INTRODUCTION

For optogenetic manipulation of membrane potentials neurons express opsins (light-sensing channels or pumps); applications of the appropriate wavelength of light then activates the opsin. Depending on the biophysical properties of the opsin, activation can result in an increase or decrease of membrane potential. Exchanging the physical light source (laser, arc lamp, or light-emitting diode) (Gradinaru et al., 2010; Kim et al., 2013) for a biological one (a luciferase emitting bioluminescence of the matching wavelength) expands optogenetics to non-invasive and circuit-wide applications. For such Bioluminescence-driven Optogenetics (BL-OG) the luciferase is fused to the opsin (luminopsin, LMO); application of the luciferin

Significance

Bioluminescent Optogenetics, BL-OG, utilizes luciferases, luciferins, and opsins for neuronal manipulation. It is important to be aware of specific versus potential non-specific, off-target effects of individual components. Thus, we tested the effects of BL-OG components systematically in neuronal populations grown on multi electrode arrays (MEAs). We define experimental parameters for achieving specific BL-OG effects and provide protocols for utilizing MEAs for efficient testing of neuronal actuators.
(the luciferase substrate) leads to an enzymatic reaction with emission of photons that in turn activate the opsin (Berglund, Birkner, Augustine, & Hochgeschwender, 2013; Berglund, Clissold, et al., 2016; Berglund, Tung et al., 2016; Park et al., 2017; Tung, Gutekunst, & Gross, 2015).

In principle, any luciferase emitting sufficient light of the matching wavelength can be combined with an optogenetic element. Our LMOs fuse variants of *Gaussia* luciferase (GLuc, sGLuc, sbGLuc, GLucM23) to activating or silencing opsins (for example: ChR2, VChR1, Mac, iChloC, hGtARC2). The specific substrate for *Gaussia* luciferase is coelenterazine (CTZ). CTZ is the molecule for light energy storage in over 75% of luminous marine organisms (Haddock, Moline, & Case, 2010; Markova & Vysotski, 2015) and is the most abundant naturally occurring luciferin. It is the substrate for many common luciferases and photoproteins, including *Renilla reniformis* luciferase (RLuc) (Jeon et al., 2010; Zhao et al., 2004) and aequorin (Lark, Kitamoto, & Martin, 2016). The bioluminescence reaction involving CTZ follows the conventional scheme: The luciferase catalyzes the oxidation of the CTZ molecule by O$_2$, resulting in formation of oxyluciferin (coelenteramide) and CO$_2$, as by-products (Kaskova, Tsarkova, & Yampolsky, 2016); no other co-factors are required. A diverse range of in vitro analytical techniques are based on bioluminescence, such as immunological and gene expression assays. Bioluminescence can also be found in applications ranging from investigations of infectious disease to environmental studies (Badr & Tannous, 2011; Roda, Pasini, Mirasoli, Michellini, & Guardiglio, 2004). In vivo rodent cancer studies have extensively used CTZ for live bioluminescence imaging to monitor tumor growth or shrinkage. For all these applications, no toxic or adverse side effects have been reported. However, in none of these applications has CTZ been used to control neural activity.

In the presence of CTZ BL-OG effects are generally as expected (i.e., neurons are activated or silenced), regardless of whether the LMO-expressing neurons are in culture, in slices, or in behaving animals. However, different CTZ solvents, concentrations, and varying types of controls (vehicle, saline, or CTZ with non-LMO-expressing cells) are being used. As BL-OG utilizes combinations of luciferins, luciferases, and opsins in novel ways that have not been tested in the context of neuronal activation, we wanted to systematically explore specific and potential non-specific effects of BL-OG components.

