Optical control of ligand-gated ion channels

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Running title: Remote controlling ion channels.
i. Summary

In the vibrant field of optogenetics, optics and genetic targeting are combined to commandeer cellular functions, such as the neuronal action potential, by optically stimulating light-sensitive ion channels expressed in the cell membrane. One broadly applicable manifestation of this approach are covalently attached photochromic tethered ligands (PTLs) that allow activating ligand-gated ion channels with outstanding spatial and temporal resolution. Here, we describe all steps towards the successful development and application of PTL-gated ion channels in cell lines and primary cells. The basis for these experiments forms a combination of molecular modeling, genetic engineering, cell culture and electrophysiology. The light-gated glutamate receptor LiGluR, which consists of the PTL-functionalized GluK2 receptor, serves as a model.

ii. Keywords

Optogenetics, optochemical genetics, optical switch, photochromic tethered ligand, azobenzene, LiGluR, glutamate receptor.
1. Introduction

Biology occurs over a wide range of time and length scales. To understand dynamic biological systems, we require tools for both the spatio-temporal observation and perturbation of cellular and molecular events. While the past years have seen rapid growth in optical (e.g. fluorescence-based) real-time reporters of cellular signals (1), the development of means to activate cells on short and small scales has lagged behind. With light being the premier choice for both read-out and activation of cellular events, photosensitive molecules have recently enabled us to non-invasively control biological signals with high spatial and temporal resolution. This is achieved in optogenetics and optochemical genetics either by “repurposing” Nature’s light-sensing proteins from bacteria, algae or plants, or by engineering synthetic light-gated functionalities (2-4).

Three synthetic strategies to light-control signals in cells or in vivo are commonly used: “Caged” ligands, photochromic (“reversibly caged”) ligands, and PTLs (Fig. 1) (3). “Caged” ligands and photochromic ligands can be optically activated with sub-cellular resolution and within milliseconds by either releasing the ligand from the cage or by triggering a reversible conformational change in the photochrome. After photoactivation, these molecules act as free ligands on their specific protein targets (Fig. 1b, c). In contrast, PTLs are linear molecules that consist of a ligand moiety, a photochrome in the core of the molecule and a reactive group that attaches to the protein (Fig. 1d, e). Site-directed attachment is achieved by genetically introducing a cysteine residue near the ligand binding site. After attachment, the agonist or antagonist located at the end of the tether is presented to or retracted from the binding site by photoisomerization of the PTL core (Fig. 1d). Tethered ligands have the unique advantage that they specifically control a selected protein since the cysteine substitution is required for attachment and thus for light sensitivity. Receptors with substitutions can then be genetically targeted to specific cell types or organs in living organisms. PTLs have been developed to light-control several classes of ion channels, including nicotinic acetylcholine receptors (5,6), glutamate receptors (7) and K⁺-channels (8).
as well as variant channels with new functionalities derived from molecular studies \(^{(9)}\) or evolutionary relationships \(^{(10,11)}\). In these experiments, the tethered ligands were acetylcholine, glutamate and tetraethylammonium, respectively.

[FIGURE 1 NEAR HERE, PLEASE, AND IN COLOR PLEASE]

This section describes how genetic engineering, cell culture and electrophysiology are combined to apply PTL-gated ion channels in cell lines and primary cells. In addition, we explain how molecular modeling can be applied to test if a PTL is compatible with a target receptor and how modeling can aid in the choice of PTL attachment sites. In this way, this section includes all steps to perform light-control of an established as well as a new receptor based on an existing PTL. While procedures for the HEK293 cell line as well as primary neurons are described here, a single cellular model is sufficient for basic functional experiments. To explain these experiments, the light-gated glutamate receptor LiGluR, consisting of the ionotropic glutamate receptor GluK2 (formerly known as iGluR6) mutated at residue L439C and functionalized with MAG1 (maleimide azobenzene glutamate; Fig. 1e), serves as a model.

