SECOND-GENERATION OPTOGENETIC CONTROL OF NEURONS: BISTABLE AND NEAR-INFRARED-ACTIVATED CHANNELS FOR IN VIVO APPLICATIONS

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Abstract: The first decade of optogenetics, anchored on channelrhodopsin-2 and its early variants, established the basic capability of millisecond-precision genetic control over identified neurons but exposed three persistent technical constraints: shallow tissue penetration of blue light, the requirement for high light intensities that produce phototoxicity in chronic protocols, and the obligatory presence of an implanted intracranial fibre. Between 2016 and 2022, a second generation of optogenetic actuators substantially relaxed each of these constraints. Ultra-sensitive bistable step-function opsins (SSFO derivatives, SOUL) lowered the photon flux required for action potential generation by approximately three orders of magnitude and extended the depolarisation lifetime to tens of minutes. Red-shifted and near-infrared (NIR) activated channels (ReaChR derivatives, ChrimsonR, ChRmine and its engineered variants) shifted the action spectrum into the optical tissue window where blood and water attenuation are minimised. Upconversion-nanoparticle (UCNP) hybrid systems coupled tissue-penetrating NIR illumination to visible-light-responsive opsins through molecularly tailored lanthanide-doped nanocrystals. Combined with soma-targeting motifs (ST-ChroME, somBiPOLES) and two-photon holographic illumination, these tools enabled, by 2022, transcranial deep-brain stimulation of identified circuits at depths exceeding 7 mm in mice and single-cell-resolution simultaneous control of dozens of neurons in cortex. The accelerating pace of new tool publications has, however, made comparative tool selection for a specific in vivo application increasingly difficult. In this article I review the technical landscape of second-generation actuators along five performance dimensions — light sensitivity, kinetics, spectral red-shift, soma-specificity, and depth-reachability — and propose the In Vivo Optogenetic Tool Selection Index (IOTSI), a single normalised composite metric — bounded on [0,1] — that integrates the five dimensions and predicts the optimal actuator class for a defined experimental design. IOTSI applied to ten representative second-generation tools returns a tool-by-application ranking that, on the basis of currently published in vivo data, prefers ChRmine-class actuators for transcranial deep-brain stimulation, SOUL-class step-function opsins for chronic minimally-invasive protocols, and ST-ChroME or somBiPOLES for two-photon holographic single-cell control.

Keywords: *optogenetics, second-generation channelrhodopsin, step-function opsin, ChRmine, near-infrared optogenetics, upconversion nanoparticles, BiPOLES, soma-targeted opsin, in vivo neural control.*

INTRODUCTION

The introduction of channelrhodopsin-2 into mammalian neurons in 2005 made it possible, for the first time, to drive identified neurons to spike on a millisecond timescale through a genetically encoded photoswitch. The subsequent decade of work established three durable empirical facts. The first is that targeted optogenetic activation can causally drive behaviour in a wide range of vertebrate and invertebrate model systems. The second is that the original ChR2 toolbox, while sufficient to establish the proof of principle, was constrained by three technical limitations: blue-light excitation that scatters and absorbs strongly in brain tissue, photon-flux requirements that produce local heating and phototoxicity under sustained illumination, and the obligatory use of implanted intracranial optical fibres. The third is that overcoming each of these three limitations requires a different class of engineered opsin — and that the search for those classes would constitute the agenda of the field's second generation (Kim, Adamantidis & Deisseroth, 2017; Emiliani et al., 2022).

The second generation of optogenetic actuators, broadly the work of the 2016-2022 window, addressed the three constraints along three converging research programmes. The first programme produced ultra-sensitive bistable step-function opsins (SFOs). Building on the stabilised step-function opsin (SSFO) developed by the Deisseroth laboratory in 2011, the 2020 introduction of SOUL — the Step-function Opsin with Ultra-high Light sensitivity — by Gong and colleagues reduced the light intensity required to drive action potentials by approximately three orders of magnitude compared with ChR2 and extended the depolarisation lifetime to tens of minutes (Gong et al., 2020). The bistability property has two practical consequences: it permits transcranial activation of deep brain structures at light intensities that pose no risk of phototoxicity, and it converts the experimental paradigm from "continuous illumination required" to "single light pulse triggers minute-scale modulation," which substantially simplifies chronic in vivo protocols in mice and non-human primates (Gong et al., 2020).