Testing genetically encoded neuronal actuators, including LMOs, in vitro is usually carried out by patch clamp studies in primary neurons (Berglund, Clissold, et al., 2016; Berglund et al., 2013; Park et al., 2017; Tung et al., 2015), requiring extensive equipment and expertise. While there are membrane potential measurements that can only be carried out in single cells, assessment of neuronal populations can be highly informative as well. Multi-electrode arrays (MEAs) allow recording of the electrical activity of neuronal networks grown on multiple extracellular electrodes (Berendonni et al., 2009; Chiappalone, Bove, Vato, Tedesco, & Martinoa, 2006; Illes, Theiss, Hartung, Siebler, & Dihné, 2009; Novellino et al., 2011; Otto, Go, Fleischer, & Siebler, 2003; Quasthoff et al., 2015; Schock et al., 2012). The advantages of this technology are its relative simplicity, compactness and ease of monitoring, as well as its potential for high throughput (Hales, Rolston, & Potter, 2010; Pine, 1980; Spira & Hai, 2013). Thus, this study also serves as an example of the benefits of MEA experiments for initial screening of LMOs, as well as other neuronal manipulation tools, before using them in ex-vivo or in-vivo experiments.

### 2 MATERIALS AND METHODS

#### 2.1 Coelenterazine (CTZ) and solvents

The luciferase substrate, coelenterazine (CTZ), was purchased from Nanolight Technology (Pinetop, AZ): Coelenterazine free base, the natural form of CTZ (Nanolight cat. no. 303); water soluble CTZ (Nanolight cat. no. 3031); CTZ Inject-A-Lume (Nanolight cat. no. 3031NJ). The following solvents were used: NanoFuel (Nanolight cat. no. 399); acetylated ethanol (0.06N HCl in ETOH); methanol (Fisher); 2-Hydroxypropyl-β-cyclodextrine (Sigma H5704, 20 mM in PBS/2% acetylated ETOH); water (buffer only without CTZ, Nanolight cat. no. 3031C); Fuel-Inject (Nanolight cat. no. 3031NJ).

#### 2.2 Neuron culture on MEAs

Primary neurons were collected from day 18 rat embryo cortex of both sexes obtained from pregnant Sprague Dawley females (RRID:RGD_5508397) from Harlan or BrainBits, LLC. Cortical neurons (4 × 10⁴ cells/5 µl/well) were plated on the electrode area of 6-well or 1-well MEA dishes (60-6wellMEA200/30iR-Ti; 60MEA200/30iR-Ti; Multi Channel Systems, Germany) coated with PEI (0.1%) and laminin (50 µg/ml) in culture medium consisting of Neurobasal Medium (Invitrogen), B-27 supplement (Invitrogen), 2 mM Glutamax (Invitrogen) and 5% Fetal Calf Serum (FCS). The following day, the medium was replaced with culture medium without serum (NB-Plain medium). The neurons were left either un-transduced or transduced with adeno-associated virus (AAV2, serotype 9) on days in vitro 1 (DIV 1). The medium was replaced with NB-Plain medium every 3–4 days thereafter. All-trans retinal (R2500; Sigma-Aldrich, St. Louis, MO) was added to the culture medium to 1 µM final concentration before electrophysiological recordings. Neurons were used for recordings on DIV 12–19.

#### 2.3 Nucleofection

Nucleofection of E18 primary rat cortical neurons was carried out per manufacturer’s instructions (Amaxa® Rat Neuron Nucleofector® Kit; LONZA) (Zeitelhofer, Vessey, Thomas, Kiebler, & Dahm, 2009; Zeitelhofer et al., 2007). Briefly, 1 - 5 × 10⁵ primary neurons were collected and resuspended in 100 µl of Nucleofector® Solution at room temperature. The cell suspension was combined with 1–3 µg plasmid DNA and transferred to the nucleofection cuvette. The Nucleofector™ 2b Device (LONZA) was used for nucleofection with Nucleofector® Program “G-013”. Cells post-nucleofection were counted and plated in appropriate numbers in 1 well MEA dishes. The following day, the medium was
replaced with NB-Plain medium. Expression from nucleofected plasmids was evident as early as 12 hr post-nucleofection. The cells were maintained and used for electrophysiological recordings as described below.

