FLEx technology and optogenetics: Flipping the switch on gene expression with high spatial and temporal resolution

Commentary by Alessia Armezzani, PhD 15/10/2019

A few decades ago, the brain remained elusive, not from a lack of intellectual curiosity on the part of scientists but, rather, from the limited technologies available. Over the past few years, however, remarkable technological advances have taken researchers to the threshold of a revolution in modern neuroscience, an era where technologies like FLEx and optogenetics meet, allowing scientists to investigate the fundamental bases of both brain function and dysfunction.

First, let's start from the basics: What is optogenetics?

Optogenetics: Controlling neurons with light

In 2005, a team from Stanford University, led by the neurobiologist Karl Deisseroth, combined optical and genetic methods to photo-stimulate mammalian neurons and alter synaptic events: a new technology was born, and the term "optogenetics" was coined (Boyden et al., 2005; Deisseroth et al., 2006).

Despite its relatively recent development, the history of optogenetics begins almost 50 years ago with the discovery of microbial rhodopsins, light-sensitive proteins that absorb photons for energy conversion or the induction of intra- or intercellular signaling pathways (Ernst et al., 2014). Microbial rhodopsins are encoded by opsin genes, and include bacteriorhodopsins, halorhodopsins, and channelrhodopsins, the first two found in the Archaea, the third in green algae (Oesterhelt and Stoeckenius, 1971; Matsuno-Yagi and Mukohata, 1977; Foster et al., 1984; Harz and Hegemann, 1991). These proteins control the flow of electric charges across cell membranes and maintain membrane potential in response to visible light (Zhang et al., 2011).

What do microbial rhodopsins and neurons have in common? Neuronal stimulations are triggered by the movement of ions across the axon's membrane: once a certain number of positive ions crosses the cell membrane, a threshold is reached and the neuron fires, sending the electrical signal down the axon (Lodish et al., 2000). Most neurons in the brain are not naturally light-sensitive, so the selective expression of opsin genes in targeted cells makes it possible to control neuronal activity with a specificity far greater than the one that can be achieved using pharmacological or electrophysiological methods (Deisseroth, 2011; Mei and Zhang, 2012; Towne et al., 2016).

Optogenetic neuromodulation is made possible by the light: when hit with the correct wavelength, microbial rhodopsins enable ions to flow across the axon's membrane, thereby controlling neural activity (Bernstein and Boyden, 2011; Mei and Zhang, 2012). For example, blue light activates channelrhodopsins that, in turn, trigger neural excitation, whereas yellow light activates halorhodopsins to silence neuronal activity (Figure 1) (Wiegert et al., 2017).

How to get an opsin into the brain?

In order to express opsins within the brain, researchers inject genetically modified viruses encoding microbial rhodopsins into specific cerebral regions. The resulting viral-infected neurons are subsequently photo-stimulated through fiber-optic cannulas directly implanted in the injection site and connected to a laser. The laser flashes light of specific wavelengths, selectively turning neuron activity on or off (Atasoy et al., 2008; Taylor et al., 2016; Hooper and Maguire, 2016).

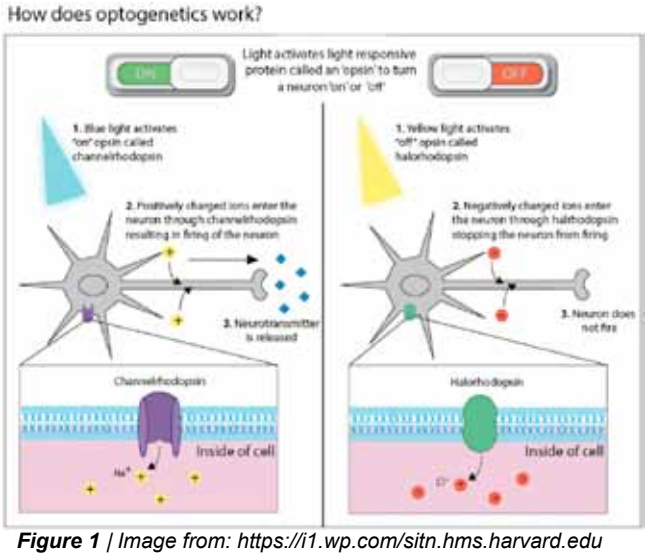

Figure 1 *| Image from: https://i1.wp.com/sitn.hms.harvard.edu*

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Recombinant adeno-associated viruses (rAAVs) have become increasingly popular for gene delivery in the central nervous system due to their relatively stable extra-chromosomally long-term expression and low ability to induce innate immune responses (Fenno et al, 2011). However, rAAVs lack the ability to specifically infect defined neuronal subpopulations, and this represents a major downside in optogenetics (Belzung et al, 2014). This could be overcome by fusing opsin genes to cell-type specific promoters; however, those usually drive weak expression of downstream genes. Strong cell-specific promoters, on the other side, are too long for rAAVs, which can only package sequences shorter than 5 kb (Hirsch et al, 2016; Hudry and Vandenberghe, 2019). How to achieve strong opsin expression in specific neuronal cell types then? This is where FLEx enters the scene!