The second programme produced red-shifted and near-infrared-activated channels. ChrimsonR, the fast variant of Chrimson developed by Mager and colleagues in 2018, enabled high-frequency neural spiking and auditory signalling with red-shifted illumination at 590 nm (Mager et al., 2018). ChRmine, discovered through structure-guided genome mining and reported by Marshel and colleagues in 2019 in the context of cortical layer-specific perception experiments and by Chen and colleagues in 2021 in the context of transcranial deep-brain stimulation, combined the red-shifted spectrum with a hundred-fold improvement in operational light sensitivity over previous fast red-shifted variants, enabling transcranial photoactivation at depths up to 7 mm without intracranial surgery (Marshel et al., 2019; Chen et al., 2021). The 2022 cryo-EM structure of ChRmine by Kishi and colleagues revealed an unusual trimeric architecture and provided the template for three subsequent engineered variants (rsChRmine, hsChRmine, frChRmine) with further-red-shifted, faster, and combined-property profiles, respectively (Kishi et al., 2022).

The third programme produced upconversion-nanoparticle (UCNP) hybrid systems. Chen and colleagues in 2018 reported that lanthanide-doped NaYF4:Yb/Tm nanoparticles, surface-functionalised with silica for biocompatibility and injected stereotactically into deep brain regions, absorb tissue-penetrating NIR (980 nm) light and re-emit visible photons at wavelengths that match the action spectra of ChR2 or its red-shifted variants (Chen et al., 2018). The UCNP approach has the conceptual advantage that it is opsin-class-agnostic: the same NIR illumination paradigm can drive any visible-wavelength-responsive opsin, including the bistable SFOs and the red-shifted ChRmine, provided the matched emission spectrum is engineered into the nanoparticle (Lin et al., 2017). The 2018 Science demonstration that transcranial NIR-UCNP-

mediated optogenetics could evoke dopamine release in the ventral tegmental area, induce hippocampal gamma oscillations, silence seizures and trigger memory recall established the technique as a complementary route to deep-brain control alongside the directly red-shifted opsin engineering (Chen et al., 2018).

Parallel to these three actuator-engineering programmes, soma-targeting and two-photon holographic illumination matured into routine techniques. The ST-ChroME variant introduced by Mardinly and colleagues in 2018 demonstrated single-cell-resolution sub-millisecond control of up to 50 neurons distributed in three dimensions over a 550 × 550 × 100 μm cortical volume, by combining the high-photocurrent ChroME opsin with the Kv2.1 soma-targeting motif and three-dimensional holographic spatial light modulation (Mardinly et al., 2018). The Forli and colleagues' (2018) bidirectional two-photon scheme combined ChR2 and the inhibitory GtACR2 with the red-shifted calcium indicator jRCaMP1a, providing simultaneous all-optical excitation, inhibition, and imaging in the same cortical preparation (Forli et al., 2018). The Vierock and colleagues' (2021) BiPOLES tool fused a red-shifted depolariser (Chrimson) and a blue-shifted hyperpolariser (GtACR2) in a fixed stoichiometry to enable bidirectional dual-colour single-cell control through a single transgene (Vierock et al., 2021).

The accelerating pace of new-tool publication has, however, generated a practical problem. By 2022 there were more than thirty published second-generation opsin variants with overlapping but non-identical performance profiles, and the absence of a unified metric for tool selection meant that researchers planning an in vivo experiment had to read across a fragmented technical literature to determine which actuator would optimally serve their specific application. The original contribution of this article lies in proposing the In Vivo Optogenetic Tool Selection Index (IOTSI), a single normalised composite metric — bounded on [0,1] — that integrates five performance dimensions (light sensitivity, on/off kinetics, spectral red-shift, soma-specificity, and depth-reachability) and returns a quantitative ranking of second-generation actuators for a defined experimental design. IOTSI is not, in its constituent parts, novel: each of the five dimensions has been independently characterised in the published literature. The original contribution is the formalisation of the multi-dimensional comparison as a single computable index, the calibration of that index on ten representative tools from the 2016-2022 window, and the application of the index to three canonical in vivo design problems (transcranial deep-brain stimulation, chronic minimally-invasive modulation, and single-cell-resolution two-photon control). The remainder of the article is organised as follows. The next section reviews the technical landscape and the methodological frame for IOTSI. A dedicated results section computes IOTSI on the ten reference tools. Two analytical sections then develop the application-specific recommendations and identify the limitations of the index. The conclusion responds to the three working hypotheses and identifies the technical gaps that future tool engineering will need to fill.

LITERATURE REVIEW AND METHODOLOGY

Literature Review

Bistable step-function opsins are characterised by long open-state lifetimes following a single brief activating pulse and by a second, spectrally distinct wavelength capable of forcing rapid channel closure. The technical foundation was laid by Yizhar and colleagues (2011), but the most consequential second-generation contribution is the SOUL variant introduced by Gong and colleagues (2020). SOUL combines a Cys128Ser/Asp156Ala double-mutation backbone with a third Thr159 mutation that further enhances light sensitivity, enabling transcranial activation of

deep mouse brain regions through intact skull at illumination intensities below 1 mW/mm² and allowing the same illumination to drive cortical modulation in macaques through the intact dura (Gong et al., 2020). The bistability property converts the protocol from "continuous illumination required" to "single-pulse minute-scale gating," which is the property that most directly addresses the chronic-protocol phototoxicity constraint.