2.4 | MEA recordings

Consistently spiking neurons were used for recordings between DIV 12–19; only wells showing spontaneous electrophysiological activity were used. The stock solutions of all solvents were pre-diluted in NB-Plain medium to a final dilution of 1:50, achieved by adding 5 µl to 250 µl of MEA culture for 6-well MEAs, or by adding 10 µl to 500 µl of MEA culture for 1-well MEAs. Prior to recording, all reagents were pre-warmed to 37°C. MEAs were transferred from the CO₂ incubator to the heated MEA2100 head stage maintained at 37°C, and the cultures were allowed to equilibrate for 5–10 min. A micropipette was used to add reagents with the reagent drop gently touching the liquid surface. Recordings were carried out with a sample rate of 10,000 Hz. After recording, the media in the wells was replaced with fresh NB-Plain media, and cultures were used for another round of recording the next day. MC Rack software was used for data acquisition. All MEA analysis was done offline with MC Rack software (MultiChannel Systems; RRID:SCR_014955) and NeuroExplorer (RRID:SCR_001818). Spikes were counted when the extracellular recorded signal exceeded 5 standard deviations of the baseline noise. For all 6-well MEA recordings, 200 s intervals before (pre) and after (post) addition of test solution were assessed for spike counts. For the 1-well MEA recordings, 100 s intervals before (pre) and after (post) addition of test solution and 10 s before and during blue light exposure were assessed for spike counts. For assessing the effects of CTZ on neurons expressing activating or inhibiting LMOs, only electrodes displaying the expected change in spiking activity with LED light were evaluated. Pooled data was obtained from different electrodes (a) of the same culture, (b) from different cultures, and (c) over different DIVs.

2.5 | IVIS imaging

For measuring luminescence from neurons cultured on MEAs, reconstituted and diluted CTZ was added to the culture medium immediately before imaging to the final concentration indicated in the experiment. Luminescence was measured using an IVIS Lumina LT In Vivo Imaging System (PerkinElmer, Waltham, MA) with Living Image 4.5.2 software (RRID:SCR_014247). Images were displayed as a pseudo-color photon counted image. Regions of interest were defined using an automatic intensity contour procedure to identify bioluminescent signals with intensities significantly greater than background. The sum of the photon counts in these regions was then calculated.

2.6 | Statistics

Data are presented as mean ± SEM. All statistical tests were performed in SPSS (IBM). Neural cell cultures were monitored for variation in electrophysiological responses to solvent control across all cultures. This variation in spiking from solvent application was measured as ±20%. Conditions where changes in spikes fell within the 20% range from pre to post treatment were considered not different, and were not analyzed for statistical differences. Comparisons of spiking in neural cell cultures were done using a paired t-test to compare spikes before and after treatments. \( p < 0.05 \) was considered to be statistically significant.

3 | RESULTS

3.1 | MEA for assessment of BL-OG effects in neuronal populations

We used MEAs for assessment of specific effects, i.e., bioluminescence-induced activation of opsins, and potential non-specific off-target effects of BL-OG (Figure 1). We cultured embryonic rat cortical neurons on 1-well (64 electrodes) or 6-well (6 × 9 electrodes) MEAs. Neurons were transformed to express constructs either by transduction with AAV2/9 vectors a day after plating (Figure 1a,b) or by nucleofection of neurons just before plating (Figure 1c). Either mode of transformation yielded cultures with over 80% of neurons expressing the constructs. Cultures with healthy neurons spontaneously spiking over most electrodes were best achieved from freshly dissociated neuron preparations. Electrical recordings were combined with simultaneous stimulation of neurons either by exposing the culture to LED light or by adding a small amount of liquid containing the luciferase substrate coelenterazine (CTZ; Figure 1d–f). We found that adding the substrate as a 5 µl drop that dispersed into the culture medium through surface tension on the top of the culture surface (Figure 1e) mostly avoided electrical artifacts and resulted in reproducible emission of light from neurons growing on top of the electrode array (Figure 1f). Recordings were carried out at 37°C and the reagents were pre-incubated at 37°C before addition to the MEA cultures to avoid possible temperature effects. Recordings were performed outside the humidified incubator, and thus were limited to 10–15 min to avoid major pH variations. We placed MEAs in an IVIS imager to determine light emission from neurons using different concentrations of CTZ (Figure 1g). Analysis of photon emission over regions of interest across different MEAs showed CTZ concentration dependent radiance (Figure 1h). Our MEA cultures showed a robust spontaneous baseline activity (Figure 1i) that could be reliably changed in cultures expressing activating or inhibiting LMOs following stimulation with physical (Figure 1j,k) or biological (Figure 1l,m) light.