FLEx: A light when night is about you!

The FLEx (for flip-excision) switch, also known as DIO (Double-floxed Inverse Orientation) or DO (Double-floxed Orientation), is a very powerful tool that provides precise temporal and spatial control of gene expression *in vivo* (Schnütgen et al., 2003). This is achieved through site-specific recombinases such as Cre or Flp that induce DNA recombination at defined recognition sites (i.e., loxP and FRT, respectively) (Abremski and Hoess, 1984; Van Duyne, 2001; Christenson Wick and Krook-Magnuson, 2018).

Optogenetic FLEx vectors contain a strong promoter upstream an opsin gene (e.g., channelrhodopsin-2, ChR2) fused to a reporter gene (e.g., mCherry) to easily detect opsin-expressing cells. The resulting fusion gene is inserted in the antisense orientation with respect to the promoter to prevent its expression, and is flanked ("floxed") by two sets of incompatible recognition sites (e.g., loxP and lox511) in opposite orientations. Since Cre does not cause recombination between mismatched recognition sites, its presence induces first opsin inversion, and then lox sites excision, therefore locking the opsin into the correct orientation to be transcribed (Figure 2) (Sharma and Zhu, 2014).

How does FLEx enable strong opsin expression in specific neuronal cells? This can be achieved using Cre-dependent viruses such as the optogenetic FLEx vectors in combination with transgenic animals or rAAVs expressing Cre under a specific cell-type promoter. Once injected into the brain, the viruses infect all the cells with an inactive opsin gene; in Cre expressing cells, however, Cre induces the recombination of the double floxed opsin construct thereby enabling its expression under a strong promoter only in specific neuronal cells. FLEx ensures therefore spatial accuracy and strong opsin expression, both essential in optogenetics to study physiological and behavioral processes (Abdallah et al, 2018; Deubner et al., 2019).

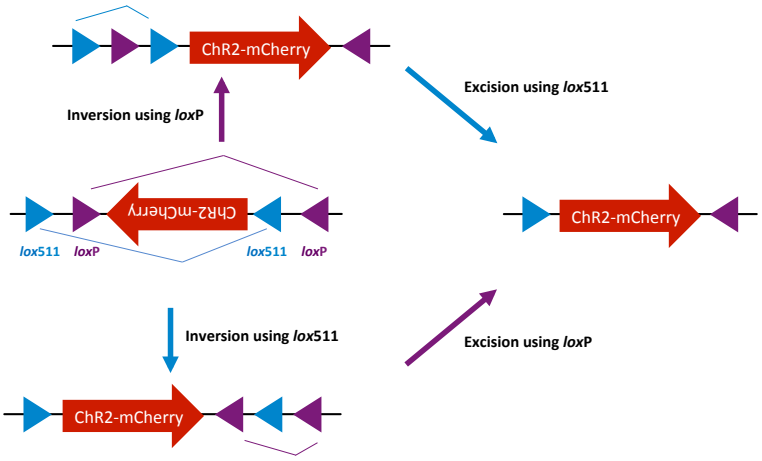

***Figure 2** | The FLEx switch strategy. The expression of ChR2-mCherry is a Cre-mediated event: in the absence of the recombinase, the reverse-oriented ChR2-mCherry is not expressed. Oppositely oriented loxP (purple triangles) and lox511 (blue triangles) sites permits Cre-mediated recombination and inversion of the ChR2-mCherry sequence with respect to the promoter, thereby allowing its expression. ChR2, channelrhodopsin-2. Figure adapted from Atasoy et al., 2008.*

For example, Taylor and colleagues used rAAV FLEx-based vectors to study the neural circuitry behind the mechanisms of sleep and anesthesia. In Cre transgenic mice, they targeted the dopamine (DA) neurons of the ventral tegmental area (VTA) of the brain, a region previous found to be important in regulating sleep. In particular, they found that optogenetic stimulations of the DA neurons produce behavioral and electroencephalography evidences of arousal in mice previously subjected to steady-state general anesthesia (Taylor et al., 2016).

Interestingly, these very same neurons play also a central role in motivated behaviors (Juarez and Han, 2016). Not so long ago, however, it was completely unclear which subpopulation of DA neurons could activate appetitive rather than aversive stimuli. In a recent paper, de Jong and colleagues used rAAV FLEx based vectors to answer this question, mapping and characterizing the activity of the DA neurons of the VTA. Using *in vivo* optogenetic stimulations, they simultaneously recorded the electrical impulses of discrete subpopulations of this brain area, demonstrating that it is possible to separate neuronal inputs to induce aversion- or reward-related behaviors. The high spatio-temporal precision and reversible modulation of FLEx vectors combined with the use of several Cre transgenic mice targeting different brain areas enabled the drawing of a detailed and functional topography of the neural circuit architecture of the brain regions associated with motivated behaviors (de Jong et al, 2019). FLEx vectors represent therefore an ideal partner to optogenetics to understand the cellular and molecular mechanisms of the brain *in vivo*.

Alessia Armezzani is scientific communication manager at genOway.

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