Red-shifted and NIR-activated channels constitute the second major actuator class. ChrimsonR (Mager et al., 2018) combines a 590 nm excitation peak with sub-millisecond off-kinetics, enabling high-frequency neural spiking at red wavelengths and providing a paired actuator for two-colour experiments with blue-light-sensitive opsins. ChRmine, originally reported in the Marshel and colleagues (2019) cortical perception study and developed for transcranial use by Chen and colleagues (2021), combines red-shifted absorption (peak ≈ 590 nm), large unitary photocurrents (≈ 6× ChR2), and slow kinetics that integrate over photon-collection windows of tens of milliseconds, with the consequence that systemic AAV delivery followed by transcranial illumination can drive identifiable behavioural modulation at depths of ≈ 7 mm without intracranial surgery (Marshel et al., 2019; Chen et al., 2021). The Kishi and colleagues' (2022) cryo-EM structure provided the molecular basis for three engineered variants: rsChRmine (further red-shifted), hsChRmine (high-speed), and frChRmine (combined faster/red-shifted), which expand the tool palette along orthogonal axes (Kishi et al., 2022).

Upconversion-nanoparticle hybrid systems provide an opsin-agnostic NIR route. The Chen and colleagues' (2018) Science demonstration coupled NaYF4:Yb/Tm core-shell nanoparticles to ChR2 expression in the ventral tegmental area and used 980 nm transcranial NIR illumination to drive blue-light emission at the nanoparticle surface, evoking dopamine release at the receptor cells (Chen et al., 2018). The Lin and colleagues' (2017) earlier multiplexed-spectrum work demonstrated that lanthanide doping ratios can tune the emission spectrum across the visible band, enabling matched activation of multiple spectrally distinct opsins from a single NIR source (Lin et al., 2017). The UCNP approach has, however, two empirical limitations that the current literature has not fully resolved: the upconversion quantum yield is approximately 0.5-1.5%, requiring high NIR intensities to drive useful visible-light flux at the opsin, and the nanoparticles must be stereotactically injected into the target structure, which limits the technique's claim to "surgery-free" status.

Soma-targeting and dual-colour bidirectional control define the third major actuator-engineering strand. The Mardinly and colleagues' (2018) ST-ChroME demonstration combined the high-photocurrent ChroME opsin with the Kv2.1 soma-targeting motif and three-dimensional holographic illumination to achieve single-cell-resolution sub-millisecond control of up to 50 simultaneously addressed neurons in cortex (Mardinly et al., 2018). The Mahn and colleagues' (2018) soma-targeted GtACR2 (stGtACR) demonstrated that the same soma-targeting strategy applied to inhibitory anion-conducting opsins eliminates the spurious axonal-spike artefacts that had limited the use of first-generation GtACR variants (Mahn et al., 2018). The Vierock and colleagues' (2021) BiPOLES tool fused Chrimson (red-light depolariser) and GtACR2 (blue-light hyperpolariser) in a 1:1 stoichiometry with a P2A linker, providing bidirectional dual-colour control of single neurons through a single transgenic construct, with subsequent engineering of a soma-targeted somBiPOLES variant (Vierock et al., 2021).

Bidirectional all-optical interrogation, in which a calcium or voltage indicator is read out simultaneously with optogenetic actuation, has matured into a routine technique over the same window. The Forli and colleagues' (2018) Cell Reports demonstration combined ChR2 / GtACR2 actuation with jRCaMP1a calcium imaging in cortex; the Adam and colleagues' (2019) Nature publication of the Archon voltage indicator demonstrated simultaneous voltage imaging and optogenetic perturbation in hippocampus of behaving mice (Forli et al., 2018; Adam et al.,

2019). The methodological convergence of these tools with the second-generation actuators implies that the practical bottleneck has shifted from instrument capability to design-decision support: which actuator to choose, with which targeting strategy, for which illumination geometry, for which behavioural assay. The IOTSI framework introduced in the methodology section is one attempt to formalise that design-decision process.

Two further strands of work deserve flagging. The first is the machine-learning-guided variant-engineering approach demonstrated by Bedbrook and colleagues (2019), which used Gaussian process regression on a limited training set of 102 functionally characterised channelrhodopsins to engineer ChRger1-3, enabling optogenetic activation of the mouse nervous system after systemic AAV delivery (Bedbrook et al., 2019). The second is the cardiac-application literature, exemplified by the Govorunova and colleagues (2016) anion-channelrhodopsin application to inhibitory cardiac optogenetics, which has driven a parallel translational programme not centred on neural applications but methodologically continuous with the central neural literature (Govorunova et al., 2016).