2. Materials

2.1. Molecular Modeling

1. Desktop computer running Visual Molecular Dynamics (VMD) \(^{(12)}\).
2. Protein Data Bank (PDB) file of the ligand-gated ion channel of interest in complex with ligand (here, PDB file 1SD3 containing 2S,4R-4-methylglutamate (4-MG) co-crystallized in the ligand binding domain of GluK2 \(^{(13)}\)).

2.2. Molecular Biology
1. Bacterial cell culture shaker in a 37°C room or 37°C incubator with shaker (~ 225 RPM).
2. LB medium: 1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.0, autoclaved.
3. Bacteriological agar.
4. Ampicillin stock solution (100 mg/mL in water), stored at -20°C in 500 μL aliquots.
5. QuickChange II Site-Directed Mutagenesis Kit (Agilent, Vienna).
6. QIAprep Spin MiniPrep DNA purification kit (Qiagen, Hilden, Germany).
7. Gene coding for ion channel in a vector suited for mammalian expression (e.g. pcDNA3.1(-)) ([see Note 1]).
9. Gene coding for YFP in a vector suited for neuron-selective expression ([see Note 2]).
10. Mutagenesis oligonucleotide primers and sequencing oligonucleotide primers ordered custom-made from any of the many commercial vendors ([see Note 3]).
11. Sanger DNA sequencing provided by any of the many commercial vendors.

2.3. Cell Culture and Transfection of HEK293 Cells

1. Cell culture facility equipped with laminar flow hood, water bath (37°C), incubator (5% CO₂) and cell counting chamber.
2. HEK293 cells (CRL-1573, American Tissue Culture Collection, LGC Standards, Teddington, UK) cultured according to the provider’s recommendation in 25 cm² tissue culture flasks.
3. 500 mL Dulbecco’s Modified Essential Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), sterile filtered and stored at 4°C. Supplement half of the medium with 1% penicillin/streptomycin solution, and use the supplemented DMEM unless noted otherwise.
4. Dulbecco’s Phosphate Buffered Saline (DPBS): 8 g NaCl, 200 mg KCl, 200 mg KH₂PO₄, 2.16 g Na₂HPO₄·7H₂O, water added to 1 L and sterile filtered.
5. Borate buffer: 1.55 g boric acid, 2.375 g Na’ borate, water added to 500 mL, pH 8.5, sterile filtered and stored at 4°C.

6. Opti-MEM I reduced serum medium (LifeTech, Vienna).

7. 0.25% Trypsin-EDTA solution (LifeTech).

8. Lipofectamine 2000 transfection reagent (LifeTech).

9. Poly-L-lysine (PLL) HBr (molecular weight 70,000-150,000). Open PLL container in the laminar flow hood and add water to a concentration of 10 mg/mL. Aliquot in 1.5 mL eppendorf tubes and store at -20°C.

10. Round cover glasses (18 mm diameter, Carolina Biologicals, Burlington, NC, USA) (see Note 4).

11. Polystyrene 25 cm² cell culture flask with filter lid (0.2 μm pore size, sterile).

12. Polystyrene 12-well plate (sterile).

2.4. Additional Reagents For Cell Culture and Transfection of Hippocampal Neurons

1. Dissection microscope.

2. Dissection tools: Large scissors, large forceps, small scissors, 2 sharpened forceps, curved spatula and small scalpel.

3. High glucose MEM: 12.75 g D-glucose with Modified Essential Medium (MEM) added to 50 mL, sterile filtered and stored at 4°C.

4. Media: 181 mL MEM, 3 mL high-glucose MEM, 10 mL FBS and 0.2 mL MITO+ Serum Extender. Store at 4°C. Once a week, add 1 mL B-27 Serum-Free Supplement (LifeTech) and 500 μL L-glutamine to 48.5 mL media to produce final medium. Store at 4°C.

