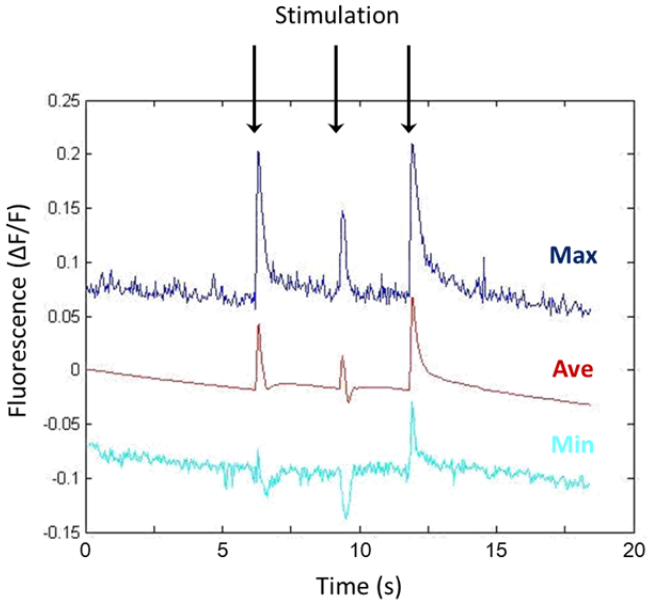

Fig. 3: Electrically-evoked depolarization detected by fluorescence changes of voltage-sensitive dyes.

We have made substantial progress in Task 4.1. We have fabricated neural fiber tracts from DRG explants and shown that we can record compound action potentials (cAPs) when stimulated electrically, as described in Fig. 4. The neural fiber tracts grow with high axon density, which was apparent when imaged with transmission electron microscopy (TEM), as shown in Fig. 4B. The 3D arrangement of neurites in high density enables reproducible recording of cAPs, which remain consistent with high-frequency stimulation (Fig. 4D). The cAPs display an expected increased latency when stimulated distally vs. proximally (Fig. 4F). The increased latency can be used to calculate the conduction velocity, which we found to be approximately 0.5 m/s. This is much slower than would be found in peripheral nerve in vivo, which is not surprising given we have a majority of small-diameter, unmyelinated axons at room temperature. Further, the amplitude is decreased for distal stimulation, reflecting the lower fiber density seen in distal segments, as shown in Fig. 4E. We are in the process of drafting a manuscript to report these results.

We have not yet been able to show induction of myelination, due to some unfortunate delays, including the loss of productivity by one researcher as mentioned before. We have observed spontaneous axon ensheathment with Schwann cells (Fig. 4B), which indicates myelin formation is feasible. The myelination protocols we have tried at first were not successful, perhaps due to the 3D nature of our constructs. We had thought that the Schwann cells present in the DRG explants would be capable of producing myelin, but we have determined that we will need to introduce additional myelin-forming cells to these cultures. This is desirable anyway, as it will allow incorporation of different types of myelin forming cells, e.g. oligodendrocytes vs. Schwann cells and human vs. rodent. We remain confident that the combination of higher enriched populations of oligodendrocytes with updated myelination protocols will enable us to produce myelinated cultures. We will continue to pursue these efforts with other funding sources.

The problems associated with obtaining robust neurite growth from iPS-derived cells, and the technical difficulties identified in performing optical stimulation in conjunction with optical recording, all combined to preclude us from accomplishing Task 5, which is unfortunate. Still, we were able to demonstrate significant contributions toward the overall goal, which was to optically measure differences in conduction velocity in myelinated vs. unmyelinated human cultures. We were able to produce high-quality human neurons and glia that will serve as excellent sources of cells for these model systems. We were able to measure conduction velocity of cAPs in 3D microengineered primary cell cultures. We were able to detect depolarization events in 3D cultures with fluorescence measurements using voltage-sensitive dyes. Most importantly, we now have a thorough understanding of the capabilities and limitations of the numerous technologies that must merge to make possible the completion of the goal. Though more difficult than originally envisioned, we remain confident that we will be able to achieve our goal of a high-throughput, functional model of myelination in human cell cultures. We are in the process of preparing a number of grant proposals to the support the continuation of this work in various forms.