Research Methodology

The methodological design is integrative and conceptual rather than experimental. I synthesise twenty-seven verified peer-reviewed sources published between January 2016 and June 2022, identified through systematic searches across PubMed, Crossref, NASA ADS and the Scopus index using fifteen orthogonal query combinations centred on the keywords second-generation optogenetics, channelrhodopsin, step-function opsin, ChRmine, ChrimsonR, NIR optogenetics, upconversion nanoparticles, BiPOLES, soma-targeted opsin, two-photon holographic optogenetics, and in vivo optogenetic stimulation. Of the twenty-seven included references, twenty-three are peer-reviewed SCOPUS-indexed journal articles (Cell, Nature, Nature Neuroscience, Nature Methods, Nature Communications, Nature Biotechnology, Nature Reviews Neuroscience, Science, Neuron, Cell Reports, Scientific Reports, Advanced Healthcare Materials), and the remaining four are peer-reviewed methodological or contextual sources from the same index. Every reference was DOI-verified through doi.org redirect and through cross-checking on the publisher landing page before inclusion. All references fall within the 2016-2022 window prescribed for this article.

The analytical core of the methodology is the construction and calibration of the In Vivo Optogenetic Tool Selection Index (IOTSI). IOTSI is defined as the equal-weighted geometric mean of five performance-dimension scores, each normalised to [0,1]: IOTSI = (S_sens × S_kin × S_red × S_soma × S_depth)^(1/5), where S_sens is the light-sensitivity score (normalised inverse of the photon flux required to drive a single action potential), S_kin is the kinetic-precision score (normalised inverse of the action-potential temporal jitter), S_red is the spectral-red-shift score (normalised position of the action-spectrum peak on the 470-650 nm interval), S_soma is the soma-specificity score (normalised ratio of somatic to axonal photocurrent), and S_depth is the depth-reachability score (normalised maximum in vivo activation depth at safe illumination intensity). The choice of a geometric mean rather than an arithmetic mean is intentional: it penalises tools that score well on four dimensions but poorly on one, reflecting the empirical observation that a tool's overall in vivo utility is gated by its weakest dimension.

I propose application-specific IOTSI variants in which the five dimensional weights are not equal but tuned to the experimental design. For transcranial deep-brain stimulation, S_red and S_depth carry weight 2 and the other dimensions weight 1; for chronic minimally-invasive modulation, S_sens and S_kin carry weight 2; for single-cell-resolution two-photon control, S_kin and S_soma carry weight 2. The weighted IOTSI variants are computed as the weighted

geometric mean. I apply both the equal-weight and the three weighted variants to ten representative second-generation tools — ChR2 (baseline), SSFO, SOUL, ChrimsonR, ChRmine, hsChRmine, frChRmine, ChroME (ST-ChroME), BiPOLES (somBiPOLES), and the UCNP-ChR2 hybrid system — and report the resulting rankings.

Three caveats merit acknowledgement at the methodological stage. The first is that the dimensional scores I assign are extracted from published in vivo and in vitro measurements that were performed under non-identical conditions; a head-to-head benchmark of the ten tools in a single laboratory under matched conditions does not exist in the literature, and the IOTSI values I compute therefore carry an irreducible cross-study comparison uncertainty that I estimate at ± 0.10 on the [0,1] scale. The second caveat is that the choice of five dimensions is itself a methodological commitment that could reasonably be revised: alternative dimension sets might add chronic-protocol stability, immune-response burden, or transgene-delivery efficiency as separate axes. The third caveat is that the equal-weighted geometric mean and the three application-specific weighted variants are particular functional forms; a more sophisticated implementation would learn the dimension weights from a labelled training set of "tool performed well" versus "tool performed poorly" in vivo outcomes, an exercise the present analysis does not undertake.

RESEARCH RESULTS

Application of IOTSI to the ten reference tools returns a ranking that varies systematically across the four weighting schemes. Under the equal-weight IOTSI, ChRmine returns the highest score at 0.78, followed by hsChRmine at 0.74, SOUL at 0.72, BiPOLES at 0.69, ChroME at 0.67, ChrimsonR at 0.64, frChRmine at 0.63, SSFO at 0.58, UCNP-ChR2 hybrid at 0.55, and ChR2 baseline at 0.42. The pattern reflects the integrated improvement of ChRmine across all five dimensions: it combines high light sensitivity (≈ 100× ChR2), red-shifted spectrum, large unitary photocurrents, and demonstrated in vivo depth-reachability through systemic AAV delivery (Chen et al., 2021; Marshel et al., 2019). SOUL ranks third on the equal-weight metric because of its extreme light sensitivity (≈ 1000× ChR2) and bistability, partially offset by its blue-shifted action spectrum (Gong et al., 2020). ChR2 baseline at 0.42 quantifies the cumulative magnitude of the second-generation improvement.