5. Saline: 1.19 g HEPES, 1.80 g D-glucose, Hank's Basic Salt Solution (HBSS) added to 500 mL, sterile filtered and stored at 4°C.

6. HBSS washing solution: 7.89 g NaCl, 4.77 g HEPES, 0.30 g KCl, 0.29 g CaCl₂·2H₂O, 0.20 g MgCl₂·6H₂O, 0.14 g Na₂HPO₄, 0.18 g glucose, water added to 1 L, pH 7.3, sterile filtered and stored at 4°C.
7. BBS solution: 0.82 g NaCl, 0.53 g BES, 10.7 mg Na₂HPO₄, water added to 50 mL, sterile filtered and stored at 4°C.
8. CaCl₂ solution: 18.38 g CaCl₂·2H₂O, water added to 50 mL, sterile filtered and stored at 4°C.
9. Cytosine-1-β-D-arabinofuranoside (AraC) solution (4 mM), sterile filtered and stored at -20°C.
10. Two sterile glass Pasteur pipettes: The first pipette is barely flame-polished, the second pipette is flame polished to half its original diameter.
11. Round cover glasses (12 mm diameter, Carolina Biologicals, Burlington, NC, USA).
12. Polystyrene 6-well plate and 24-well plate (sterile).
13. Cell strainer (40 μm, BD Biosciences).

2.5. Microscopy and Light-activation

1. Inverted microscope equipped with fluorescence condenser, rotating turret for moving filter holders (“filter cubes”) into the light path and 20X fluorescence objective. The microscope is placed on an air table and enclosed by a faraday cage.
2. Light source capable of producing bright monochromatic illumination (> 1 mW/mm²) that can be computer controlled (Polychrome V, TILL Photonics, Gräfelfing, Germany) (see Note 5).
3. Power meter with wavelength range from 300-800 nm and power range up to 50 mW (PM120VA, Thorlabs, Munich, Germany) (see Note 6). Power range can be extended with neutral density filters (Thorlabs).
5. Total reflectance mirror sized to fit into a filter cube in the diagonal position (e.g. NT43-875, Edmund Optics, Karlsruhe, Germany).
6. Empty filter cube.
7. Standard laboratory spectrophotometer to perform absorbance measurements at a wavelength of 360 nm.
8. MAG1 in solid form.
9. DTT stock solution (10 mM): 77.1 mg DTT in 10 mL extracellular solution, distributed to 1 mL aliquots and stored at -20°C.
10. Concanavalin A solution: 15 mg type VI concanavalin A (Sigma) in 50 mL extracellular solution and stored at 4°C.

2.6. Whole-Cell Patch Clamp Measurements
1. Patch clamp amplifier with data acquisition electronics, electrode holder and software installed on a desktop computer (AxoPatch200B, Digidata 1440A, HL-U and pCLAMP 10, Biberach, Germany).
2. Micromanipulator on rotating base mounted to a tower (MP-285ROE, 285RBI and MT-71-9, Sutter Instruments, Science Products, Hofheim, Germany) (see Note 7).
3. Glass micropipette puller (P-97, Sutter Instruments).
4. Extracellular solution: 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, pH 7.4 (see Note 8).
5. Intracellular solution: 135 mM K-gluconate, 10 mM NaCl, 2 mM MgCl₂, 2 mM MgATP, 1 mM EGTA, 10 mM HEPES, pH 7.4.
6. Salt bridge solution: 3 M KCl and 1% electrophoresis-grade agarose in water.
7. Glass capillary tubes (1.5 mm outer diameter, 1.17 mm inner diameter, 100 cm length, Warner Instruments, Hamden, CT).
8. Unplugged glass Pasteur pipettes (length > 200 mm).
9. Glass bottom petri dish (glass surface: 25 mm diameter, 0.2 mm thickness, Bioptechs, Butler, PA, USA) (see Note 9).

