The Lego-logic of optogenetics

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Daisy-chaining light-sensitive ion channels, pumps and fluorescent proteins extends the possibilities for control of neuronal activity.

The discovery of light-gated ion channels and ion pumps in green algae and bacteria began a new era in neuroscience, now yielding a bumper crop of exciting discoveries every year. As it turns out, these microbial light sensors work very well when expressed in heterologous systems such as mammalian neurons. They do not need any exogenous cofactors for proper operation and do not affect neuronal activity in the absence of light. Light-gated cation channels, usually variants of the famous channelrhodopsin-2 (ChR2)[1], are now routinely used by many laboratories to trigger action potentials in neurons with millisecond precision, whereas proton and chloride pumps such as halorhodopsin (NpHR) hyperpolarize cells[2] and efficiently keep neurons from firing action potentials. As a consequence, questions that used to be completely in the realm of 'thought experiments'—for example, studying the impact of a specific population of neurons on the behavior of freely moving animals—have now become experimentally accessible[3]. In a paper in this issue Kleinlogel *et al.*[4] put a new twist on optical control of neurons.

To investigate the effect of certain neurons on network function and behavior, it would be optimal if one could excite and shut off a specific set of neurons with light of different color. In practice, getting a bidirectional 'push-pull' system to work proved to be quite difficult as expression of two different optogenetic actuators at variable levels leads to variable excitation or inhibition of individual cells. Under these conditions, it might be possible to find a neuron that shows bidirectional responses in a patch-clamp recording[2], but it is impossible to predict the effect of illumination on a specific transgenic cell a priori, which defeats the purpose of optical activity control. In addition, some optogenetic tools that reportedly work quite well in *Xenopus laevis* oocytes express rather poorly in neurons, a disappointment for many early adopters that were eager to put these new tools to use.

In this issue of *Nature Methods*, Kleinlogel *et al.*[4] demonstrate the power of a new expression strategy. Instead of expressing two light-gated ion channels from two separate or one bicisitronic self-cleavable construct, they experimented with very large fusion proteins, containing two different optogenetic actuators coupled by a fluorescent protein and a transmembrane helix taken from a gastric proton pump.

This conceptually simple idea turns out to solve several problems that plagued optogenetics from the start. First, it forces equal numbers of the tandem partners into the membrane, ensuring identical excitation-to-inhibition ratios in every transgenic cell. Second, and somewhat surprising, the tandem construct is often better expressed than its individual components, allowing even notoriously problematic tools such as *Volvox carteri* ChR1 to become useful in neurons.

Tandem constructs of synergistic (that is, depolarizing) actuators can extend the spectral sensitivity of the fusion protein (**Fig. 1a**), resulting in 'white' light sensors that hold promise for vision restoration. Combining actuators with opposing currents results in wavelength-dependent, bidirectional photocurrents (**Fig. 1b**), ideally large enough to induce or suppress action potentials in neurons.

For bidirectional control of neuronal spiking using these constructs *in vivo*, two requirements have to be met: first, owing to the broad and overlapping excitation spectra, excitatory and inhibitory photocurrents have to have similar amplitude. Second, even a well-balanced excitatory-inhibitory pair (**Fig. 1b**) is useless if the peak-to-peak current amplitude remains on the order of tens of picoamperes. In large pyramidal cells, for instance, it takes up to one nanoampere to reliably prevent spiking under conditions of strong excitatory synaptic input. As an example, the neuronal silencer archaerhodopsin-3 (Arch) produces sufficiently large inhibitory currents, but because of overlap of its excitation spectrum with that of ChR2,

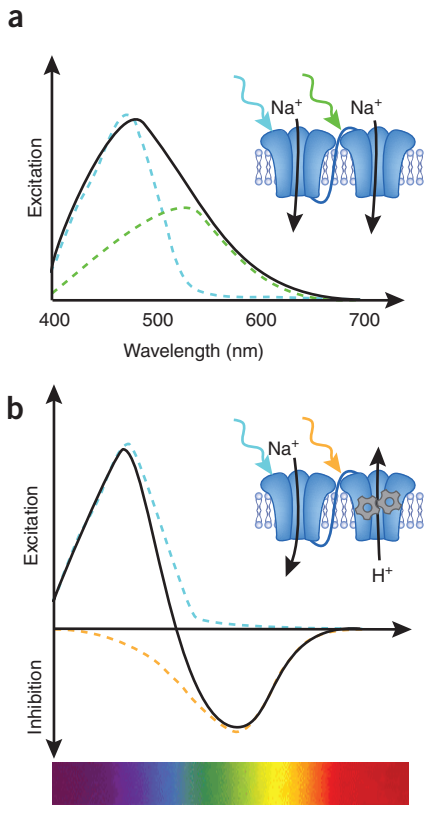

Figure 1 | Photocurrent amplitude depends on the wavelength of light. (**a**) Expression of synergistic optogenetic actuators in tandem (ChR2-VChR1) results in extended spectral sensitivity. A flexible linker spans the plasma membrane to connect the C- and N-terminal ends of the two membrane proteins. (**b**) Tandem constructs of antagonistic actuators (for example, ChR2-NpHR) ideally allow for bidirectional control of membrane potential.

