

Making light work of fine-tuning channelrhodopsins

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The development of genetically engineered proteins that can control cell excitability with light have revolutionized our understanding of the nervous system. The most widely used of these optogenetic tools is the light-gated ion channel, channelrhodopsin 2 (ChR2). A new study by Cho *et al.* describes the development of ChR2 variants with improved photocurrents and more selective ion permeability using an automated multifaceted fluorescence-based screening. This methodological framework holds promise not only in refining features of ChR2, but also for other proteins in which fluorescence phenotyping is possible.

The application of light-activated proteins to manipulate cellular excitability and signaling pathways is a great example of translating basic biological discoveries into scientific tools that can probe the functions of specific cell populations. The most broadly utilized group of light-activated proteins are the channelrhodopsins (ChRs)² — transmembrane proteins with a bound opsin molecule that isomerizes in response to a photon to trigger the opening of an integral ion channel pore, with ChR2 being permeable to protons, sodium, potassium, and calcium (1). The expression of these ChRs in specific cell populations enables light-induced control of electrical activity, and this optogenetic control of specific neural circuits has revolutionized neuroscience. Similarly, the advances made in neuroscience have stimulated new work on ChRs to understand their behavior in more detail and to identify more precise and robust tools. The ability of ChR to spatially and temporally control neural activity depends on achieving good expression of the construct and on properties of the ChR itself, including high sensitivity to desired wavelengths, a large conductance, rapid kinetics, minimal desensitization, and appropriate ionic selectivity (2). Significant effort has been invested into optimizing these properties, for example, producing a spectrally shifted ChR that can be activated by longer and more penetrant wavelengths, or mutating ChR to create an anion-selective inhibitory ChR (reviewed in Ref. 3). In this issue of JBC, Cho *et al.* (4) expand this toolbox further, describing a random mutagenesis and semi-automated screening approach to generate a ChR2 variant with improved photocurrents and reduced Ca²⁺ and proton permeability.

In many previous ChR engineering studies, researchers have focused on mutating residues within the transmembrane pore,

with the expectation that this will yield the biggest changes to channel properties. To look for ChRs with new properties, Cho *et al.* (4) needed to develop an approach that would allow them to cast a wider net. Their approach is illustrated in Fig. 1. They initially used a high-throughput screening strategy to evaluate point mutations introduced into each of the first 298 of the 737 amino acids in ChR2 using site-saturated random mutagenesis. These residues comprise the more functionally relevant ChR2 transmembrane domains, but notably also include loops and residues pointing away from the channel pore (5). Mutant constructs for each position were transfected into HEK293 cells and screened for light-evoked voltage responses using a voltage-sensitive fluorescence dye. From this first “residue map” stage, about 50 amino acid positions were identified where random mutations produced an increased light-evoked voltage response. Cho *et al.* (4) then selected 27 of these positions (with and around those showing the largest increase) for “target mutation” and “target checking” stages, involving the introduction of each alternative amino acid and subsequent evaluation of the functional effect. This further identified and confirmed point mutations with increased voltage-sensitive fluorescent responses.

The next stage was to identify point mutations with reduced Ca²⁺ signals and proton flux (acidification), and they did this largely using Ca²⁺- and pH-sensitive fluorescent dyes. The subsequent steps validated selected mutants using patch clamp recordings, and then combined point mutations to drive relative Ca²⁺ and proton permeability further down, ultimately deriving the ChromeT (triple) and ChromeQ (quad) constructs, both of which had similar permeation properties. Finally, the authors compared the functional properties of ChromeQ to WT ChR2, in both HEK293 and cultured neurons. The new ChromeQ construct demonstrated larger photocurrents, improved expression and photosensitivity, and had the capacity to induce spikes with improved temporal fidelity as compared with ChR2. By using this sequential screening strategy, Cho *et al.* (4) have produced a construct potentially more useful for specific light-induced neuronal depolarization, with less Ca²⁺ and proton influxes that can occur with traditional ChRs (*e.g.* Ref. 6). Concurrent Ca²⁺ and proton fluxes can falsely activate or modify fluorophores designed to quantify neuronal activity in optogenetics experiments, or may result in activation or modification of cellular signaling pathways.

Importantly, this new random mutagenesis and staged screening approach provides a framework for future studies to optimize the properties of ChR and related opsins. This *tour de force* approach, facilitated by semi-automated fluorescent

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² The abbreviation used is: ChR, channelrhodopsin.