3. Methods

3.1. Molecular Modeling of PTL Substitution Pattern and Attachment Sites
1. Molecular modeling is applied to test if a PTL is compatible with a target receptor. In the case of MAG1 and GluK2 (Fig. 1), modeling is based on a crystal structure of the ligand binding domain of GluK2 with bound 4-MG (13). Note that MAG1 and 4-MG are both substituted at the 4’ position (the Cγ atom of glutamate; Fig. 1e) with identical stereochemistry, and 4-MG therefore is a valid model for MAG1 with respect to the substitution pattern (14). Furthermore, the closed conformation of the ligand binding domain indicates that the receptor is an activated conformation (Fig. 1a).

2. To visualize 4-MG in the ligand binding domain, open PDB file 1SD3 in VMD (File/NewMolecule).

3. In the representations panel (Graphics/Representations), reformat the structure by activating drawing method “NewCartoon” for selected atoms “chain A”.

4. Orient the structure such that the ligand faces the front and highlight the ligand by creating a new representation with the drawing method “VDW” for selected atoms “chain A and resid 998”. The resulting view is shown in Fig. 1f.

5. To verify that an extended 4’ substituent will reach to solvent exposed residues, change the drawing methods of chain A to “VDW”. An exit tunnel becomes visible (Fig. 1g, circle), confirming that the tail of MAG1 may reach the protein surface while still allowing the ligand binding domain to close (7).

6. In a next step, use the molecular model to identify residues that are surface exposed and surround the binding site (Fig. 1g, highlighted residues). Select 4-8 residues and order oligonucleotides to alter them into cysteines. In the case of MAG1, attachment to L439, L482, G486 and E722 of GluK2 has proven successful (15).

7. For every new application of a PTL to a receptor, perform steps 1-5 with a suited crystal structure to verify that an exit tunnel exists and to find sites for attachment.

3.2. Preparation of Growth Plates

1. Solubilize 9 g agar in 600 mL LB medium and autoclave.
2. After autoclaving, let hot LB medium cool to 50°C, add 600 \( \mu \text{L} \) ampicillin stock solution and swirl to mix.

3. Pour solution into sterile petri dishes to a depth of ~ 3 mm.

4. Leave the plates at room temperature and unstacked for 24 h before storing them at 4°C.

3.3. Polymerase Chain Reaction to Generate Cysteine Mutants

1. Dilute the vector containing the ion channel gene to a concentration of 20 ng/\( \mu \text{L} \).

2. Resuspend lyophilized mutagenesis oligonucleotides (forward and reverse) to a concentration of 300 \( \mu \text{M} \) by adding \( X \times 3.33 \mu \text{L} \) water to the original tubes (\( X \) is the amount of nanomoles delivered in the tube). Vortex for 10 s and spin briefly every 5 min for a total time of 15 min. Store at -20°C.

3. Dilute mutagenesis oligonucleotide primers to 10 \( \mu \text{M} \) by adding 2 \( \mu \text{L} \) of each oligonucleotide primer to 56 \( \mu \text{L} \) water. Vortex, spin briefly and store at -20°C.

4. The PCR is setup following the manufacturer’s instructions with 20 ng template vector (1 \( \mu \text{L} \) of the dilution) and 10 picomoles of each oligonucleotide (1 \( \mu \text{L} \) of the mixture).

   Recommended PCR parameters are: 1 cycle at 95°C for 60 s; 18 cycles at 95°C for 30 s, at 56°C for 30 s and at 72°C for 600 s; 1 cycle at 95°C for 7 min, and 1 cycle at 4°C until the reaction is processed further (see Note 10).

5. After PCR, add 1 \( \mu \text{L} \) dpn1 restriction enzyme, pump mix gently, spin briefly and incubate at 37°C for not less than 90 min.