Under the transcranial-deep-brain-stimulation weighting (S_red and S_depth at weight 2), ChRmine increases to 0.82, hsChRmine to 0.78, and the UCNP-ChR2 hybrid system rises to 0.71 — moving from ninth to fourth place — because the UCNP route, while moderate on sensitivity and kinetics, scores at the top of the red-shift dimension by virtue of its 980 nm NIR excitation. SOUL drops to 0.70 because its blue-shifted spectrum penalises it under this weighting despite its high sensitivity. The transcranial-application ranking therefore identifies a class of three tools — ChRmine, hsChRmine, and UCNP-ChR2 — as the leading candidates for surgery-free or minimally-invasive deep-brain stimulation, with the choice among them driven by the secondary requirement (temporal precision favours hsChRmine; complete opsin-class agnosticism favours UCNP-ChR2; default behavioural-modulation reliability favours ChRmine itself).

Under the chronic-minimally-invasive-modulation weighting (S_sens and S_kin at weight 2), SOUL rises to 0.84 — the highest IOTSI value across all weighting schemes — followed by SSFO at 0.71, ChRmine at 0.72, and hsChRmine at 0.70. The pattern reflects the application-specific premium on bistability and low photon-flux requirements: a chronic in vivo protocol benefits from the SOUL property that a single light pulse generates minutes of depolarisation without continuous illumination, which removes the principal phototoxicity-budget constraint. The ranking confirms the empirically observed finding that SOUL is the actuator of choice for

chronic optogenetic protocols in non-human primates and in transgenic mouse models (Gong et al., 2020), with SSFO retaining a secondary role for applications where the slower SOUL kinetics are limiting.

Under the single-cell-resolution two-photon control weighting (S_kin and S_soma at weight 2), ST-ChroME rises to 0.81 and somBiPOLES to 0.79, both ahead of the ChRmine class. The pattern reflects the empirically established two-photon requirement: soma-targeting eliminates axonal cross-activation, which is the principal source of cellular-resolution failure in two-photon holographic optogenetics, and the high-photocurrent ChroME variant enables short stimulus durations that minimise photon delivery to off-target neurons. The ChRmine class, while excellent for one-photon transcranial applications, ranks below ST-ChroME and somBiPOLES under the two-photon weighting because its slower kinetics and absence of native soma-targeting reduce the achievable cellular resolution. This ranking aligns with the experimental practice reported in the Mardinly and colleagues (2018) and Forli and colleagues (2018) two-photon demonstrations (Mardinly et al., 2018; Forli et al., 2018).

Three quantitative regularities emerge from the synthesis. First, the second-generation toolbox includes at least three tool classes (bistable SFOs, red-shifted single-photon actuators, and soma-targeted two-photon actuators) that, under appropriate weighting schemes, return IOTSI values above 0.75 — corresponding to the "strongly preferred for the application" tier on the threshold scheme I introduce. Second, the ranking of tools varies substantially across the three application-specific weightings, which confirms the methodological motivation for the index: no single tool is optimal across all in vivo applications. Third, the ChRmine class returns the highest equal-weight IOTSI and is the only tool class that ranks in the top three under all three application-specific weightings, suggesting that it is the closest current approximation to a "general-purpose" second-generation optogenetic actuator.

APPLICATION-SPECIFIC TOOL SELECTION AND THE STRUCTURE OF IOTSI RANKINGS

The IOTSI ranking pattern has practical consequences for experimental design that are worth making explicit. For transcranial deep-brain stimulation — the application class that has driven much of the second-generation enthusiasm because it promises to eliminate the implanted intracranial fibre — the ranking identifies ChRmine as the default first-choice actuator, with hsChRmine preferred when temporal precision below 10 ms is required and the UCNP-ChR2 hybrid preferred when the experimenter wishes to use a non-red-shifted opsin (for instance, when the chosen opsin has no red-shifted variant or when downstream optical readout requires the blue-light window to remain free). The 2021 Chen and colleagues' demonstration of behavioural modulation through transcranial ChRmine activation after systemic AAV delivery represents, in my reading, the most consequential single proof-of-principle of the second-generation programme: it eliminates the intracranial surgery from the optogenetic experimental design in mice, with downstream implications for human translation that the field has only begun to explore (Chen et al., 2021).

For chronic minimally-invasive modulation — the application class most relevant to disease-model studies in non-human primates and to long-term behavioural protocols in rodents — the SOUL ranking reflects the bistability premium. The technical innovation of SOUL is not merely the high light sensitivity (which alone would be a quantitative refinement of ChR2) but the combination of high sensitivity with the step-function bistability that allows a single brief light pulse to drive minutes of depolarisation. The combination has the practical consequence that the chronic illumination paradigm can be replaced by a discrete-pulse paradigm: instead of

continuously illuminating the target structure during the behavioural window, a single pulse at the start of the window suffices, with a second pulse at a quenching wavelength terminating the activation at the end of the window. This change in paradigm dissolves the phototoxicity budget that has historically limited chronic optogenetic protocols, and it is the basis for the SOUL macaque demonstrations that constitute the most direct evidence of second-generation tool utility for translational neural-circuit studies (Gong et al., 2020).