3.2 | Effects of solvents ± CTZ

CTZ is chemically synthesized and soluble in organic solvents. Recently, a water-soluble version of CTZ became available. In addition, proprietary solvents for specific in vitro and in vivo applications exist, such as NanoFuel and Inject-A-Lume respectively. To test for possible luciferase-independent, non-specific effects of some of
the most commonly used CTZ preparations, we first recorded from un-transduced primary rat cortical neurons using MEAs. Cultures were treated with six different solvents alone (vehicle controls) or with CTZ dissolved in the respective solvents, in both cases at a final concentration of 100 µM (see Table 1). Solvents were assigned randomly to MEAs initially, with subsequent recordings arranged so that the same cultures were not exposed to the same treatments repeatedly. Thus, any solvent was tested in multiple independent cultures, and any culture was treated with multiple different solvents. Recordings of electrical activity were continuous and spiking activity before and after addition of solutions was compared (Figure 2); only data collected from spontaneously spiking neurons were used. Figure 2a shows the effects on fold change of spiking activity for each of the six solvents, without CTZ. Four of these solvents caused reductions in spiking; acidified ethanol and Inject-A-Lume caused significant reductions in spiking activity (Paired t-test: Acidified Ethanol—t(51) = 5.51, p = 1.173 × 10⁻⁶, N = 52; Inject-A-Lume—t(106) = 12.14, p = 8.904 × 10⁻²², N = 107). Only Cyclodextrin produced a clear increase in spiking (Paired t-test: t(123) = −3.7333, p = 0.0003, N = 124). Figure 2b shows real time screenshots of representative recordings for the six conditions graphed in Figure 2a. We then recorded from a different set of un-transduced neurons, this time with CTZ present in the respective solvents (Figure 2c,d). Figure 2c displays the effects on fold change of spiking activity of 100 µM CTZ dissolved in the six different formulations and diluted as for Figure 2a; Figure 2d shows examples of the corresponding recordings. CTZ present in the various solvents shows a qualitatively similar pattern of effects on spiking activity as the respective solvents alone. For example, acidified ethanol, Inject-A-Lume, and NanoFuel each resulted in a similar reduction in spiking with and without CTZ, each showing non-significant differences from the effect of solvent alone. Addition of CTZ did, however, significantly reduce the effect of cyclodextrin (Student’s t-test: t(164) = 3.01, p = 0.003, N = 166). The solvents with the least non-specific effects in either condition (without or with CTZ), were NanoFuel and the water soluble formulations. Overall, it appears that in un-transduced neurons the solvents by themselves, at least when applied at the concentrations shown, have an unwanted impact on the spiking activity of neurons.

### 3.3 Effects of solvent concentrations ± CTZ

For two of the solvent formulations, NanoFuel and water soluble, we recorded the effect on spiking activity over a range of concentrations in un-transduced neurons. The solvent stocks without and with CTZ (see Table 1) were diluted in NB-Plain media to achieve final concentrations of CTZ between 50 and 1 µM, resulting in additional dilution of the solvents from two-fold to a hundred-fold relative to the concentrations used in Figure 1. As can be seen in Figure 3, NanoFuel produced a dose dependent effect (Two-way ANOVA Dose by CTZ, main effect for Dose: F(5, 584) = 10.05, p = 3.05 × 10⁻⁷, N = 596), as well as a main effect for CTZ (F(5, 584) = 6.61, p = 5.397 × 10⁻⁶, N = 596). This dose effect was differential between solvent and CTZ conditions (Interaction of CTZ with
dose $F(5, 584) = 2.90, p = 0.0136, N = 596)$. Post-hoc tests indicate the change in spiking is significantly greater at 50 µM concentration compared to all other concentrations when CTZ is present (Tukey’s post-hoc: $p = 0.05$). Similarly, a 1 µM concentration produced a minimal change in spiking, significantly lower than concentrations of 10 µM and above (Tukey’s post-hoc: $p = 0.05$). No significant differences were found between concentrations when no CTZ was present. The change in spiking was significantly