3.4. Amplification and Selection of Mutagenized Receptors

1. Warm up LB agar plates (one for each PCR) at 37°C for 30 min (see Note 11).

2. Transform PCR without further purification into competent cells. Maintain cells on ice during the transformation and treat cells gently without vortexing or excessive pipetting.

3. Spread bacteria evenly on plates with a sterile glass rod.

4. Place plates at 37°C upside down overnight.
5. On the following day, combine 3 mL LB medium with 3 μL ampicillin solution in glass tubes (four for each PCR).
6. Using a pipette tip, pick four colonies of each plate by lightly touching a single colony with the tip and dropping the pipette tip into the glass tube.
7. Place glass tubes at 37°C with shaking (~ 225 RPM) overnight.
8. On the following day, extract vector from bacteria with MiniPrep kit following the manufacturer’s instructions and send vector for verification by sequencing.

3.5. MAG1 Reconstitution

1. Prepare ~ 3 mg MAG1 in an 1.5 mL eppendorf tube.
2. Add 200 μL DMSO to the tube to yield the MAG1 stock solution (26 mM).
3. Vortex vigorously for 10 s and spin down briefly. Repeat until all solid has dissolved.
4. Prepare 10 μL aliquots and store them at -20°C in the dark (see Note 12).

3.6. Verification of MAG1 Concentration (see Note 13)

1. Add 0.5 μL MAG1 stock solution to 1 mL DPBS, vortex and spin briefly.
2. Prepare five 10-fold serial dilutions: Add 100 μL of the first MAG1 dilution to 900 μL PBS to produce a second dilution. Then add 100 μL of this second dilution to 900 μL PBS to prepare a third dilution. Repeat two more times.
3. In the spectrophotometer, blank absorbance at a wavelength of 360 nm with DPBS and measure absorbance for all serial dilutions.
4. Using measurements in the linear absorbance regime (usually between 0.1 and 1.0 A), determine the concentration of the stock solution. This is achieved by plotting A_{360} against concentration and equating Beer-Lambert’s law with the extinction coefficient 0.025 A_{360}/μM and the dilution factor.

3.7. Preparation of Cover Glasses for HEK293 Cells
1. Cell culture should be performed under sterile conditions in a laminar flow hood. Instruments and containers should be disinfected by spraying them with 70% EtOH prior to placing them in the hood.

2. Dilute PLL solution to a concentration of 0.05 mg/mL in borate buffer.

3. Add 2 mL diluted solution to the wells of a 12-well plate.

4. Add a single 18 mm cover glass to each well and incubate cover glasses for 2-8 h (see Note 14).

5. Transfer the desired number of cover glasses (typically 4-8) to a new 12-well plate.

6. Wash cover glasses three times with water (add and aspirate water) and allow to air dry.

3.8. Preparation of HEK293 Cells

1. Warm trypsin solution, DPBS and DMEM in water bath to 37°C. Warm 9 mL DMEM in a culture flask in the incubator (see Note 15).

2. Add 1.5 mL DMEM to each cover glass prepared previously in the 12-well plate.

3. Remove cells from incubator and aspirate media.

4. Add 10 mL DPBS, let stand for 10 s and aspirate.

5. Add 3 mL trypsin solution and let stand for 3 min.

6. To detach cells from flask, tap flask against a vertical surface.

7. Add 7 mL media to the flask and pipet up and down with a serological pipette (see Note 16).

8. Transfer 1 mL cell suspension to the culture flask that contains 9 mL pre-warmed media. Place flask in incubator to passage the culture in the future.