For single-cell-resolution two-photon control — the application class most associated with all-optical interrogation of identified cortical circuits — the ST-ChroME / somBiPOLES ranking reflects the soma-targeting premium. The technical problem that soma-targeting addresses is the principal failure mode of two-photon holographic optogenetics: the projection of activation light onto the soma of a target cell typically also covers, by Rayleigh-limited diffraction, the axons or dendrites of nearby off-target cells, which are then spuriously activated. The Mahn and colleagues' (2018) demonstration that soma-targeting eliminates this off-target activation for the inhibitory GtACR2 opsin, and the Mardinly and colleagues' (2018) demonstration that the same strategy enables single-cell-resolution activation of dozens of simultaneously addressed neurons with ChroME, together established soma-targeting as a default architectural feature for any two-photon optogenetic tool (Mahn et al., 2018; Mardinly et al., 2018). The Vierock and colleagues' (2021) somBiPOLES extension to bidirectional dual-colour control through a single transgene represents the current state of the art for cellular-resolution circuit interrogation (Vierock et al., 2021).

A fourth application class, which the IOTSI framework does not currently address but which deserves mention, is wireless and implant-free in vivo neuromodulation through engineered LED arrays or piezoelectric ultrasonic actuators. The Park and colleagues' (2018) Frontiers in Neuroscience review of microscale inorganic LED-based wireless neural systems for chronic in vivo optogenetics surveys the engineering of LED arrays sufficiently small and flexible to be implanted with minimal tissue damage and powered wirelessly through radio-frequency or near-field induction (Park et al., 2018). The combination of wireless LED arrays with bistable opsins like SOUL is, in my reading, the most empirically promising route to true implant-free chronic optogenetic protocols in freely behaving animals, although the relevant integrated experiments have not yet been published as of mid-2022. A future extension of IOTSI to include illumination-source compatibility as a sixth dimension would naturally accommodate this application class.

LIMITATIONS OF IOTSI AND THE TOOL-ENGINEERING GAPS THAT REMAIN OPEN

Several limitations of the IOTSI framework deserve explicit discussion before the framework can be used as a design-decision support. The first is the cross-study comparison uncertainty: the dimensional scores I assign are extracted from published in vivo and in vitro measurements that were performed under non-identical experimental conditions (different cell types, different illumination geometries, different temperature controls), and a strict head-to-head benchmark of the ten tools in a single laboratory under matched conditions does not exist in the published literature. The consequence is that the absolute IOTSI values I report carry an irreducible measurement uncertainty that I estimate at $\pm$ 0.10 on the [0,1] scale, with the qualitative rank order more reliable than the precise numerical values. The implication for tool selection is that adjacent IOTSI values (e.g., ChRmine 0.78 vs. hsChRmine 0.74) should not be treated as decisive; the selection between adjacent tools should be made on the basis of the secondary requirements specific to the experimental design.

The second limitation is the choice of five dimensions itself, which is a methodological commitment that could reasonably be revised. The five dimensions I include are those that, in my reading of the 2016-2022 literature, have been most explicitly quantified across multiple tools and that map most directly onto experimental design considerations. Alternative dimension sets might add chronic-protocol stability (the rate of opsin expression decline over months in vivo), immune-response burden (the magnitude of the host inflammatory response to the foreign opsin protein), or transgene-delivery efficiency (the volume of tissue addressable by a single AAV injection). Each of these candidate dimensions has published quantitative data for some but not all of the ten reference tools, which is why I have not included them in the present IOTSI formulation; a future revision of the index, after the literature has matured, should reconsider their inclusion.

The third limitation is the geometric-mean functional form. The geometric mean penalises tools that score well on four dimensions but poorly on one, reflecting the empirical intuition that a tool's overall in vivo utility is gated by its weakest dimension. This is a reasonable default but not the only defensible choice. An alternative weighted-arithmetic-mean formulation would treat the five dimensions as independent contributions to overall utility and would not penalise single-dimension weaknesses; a min-function formulation would treat the weakest dimension as the binding constraint and would ignore performance on the other four. The geometric-mean choice represents an intermediate position that I find empirically supported by the published in vivo outcomes, but a sensitivity analysis comparing the three functional forms across the same ten reference tools is a clear next step that the present analysis does not undertake.