### Table 1: Dilutions of solvents with or without CTZ

<table>
<thead>
<tr>
<th>Solvent</th>
<th>CTZ concentration in solvent (mM)</th>
<th>Solvent dilution in NB-plain (without or with CTZ)</th>
<th>Final dilution in MEA culture</th>
<th>Final CTZ concentrations (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidified ethanol, Inject-A-Lume, Cyclodextrine, Methanol, Water soluble formulation</td>
<td>12</td>
<td>1:2.4</td>
<td>1:50</td>
<td>100</td>
</tr>
<tr>
<td>NanoFuel</td>
<td>50</td>
<td>1:10</td>
<td>1:50</td>
<td>100</td>
</tr>
</tbody>
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**Figure 2** Effects of solvents, alone or with CTZ, on untransduced primary neurons. (a, c) The effects on spiking activity of six different solvents are displayed as percent change in firing rate before and after addition of medium containing the solvent alone (a, open bars), or containing the same amount of solvent with CTZ for a final concentration of 100 µM (c, filled bars). $N > 50$ electrodes for each bar (for details see text). (b, d) Representative recording traces for the six solvents alone (b) and with CTZ (d), showing the respective time windows for the analysis of change in firing rate. pre-addition: orange window; post addition: green window
greater in the presence of CTZ at 10 and 50 µM compared to the solvent alone (Tukey’s Post-hoc: \( p = 0.05 \)). By contrast, no significant differences were found between doses of water soluble CTZ.

3.4 | Effects of CTZ on neurons expressing luciferase, opsin, or luminopsin

Using CTZ dissolved in NanoFuel at concentrations of 25 µM or lower provides strong bioluminescence as measured in an IVIS system without causing non-specific effects on neuronal spiking activity in un-transduced neurons as measured in MEAs. We next wanted to explore whether adding CTZ or vehicle has any effects on the activity of neurons expressing only an optogenetic element (without a luciferase), or expressing only a luciferase (without an opsin). Neurons expressing the activating opsin VChR1, or the inhibiting opsin hGtARC2 (Govorunova, Sineshchekov, Janz, Liu, & Spudich, 2015), or sbGluc anchored to the cell membrane by fusion to the transmembrane sequence from the human CD4-2 sequence (Kim et al., 2011) were cultured on MEAs. As each construct carries a fluorescent reporter at its C-terminal, expression for each culture was verified by fluorescence microscopy. We first recorded from the opsin-expressing neurons using LED stimulation as a control for the expected function of the opsins. There were several experiments where for some of the recording electrodes we observed an increase of spiking activity while recording from inhibitory opsin-expressing neurons, and a decrease while recording from excitatory opsin-expressing neurons, the opposite of what we expected to see. Dissociated cortical neurons re-form neuronal networks by the time we carry out recordings (2–3 weeks after plating), and these networks are comprised of both GABAergic and glutamatergic populations. Thus, when illuminating the entire culture with light, we are likely seeing a mixture of direct activation as well as synaptic inhibition of excitatory opsin-expressing neurons, and direct silencing as well as synaptic dis-inhibition of inhibitory opsin-expressing neurons. This will make it potentially difficult to tease apart specific from non-specific effects when using CTZ, so we pretreated cultures with kynurenic acid (1 mM), a glutamate receptor antagonist (Bekkers & Stevens, 1991). Under these conditions, changes in spiking activity occurred as expected under blue light stimulation, with increases in firing rate for VChR1 expressing neurons (Figure 4a; \( t(15) = -4.16, p = 0.0008, N = 16 \)), and decreases in firing rate for hGtARC2 expressing neurons (Figure 4c; paired t-test: \( t(15) = 8.22, p = 6.155 \times 10^{-7}, N = 16 \)). In contrast, addition of CTZ, or its vehicle, to these cultures resulted in responses below the solvent variation (Figure 4a,c). Thus, CTZ at this concentration does not seem to have a direct effect on neurons themselves, nor an indirect effect on neuronal activity through opsins.

Next, we asked if the emission of biological light itself alters spiking activity in neurons. MEA cultures with neurons expressing a membrane-tethered sbGluc, sbGluc-CD4, were treated as described above. Here the stimulation by LED light served as a negative control, as these neurons did not express opsins. In this experiment, neither blue light nor CTZ produced a response beyond the solvent threshold (Figure 4b).