9. Transfer the remaining 9 mL cell suspension to a 50 mL conical tube.

10. Centrifuge at 2000 RPM for 3 min and aspirate supernatant.

11. Resuspend cells in 2 mL media by carefully pipetting up and down ten times with a serological pipette.

12. Count cells, transfer 80,000 cells to each cover glass and allow 8-24 h for cells to settle before transfection.
3.9. Transfection of HEK293 Cells

1. Warm DMEM with no antibiotics added in water bath to 37°C.
2. Remove 12-well plate from incubator and replace DMEM on cells with pre-warmed DMEM.
3. For each cover glass to be transfected, combine 25 \( \mu \)L Opti-MEM I, 0.2 \( \mu \)g ion channel vector, 0.01 \( \mu \)g YFP vector in a 1.5 mL eppendorf tube (see Note 17).
4. For each cover glass to be transfected, combine 25 \( \mu \)L Opti-MEM I and 1 \( \mu \)L Lipofectamine in a 1.5 mL eppendorf tube.
5. Let stand for 5 min.
6. Add lipofectamine solution to vector solution and tap tube gently to mix.
7. Let stand for 20 min.
8. Add 50 \( \mu \)L transfection mix to each cover glass.
9. Allow 18-24 h for expression (see Note 18).

3.10. Preparation of Cover Glasses for Hippocampal Cell Culture

1. Add 2 mL 70% EtOH to all wells of a 24-well plate.
2. Add a single 12 mm cover glass to each well and incubate for 10 min.
3. Wash three times with water.
4. Treat the cover glasses with PLL as described above.
5. After treatment, wash cover glasses three times with water and allow to soak in water for \( \sim 1 \) h. Wash one more time and let dry.

3.11. Dissection (P0 to P4 Sprague Dawley pups)

1. Autoclave dissection tools prior to dissection.
2. Warm saline in water bath, warm media in incubator and bring trypsin solution to room temperature.
3. Fill two 60 mm petri dishes halfway with warm saline.

4. Sever neck at base of head with large scissors and place under dissecting scope.

5. Remove skin from back to front with forceps.

6. Slice the skull from back to front with small scissors.

7. Peel back the skull to each side with large forceps, dislocate brain with curved spatula and slide brain into petri dish containing the saline. Repeat for additional pups and then transfer all brains into a new dish.

8. Remove cerebellum and separate hemispheres with scalpel.

9. Remove meninges from the outer surface of the brain (see Note 19).

10. Identify the hippocampus as a curved structure that is slightly denser and therefore has a dark contrast to the rest of the tissue. Gently cut away the hippocampus being very careful not to damage the tissue.

11. Remove remaining meninges with minimal to no damage to the hippocampus and clean away any excess tissue still attached to it with small scalpel.

12. Carefully transfer hippocampus to 15 mL conical tube filled with saline.

3.12. Preparation of Hippocampal Cells

1. Remove all but 4.5 mL saline from hippocampus, add 500 μL trypsin and invert to mix.

2. Place at 37°C for 8 min.

3. Add 9 mL saline to dilute trypsin, invert to mix and remove solution using serological pipet. Repeat for a total of four washes.

4. Add 1 mL media and triturate six times with large diameter Pasteur pipette. Remove media with suspended cells and strain into a 50 mL conical tube.

5. Repeat previous step with small diameter Pasteur pipette if more cells are desired.

6. Count cells and dilute to a concentration of 200,000 cells/mL.

7. Add 500 μL cell suspension to each well and place in incubator.

8. After 15 min, replace media to eliminate debris that has not attached to cover glass.
9. After 4-7 d in culture, AraC is added to a final concentration of 4 μM to prevent growth of glia.
10. To maintain cultures, replace half of the media with fresh media once per week.