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it is not clear whether spike induction with blue light pulses will be possible in ChR2-Arch tandem protein–expressing neurons *in vivo*. Thus, creating the perfect tool for spike control in behaving animals might still require optimization, perhaps using high-current ChR2 mutations such as T159C (ref. 5) or L132C (ref. 6).

Simultaneous activation of a depolarizing channel and a hyperpolarizing pump, in contrast, might find useful applications outside systems neuroscience. An 'optical voltage clamp' approach could be used to develop high-throughput assays, for example, to screen the effects of chemical compounds on voltage-gated channels. For the design of such all-optical experiments, recently published blue- and red-shifted genetically encoded calcium sensors will certainly come in handy[7] and might even be built into subsequent fusion constructs as integrated reporters.

Another advantage of tandem expression is the possibility to precisely quantify the properties of a new optogenetic tool by comparing them to those of the well-characterized ChR2. This might sound rather technical, but it is in fact very helpful to understand the differences in performance between various optogenetic actuators. Previously, researchers could only report the total photocurrent generated by a new tool and could not distinguish the effects of altered single-channel properties, expression amounts or differential targeting to the plasma membrane[5].

Compared to this situation, stoichiometric comparison is a big improvement and resulted in some unexpected findings. Arch, for example, produced considerably larger currents than the original NpHR in neurons[8]. To their surprise Kleinlogel *et al.*[4] now found that Arch has better expression than NpHR but lower pumping efficiency when compared on the single-molecule level. The authors go on to show that optimization of codon usage enhances expression of all tested constructs[4], resulting in a larger increase in photocurrent than, for example, with the popular ChR2 point mutation H124R.

This kind of precise quantification is of course essential to speed up rational design and improvement of optogenetic tools. For application-oriented groups, the good news is that basic optogenetic building blocks can be put together to create new tools with enhanced functionality. Making full use of these combinatorial possibilities, your favorite thought experiment might just have become possible!

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

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Recognizing heart cells in a crowd

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A cardiac-specific reporter genetically engineered into human embryonic stem cells allows the optimization of differentiation protocols and the identification of cell-surface markers—a welcome new tool to help isolate and define cardiac cell lineages.

Access to unlimited quantities of human heart cells for research, drug discovery and therapeutic applications is one of the most exciting promises of stem-cell research. But how do you get these specialized cells in sufficient quantity and purity to be useful for these many applications? Although methodologies for generating cardiac cells from human pluripotent stem cells are rapidly improving, even these improved protocols result in mixed cell preparations with the relative abundance of different cell types varying depending on the starting cell line and on baseline culture conditions. To make matters worse, the reagents needed to identify the different cell types that may be present are often limited or nonexistent. This is particularly the case for identifying living cells. In two papers, one in this issue[1] and a related report elsewhere[2], researchers now describe a new genetically modified human embryonic stem cell (ESC) line that has an enhanced GFP (eGFP) reporter engineered into the gene of a well-studied cardiac transcription factor, Nkx2.5. Both studies confirmed the fidelity of this *NKX2-5*$^{eGFP/w}$ human ESC line for identifying cardiac precursors and cardiomyocytes, but what is more important is how the researchers used this reporter system to both optimize differentiation protocols and to identify new cell-surface markers of cardiac cell lineages (**Fig. 1**).

The use of reporter genes introduced into stem cells to identify cell lineages and define distinct cell populations is a common strategy, in particular in the mouse. Nkx2.5, one of the earliest cardiac transcription factors expressed in development, has previously been targeted in mouse ESCs, and the resulting engineered mouse ESC lines and transgenic mice revealed that the gene is expressed in mouse cardiac progenitors and in differentiated cardiomyocytes[3–5]. Genetic engineering of human pluripotent stem cells has proven more difficult. Only a handful of human transgenic ESC lines relevant for the cardiac differentiation field have been described in detail[6,7]. In the paper published in this issue, Elliott and colleagues describe a recombineering approach and homologous recombination to knock *eGFP* into the *NKX2-5* locus of a human ESC line[1].

Initial characterization of the *NKX2-5*$^{eGFP/w}$ ESCs demonstrated that they differentiated *in vitro* similarly to nongenetically modified ESCs, with sequential expression of genes in the normal cardiac developmental program. Notably, this differentiation includes the increased expression of Nkx2.5 starting on day 7 of differentiation; Elliott and colleagues found that eGFP expression faithfully represented the endogenous pattern[1]. The earliest cells expressing eGFP were not contracting cardiomyocytes but cardiac precursors, demonstrated by single-cell clonal assays to be at least bipotential: they gave rise to both cardiomyocytes and smooth muscle cells, comparable to at least one previous

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