The above experiments demonstrate that CTZ at a working concentration of 25 µM or lower does not have a significant off-target effect in neurons cultured in MEAs. Utilizing the experimental design as described in the previous section, we exposed neurons of the same culture to blue LED light, to CTZ, and to vehicle, with neurons expressing the activating luminopsin LMO3 (sbGluc-VChR1-EYFP; Figure 4d) or the inhibiting luminopsin sbGluc-hGtARC2-EYFP (Figure 4e). Exposure to LED as well as addition of CTZ to generate biological light resulted in the expected increase and decrease of spiking activity, respectively, while addition of vehicle did not
change spiking activity significantly (Figure 4d,e). As expected, both blue light and CTZ produced significant increases of spiking in neurons expressing LMO3 (Blue light: t(50) = -11.35, p = 1.914 × 10^{-15}, N = 51; CTZ: t(50) = -11.79, p = 4.748 × 10^{-16}, N = 51).

Similarly, blue light and CTZ significantly reduced the spiking activity in neurons expressing sbGLuc-hGtARC2 (Blue light: t(106) = 19.24, p = 1.149 × 10^{-36}, N = 107; CTZ: t(106) = 19.08, p = 2.287 × 10^{-36}, N = 107). Taken together, these experiments show significant effects on spiking activity of CTZ in neurons expressing luciferase-opsin fusion proteins, but not of the CTZ vehicle.

4 | DISCUSSION

Bioluminescence driven optogenetics requires oxidation of a luciferin by a luciferase, thereby delivering light to opsins for modulating
the activity of neurons expressing luciferase–opsin fusions, LMOs. As a control for the specificity of the effect on neuronal spiking, changes in neuronal activity in the presence of the luciferin are compared to those with vehicle alone. As CTZ, the luciferin for most marine luciferases, can be dissolved in various organic compounds, we wanted to compare directly effects of different CTZ preparations. Furthermore, while the intended effect is a change in membrane potential of LMO expressing neurons, we wanted to test if there are any off-target effects of CTZ or its respective solvents on neurons directly, or on the opsins itself. Lastly, we evaluated if there are effects on neuronal activity through bioluminescent light emission itself.

Using a concentration often applied in in vivo bioluminescence imaging experiments six solvent formulations both with and without CTZ showed significant effects on neuronal spiking activity in untransduced primary neurons. We can speculate as to the mechanisms that are causing the observed effects for some of the solvents. Methanol, for example, is known to cause retinal and optic nerve injury both in vivo (Eells et al., 2003) and in vitro (Treichel, Henry, Skumatz, Eells, & Burke, 2003). Methanol is sequentially metabolized to form formic acid, formaldehyde, and carbon dioxide by oxidative steps. Formate, the putative toxic metabolite of methanol inhibited cytochrome oxidase activity, a component of the electron transport chain involved in ATP synthesis, at higher concentrations (Wallace, Madeira, Cortopassi, & Jones, 1997) and showed neurotoxic effects on dissociated primary mouse neuronal cell cultures (Dorman, Bolon, & Morgan, 1993). Also, there are reports about the receptor mediated effects of ethanol in modulating the activity of primary rat cortical neurons at mM concentrations (Moriguchi, Zhao, Marszalec, Yeh, & Narahashi, 2007). The neuronal nicotinic acetylcholine (ACh) receptors (nAChRs) are present on the soma, and presynaptic regions of the GABAergic and other interneurons in the cortex and hippocampus, and their activation has been shown to enhance synaptic release of GABA, glutamate, and other transmitters (Alkondon, Pereira, Eisenberg, & Albuquerque, 2000). These receptors have been increasingly recognized as being sensitive to ethanol treatments, again at mM concentrations (Alstrup, Marszalec, & Narahashi, 1999).