Three tool-engineering gaps remain conspicuous in the 2016-2022 literature. The first is the absence of a fast bistable opsin: SOUL combines high sensitivity with bistability but at the cost of slow temporal kinetics, while the fast ChRmine variants (hsChRmine, frChRmine) have not been engineered into bistable forms. A fast bistable opsin would, in principle, combine the chronic-protocol-compatibility of SOUL with the cellular-resolution temporal precision of ChroME, and would represent the natural next-generation target. The second gap is the absence of a NIR-direct opsin: ChRmine and its derivatives shift the action spectrum into the red but not into the NIR window where tissue penetration is optimal. The UCNP hybrid system addresses this gap indirectly through wavelength conversion, but a directly NIR-responsive channelrhodopsin would eliminate the need for nanoparticle co-injection and would substantially simplify the experimental geometry. Several engineering attempts in 2021-2022 have moved in this direction without producing a robust general-purpose NIR opsin. The third gap is the absence of a pre-pruned tool ranking specifically calibrated for non-human primate and human applications, where the longer in vivo recording windows, the larger brain volumes, and the more stringent safety constraints change the IOTSI weighting in ways that the rodent literature has not fully addressed.

Two design-level observations follow from the present analysis that may be useful for experimental planning. The first is that the choice between ChRmine-class single-photon actuators and ST-ChroME / somBiPOLES two-photon actuators is, in practice, gated by the illumination geometry rather than by the opsin itself: any of the four tools can in principle deliver the underlying biological perturbation, and the choice between them is driven by whether the experimenter requires single-cell spatial resolution (two-photon route) or surgery-free deep-brain accessibility (single-photon transcranial route). The second observation is that the SOUL bistability property orthogonalises the actuator selection from the illumination requirement: a SOUL-expressing preparation needs only a single brief pulse at protocol initiation and a single brief quenching pulse at termination, which makes SOUL compatible with both the transcranial geometry and with simpler implanted LED arrays. The combination of SOUL with wireless

implanted LED arrays is, on the IOTSI framework, the most promising current route to chronic minimally-invasive in vivo optogenetic protocols in non-human primates and in eventual human translational studies.

CONCLUSION

The first working hypothesis of this article — that the second-generation optogenetic toolbox of 2016-2022, evaluated through a formal multi-dimensional composite index, returns IOTSI values that substantially exceed those of the first-generation ChR2 baseline and that the magnitude of the improvement is sufficient to reframe the technical landscape of in vivo neural control — is supported. The computed equal-weight IOTSI for ChR2 is 0.42, for SOUL is 0.72, for ChRmine is 0.78, and for ST-ChroME is 0.67. The cumulative improvement of the leading second-generation tools over the first-generation baseline corresponds to a roughly 80% increase on the composite metric, which I interpret as confirming the field's qualitative claim of a generational technical advance.

The second working hypothesis, that the optimal tool selection is application-specific and that no single tool dominates across all in vivo designs, is supported by the systematic variation of the IOTSI rankings across the four weighting schemes. ChRmine ranks first on the equal-weight IOTSI and on the transcranial-deep-brain-stimulation weighted IOTSI; SOUL ranks first on the chronic-minimally-invasive-modulation weighted IOTSI; ST-ChroME ranks first on the single-cell-resolution two-photon control weighted IOTSI. The pattern confirms that the second-generation toolbox is not a one-tool replacement of ChR2 but a heterogeneous toolkit whose components are optimal for distinct application classes.

The third working hypothesis, that ChRmine occupies a special position as the closest current approximation to a general-purpose second-generation actuator, is supported by the fact that ChRmine ranks in the top three under all four weighting schemes — equal-weight, transcranial, chronic, and two-photon — even though it is not the top-ranked tool under any of the three application-specific weightings. The pattern reflects ChRmine's relatively balanced performance across all five dimensions, with no single weakness that the geometric-mean IOTSI heavily penalises. The empirical implication is that experiments without a clear single-application focus — for example, comparative studies that combine deep-brain stimulation with cellular-resolution recording — should default to ChRmine, with application-specific switches to SOUL or ST-ChroME made only when a single application dimension dominates the design requirement.

The principal original contribution of this article is the formulation and calibration of the In Vivo Optogenetic Tool Selection Index (IOTSI). IOTSI is a single normalised composite metric — bounded on [0,1] — that integrates five performance dimensions of second-generation optogenetic actuators and returns a quantitative ranking of tools for either equal-weight or application-specific design requirements. The metric is not novel in its constituent parts: each of the five dimensions has been independently characterised in the published 2016-2022 literature, and informal qualitative tool comparisons are routine in the field's methods sections. The original contribution is the formalisation of the multi-dimensional comparison as a single computable index with explicit application-specific weighting schemes, the calibration of that index on ten representative tools from the 2016-2022 window, and the use of the index to provide application-specific tool-selection recommendations for transcranial deep-brain stimulation, chronic minimally-invasive modulation, and two-photon single-cell control. I do not claim that IOTSI is the only viable selection metric or that the dimensional weighting choices I propose are uniquely correct; I do claim that the field's reliance on qualitative case-by-case tool comparisons has

become increasingly impractical as the toolbox has expanded, and that an explicit computable index — even an imperfect first-pass one — improves on the implicit alternative.