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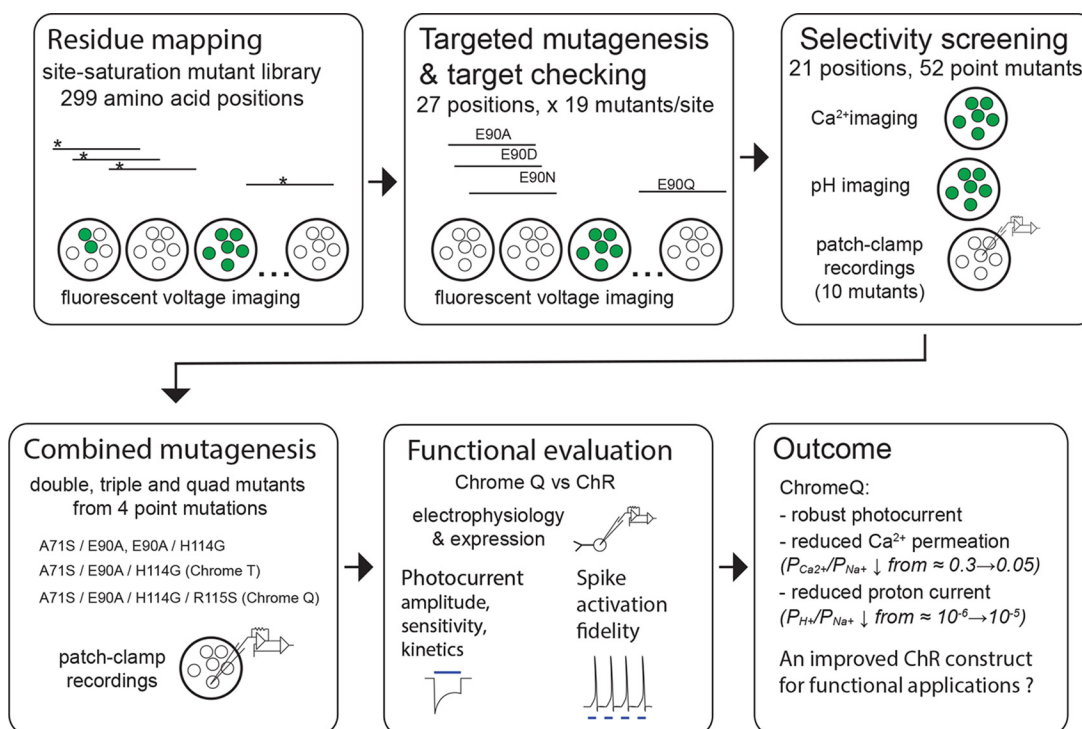


Figure 1. Flowchart of screening process and properties of the new quadruple ChR mutant.

screening of functional properties, represents an alternative pathway to a more targeted mutagenesis strategy based on the crystal structure of the chimeric ChR1ChR2 (5). Mutations targeting the putative channel permeation pathway of this structure, for instance, have also led to ChRs with reduced Ca^{2+} permeation and even allowed conversion to an anion-selective ChR (3, 7); however, the random mutagenesis strategy employed by Cho *et al.* (4) can reveal unexpected functional residues and domains. For example, the authors observed that mutations throughout many transmembrane domains could increase photocurrents, while, somewhat surprisingly, putative non-pore-facing residues influenced permeation (e.g. His-114 at the extracellular border of TM3 and Ala-71 at the intracellular end of TM1). This strategy can conceivably be applied to identify mutants with other functional aspects enhanced, for instance with altered optimal light wavelength sensitivity or, as observed in some of the screened mutants, with increased permeability to Ca^{2+} . Perhaps combining these identified point mutants with increased Ca^{2+} permeability, or further fine-tuning them as done here, could result in a selective light-gated calcium channel to optogenetically elicit Ca^{2+} signaling in cells such as glia or muscle. Furthermore, for the many opsins with less structural information (8), this strategy could be particularly valuable in resolving important functional domains. As the array of different opsins continues to be expanded, one also hopes that light-activated proteins may one day find a variety of clinical applications, and optimized specific functionality with reduced off-target side effects could help realize this opportunity. Indeed, ChR constructs are currently being trialed to restore light-activated signaling in retinal diseases (<https://clinicaltrials.gov/>, identifier NCT03326336). The paper by Cho

et al. (4) provides one such optimized ChR construct, and a framework for how others could potentially be identified.

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