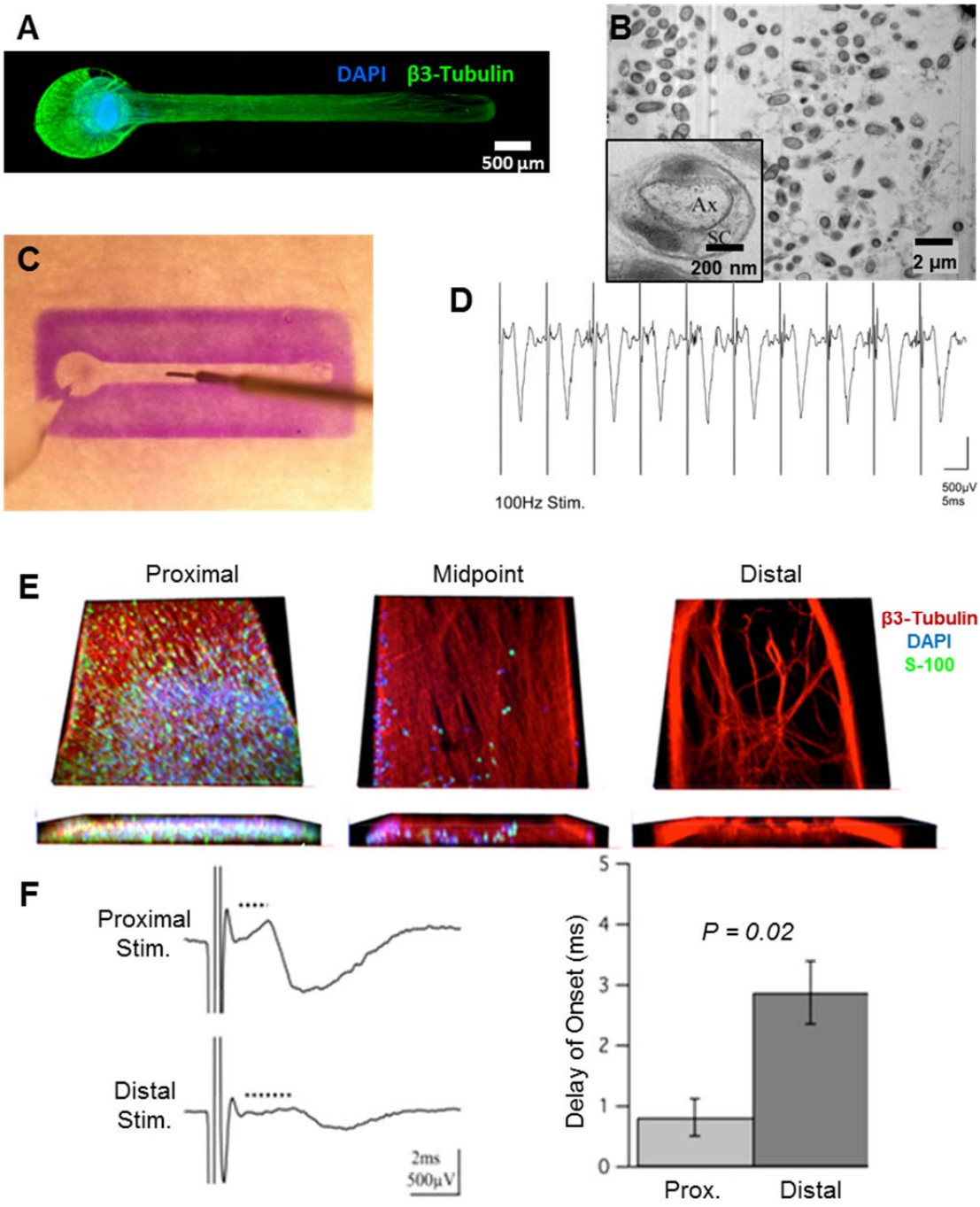

Fig. 4: Stimulation of microengineered DRG neural fiber tracts. **A)** Fluorescently-labeled construct with nuclei stained with DAPI (blue) and neurites stained with β3-tubulin (green). **B)** TEM cross-section of neural fibers; inset = closeup of Schwann cell (SC) wrapped around single axon (Ax). **C)** Field potential and stimulation electrode placement (outer PEG gel dyed for clarity). **D)** Example trace of cAP recording during 100Hz stimulation. **E)** Confocal image stacks of neural fiber tracts proximal to the ganglion, at the midpoint, and distal from the ganglion, stained with β3-Tubulin (red) stained neurites, DAPI (blue) nuclei and S-100 (green) Schwann cells. **F)** Recorded cAPs stimulated proximally show shorter latency (quantified in plot to the right) and greater amplitude than those stimulated distally.

Key Research Accomplishments

- Human iPS cells have been converted to neuronal and glial phenotypes with high differentiation efficiencies when using epigenetic memory.
- HA hydrogels and interpenetrating networks with Puramatrix have been synthesized and used to support 3D culture of neural stem cells.
- Electrically-evoked action potentials were recorded in 3D cultures with optical measurements of voltage-sensitive dye fluorescence.
- The design parameters for a novel optical recording and stimulation system have been defined.
- Compound action potential propagation was recorded in microengineered neural fiber tracts grown from primary neural cells.
- Conduction velocity of compound action potentials was confirmed as a feasible functional metric in 3D microengineered neural fiber tracts.

Reportable Outcomes

- Lines of human iPS cells have been generated that have been shown to be capable of producing highly enriched neuronal lineage for generating substantial quantities of neurons and glia.
- Renee Huval completed her Masters' thesis on the topic of recording cAPs in microengineered tissue constructs.
- The PI presented portions of this work at a Gordon Research Conference on Biomaterials and Tissue Engineering and the Society for Biomaterials:
  Moore MJ*, Huval RM, Miller OH, Fan Y, Hall BJ. Compound action potential propagation in microengineered peripheral neural tissues. Society for Biomaterials Annual Meeting, Denver, CO, April 19, 2014.
  Huval RM, Miller OH, Fan Y, Hall BJ, Moore MJ*. Sensory peripheral nerve "on-a-chip." Gordon Research Conference, Holderness, NH, July 28 - Aug 2, 2013.
- Only named Key Personnel received pay funded by this award: Dr. Michael J. Moore, Dr. Benjamin J. Hall, and Dr. Kenneth J. Eilertsen.

Conclusions

In summary, we have made substantial progress toward demonstrating the feasibility of developing microengineered human neural tissues that can be functionally assessed non-invasively. We have obtained highly enriched populations cells of neural lineage from human iPS cell lines that will serve as sources of microengineered human tissue. We have verified a micropatterned hydrogel culture system will support the maintenance of human neurons, but have determined an alternative micropatterning method may be optimal for optical stimulation and recording. We have refined methods for non-invasive monitoring of evoked electrophysiological responses in microengineered tissue constructs with voltage-sensitive dyes. This work represents a unique combination of enabling technologies, including human iPS cells, microfabrication, and optical neural recording. Though we were not able to complete fully all of the defined tasks, we have determined the capabilities and limitations of combining these technologies and identified the challenges that must be overcome. We will continue to pursue further funding toward our goal of showing how it may be possible to create a high-throughput, functional assay of human neural activity. Such an assay may prove to be useful for modeling neurological disorders, such as multiple sclerosis, and for screening potential therapies.

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Miller EW, Lin JY, Frady EP, Steinbach PA, Kristan WB, Tsien RY. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. *Proc Natl Acad Sci USA* 2012, 109(6):2114-9.