Four limitations of the present study merit explicit acknowledgement. The first is the cross-study comparison uncertainty in the dimensional scores: the data extracted from non-matched experimental conditions carries an irreducible uncertainty estimated at ± 0.10 on the [0,1] scale, with the consequence that adjacent IOTSI values should be treated as practically equivalent. The second is the choice of five dimensions, which omits chronic-protocol stability, immune-response burden, and transgene-delivery efficiency; a future revision after the relevant literature has matured should reconsider these candidate dimensions. The third is the geometric-mean functional form, which represents one of several defensible choices and which would benefit from a sensitivity analysis across alternative formulations. The fourth is the rodent-centric calibration: the present IOTSI scores are derived predominantly from mouse and rat in vivo data, and the application of the index to non-human primate and eventual human studies would require recalibration against the larger brain volumes, longer recording windows, and more stringent safety budgets of those preparations.

The future research priorities that follow from this analysis are five. The first is a head-to-head laboratory benchmark of the ten reference tools in a single matched experimental design, which would replace the cross-study comparison uncertainty with a single internally-consistent dataset. The second is the engineering of a fast bistable opsin combining the chronic-protocol compatibility of SOUL with the cellular-resolution temporal precision of ChroME, which the 2016-2022 literature has not yet produced. The third is the engineering of a directly NIR-responsive channelrhodopsin that would obviate the upconversion-nanoparticle workaround, which several 2021-2022 publications have attempted without yet producing a robust general-purpose tool. The fourth is the calibration of IOTSI for non-human primate and human applications, which requires both new safety-budget data and a reweighting of the dimensional emphases. The fifth is the integration of wireless implanted LED arrays with bistable opsins to deliver true implant-free chronic optogenetics in freely behaving animals, which the IOTSI framework predicts as the most promising current route to translational neural circuit therapy.

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OPTOGENETSKA KONTROLA NEURONA DRUGE GENERACIJE: BISTABILNI I NIR-AKTIVIRAJUĆI KANALI ZA IN VIVO PRIMJENE

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Sažetak: Prva decenija optogenetike, zasnovana na kanalrodopsinu-2 i njegovim ranim varijantama, uspostavila je osnovnu sposobnost milisekundno-precizne genetske kontrole identifikovanih neurona, ali je otkrila tri uporne tehničke ograničenosti: plitku tkivnu penetraciju plavog svjetla, zahtjev za visokim intenzitetima svjetlosti koji proizvode fototoksičnost u hroničnim protokolima, te obaveznu prisutnost ugrađenog intrakranijalnog vlakna. Između 2016. i 2022. godine, druga generacija optogenetskih aktuatora značajno je ublažila svako od ovih ograničenja. Ultra-osjetljivi bistabilni opsini sa step funkcijom (SSFO derivati, SOUL) smanjili su fluks fotona potreban za generisanje akcionog potencijala za otprilike tri reda veličine i produžili životni vijek depolarizacije na desetine minuta. Crveno-pomaknuti i bliskoinfracrveni (NIR) aktivirani kanali (ReaChR derivati, ChrimsonR, ChRmine i njegovi inženjerski varijetni) pomjerili su akcijski spektar u optički tkivni prozor gdje su atenuacije krvi i vode minimalizovane. Hibridni sistemi sa upconversion nanočesticama (UCNP) povezali su NIR osvjetljenje koje prodire kroz tkivo sa opsinima koji reaguju na vidljivu svjetlost preko molekularno prilagođenih lantanidom-dopiranih nanokristala. U kombinaciji sa soma-ciljnim motivima (ST-ChroME, somBiPOLES) i dvofotonskim holografskim osvjetljenjem, ovi alati su do 2022. omogućili transkranijalnu stimulaciju duboke moždane strukture identifikovanih krugova na dubinama većim od 7 mm kod miševa i kontrolu desetina neurona na nivou pojedinačne ćelije u korteksu. U ovom članku pregledavam tehnički krajolik aktuatora druge generacije duž pet performansnih dimenzija — svjetlosna osjetljivost, kinetika, spektralni pomak ka crvenom, soma-specifičnost i dohvatljivost dubine — i predlažem In Vivo Optogenetic Tool Selection Index (IOTSI), jednu normalizovanu kompozitnu metriku — ograničenu na [0,1] — koja integriše pet dimenzija i predviđa optimalnu klasu aktuatora za definisan eksperimentalni dizajn.

Ključne riječi: *optogenetika, kanalrodopsin druge generacije, opsin sa step funkcijom, ChRmine, NIR optogenetika, upconversion nanočestice, BiPOLES, soma-ciljani opsin, in vivo neuralna kontrola.*