Although we used these solvents well below mM concentrations, our cultures were not perfused and thus the reagents were present at a constant concentration over time. While ethanol and methanol non-specifically hyperpolarized neurons, cyclodextrin non-specifically increased the firing rate of neurons on average. Cyclodextrins are cyclic oligosaccharides composed of a hydrophilic outer surface and lipophilic central cavity and their major pharmacological use is to improve solubility, bioavailability and physical stability of the active drug components by formation of inclusion complexes (Szejtli, 1994). The cavity diameter (I.D. 7.5 Å) of β-cyclodextrin is appropriate for use with many compounds frequently used in tissue and cell culture applications. For this reason, β-cyclodextrin is most commonly used as a complexing agent. Some cyclodextrins extract cholesterol from cell membranes thereby affecting the function of receptors localized in cholesterol-rich membrane domains. Neuroprotective activity of some of the β- forms of cyclodextrin used in our experiments, such as β-methyalted and β-sulfated cyclodextrins, has been reported for cortical neuronal cultures (Abulrob et al., 2005). Nanofuel, water-soluble vehicle, and Inject-A-Lume are proprietary compositions. Whether the observed electrophysiological artifacts of these as well as the above discussed solvents are caused by binding to a common or to different receptors on neurons, or by simply off-setting the ionic balance in the medium, would be conjecture. When we used lower concentrations for two of the CTZ solvents, Nanofuel and water-soluble solvent, their non-specific effects were reduced to acceptable levels, and this might also be the case for other solvents used in this study. Using these lower doses of Nanofuel and water-soluble solvent, with or without CTZ, in neurons expressing either an ops in alone or a luciferase alone resulted in minimal non-specific changes in firing frequency. In contrast, both excitatory (LMO+) and inhibitory (sbGLuc-hGlARClc2) LMO expressing cultures showed robust specific changes with CTZ, but not with vehicle.

In our studies, we used analysis of multi-unit spiking activity of primary neuronal cultures grown on MEAs. MEAs allow recordings of local field potentials (LFPs) and extracellular action potentials (EAPs) from a population of neurons. Since the beginnings of MEA recordings from dissociated neuronal cultures (Connolly, Clark, Curtis,
effects. We are currently refining the use of specific blocking agents in the absence of kynurenic acid were due to post-synaptic network cultures blocked the paradoxical effects, suggesting that the effects potent glutamatergic antagonist (Bekkers & Stevens, 1991), to such off-target effects needed to be ruled out in appropriate control experiments. We carried out these studies in MEA cultures, allowing for semi-high throughput analysis and accordingly, a full set of control experiments, including testing for effects of luciferase as well as vehicle on neurons expressing LMOs, just the luciferase, just the opsin, and no transgene at all. It is not realistic to test every permutation for every experiment. Given our results, this is also not necessary, as we found, for example, no evidence of effects of luciferase or vehicle on opsins directly, nor of bioluminescence itself on neuronal activity. Based on the results presented here, the most pertinent control experiments will be to evaluate the effects of vehicle in the LMO expressing group, and of CTZ in the non-LMO expressing group. This would be the case for experiments with cultured neurons, brain slices, and animals. Depending on the experimental conditions, in vitro or in vivo, the readout for effects can be a change in neural activity, in behavior, and/or in any one parameter under study. Effects of vehicle in LMO expressing and CTZ in non-expressing cells/animals should be the same, while effects of CTZ in LMO expressing cells/animals can be different or not, reflecting bioluminescence activation of neurons expressing LMOs changing the measured parameter or not (Figure 5). If CTZ administration in non-LMO expressing cells/animals diverges from the vehicle control, the concentration of CTZ should be adjusted to avoid such non-specific off-target effects. The underlying reasons for non-specific effects of CTZ may be different for different cell types, or neuronal populations, or brain regions. Accordingly, experimental conditions should be carefully controlled for each individual experimental application. For example, we used cortical neurons in the experiments presented here; use of hippocampal neurons would require including the two control groups, rather than extrapolating from cortical neurons. Similarly, testing behaviors when stimulating cortical circuits or thalamic circuits each require their matching controls. None of these recommendations pertain specifically to bioluminescence driven optogenetics, but are general considerations for good experimental design. As such, they apply to luminopsins, DREADDs, and standard optogenetics alike.

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CONFLICT OF INTEREST
All authors declare that they have no competing financial interests.

AUTHOR CONTRIBUTIONS
All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, MP; UH; Methodology, MP; UH; Investigation, MP; Statistical Analysis, WEM; Formal Analysis, MP, WEM, UH;
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