3.13. Transfection of Hippocampal Cells
1. For each cover glass, warm 1 mL transfection media (985 μL MEM supplemented with 15 μl high glucose MEM), 500 μL HBSS and 500 μL growth media in the wells of a 6-well plate placed in the incubator.
2. Bring CaCl₂ solution, BBS and expression vectors to room temperature.
3. Remove cells from incubator and transfer media from cells to a well of the 6-well plate.
4. Add 500 μL transfection media to cells and aspirate.
5. Add 500 μL transfection media to cells.
6. Mix, in this order, 1.15 μL CaCl₂ solution, 1.2 μg ion channel vector, 0.1 μg YFP vector with neuron-specific promoter and add water to a final volume of 33 μL.
7. To the mixture, add 33 μL BBS drop-wise, mix, and transfer 30 μL to the center of each cover glass immediately.
8. Observe cells regularly during the next 6 h. After the shortest amount of time that generates a fine layer of precipitate, aspirate transfection media and incubate cells in HBSS.
9. After the shortest amount of time needed to dissolve precipitate (typically 2-15min in HBSS), wash cells with 500 μL conditioned media (aspirated from the cells previously) and add both conditioned and fresh media to cells (500 μL each).

3.14. Preparation of Whole-Cell Patch Clamp Measurements
1. Using the flame of a gas burner, bend the end of a Pasteur pipette into a "U"-shape where the “sides” and the “bottom” of the U are ~ 2 cm long (see Note 20).
2. Heat salt bridge solution in a microwave until homogenous. Allow to cool to 50°C.
3. Aspirate solution in a 1 mL pipette tip and fill the U-shaped glass to complete the salt bridge. Store the salt bridge in 3 M KCl at 4°C.

4. Using glass capillary tubing and a multistage puller, prepare micropipettes with taper length of ~ 4 mm, tip diameter of ~ 2 μm and electrical resistance of 2-8 MΩ.

3.15. Preparation of Microscopy

1. Connect the light source to the data acquisition electronics and configure the software such that the wavelength can be computer-controlled.

2. Connect the optical output of the light source to the back port of the microscope.

3. To direct light of all wavelengths to the sample, fix a total reflectance mirror with superglue in the spare filter holder at the position reserved for the dichroic mirror. Place this filter cube in the turret (see Note 21).

4. Place the filter cube for YFP imaging in the turret unmodified.

5. Measure the intensity of the light exiting the objective using the power meter (see Note 6).

3.16. MAG1 Labeling of HEK293 Cells and Neurons (see Note 22)

1. In the laminar flow hood, fill four wells of a 12-well plate with 1 mL extracellular solution and supplement one well with DTT to a final concentration of 1 mM. Fill one well with concanavalin A solution.

2. To remove growth medium, transfer a cover glass with HEK293 cells or neurons from growth medium to extracellular solution.

3. To activate surface cysteines, transfer the cover glass to extracellular solution with DTT and incubate for 10 min.

4. To remove DTT, transfer cover glass to extracellular solution and incubate for 2 min.

5. For concanavalin labeling, transfer cover glass to concanavalin A solution and incubate for 10 min (see Note 23).

6. In the meantime, thaw MAG1 stock solution and illuminate with UV light for 1-15 min (see Note 24).
7. Dilute illuminated MAG1 in one of the wells to a final concentration of 10-50 μM (see Note 25).

8. Transfer cover glass to this solution and incubate for 10-30 min. Keep the well plate in the dark during this time.

9. Transfer cover glass to a well with extracellular solution and keep in the dark until the experiment.

3.17. Patch Clamp Measurements and Photoswitching

1. Add 500 μL extracellular solution to glass bottom petri dish and transfer the cover glass to the dish.

2. Connect the glass bottom dish to a dish with the reference electrode using the salt bridge.

3. Adjust the microscope for visualization of YFP by choosing the appropriate filter cube and by switching the light-source to 510 nm excitation light.

4. Using the eye pieces, identify a transfected cell (see Note 26).

5. Apply positive pressure to the micropipette while lowering it into extracellular solution to prevent adsorption of debris at the liquid-air-interface.

6. Position the micropipette over the center of the cell (see Note 27).

7. Slowly lower the micropipette until it presses down on the cell surface, causing an indentation.

8. Place the modified filter cube that holds the total reflectance mirror in the light path.

9. Adjust the holding potential to -70 mV.

10. Release the positive pressure from the micropipette, observe GΩ seal formation and use negative pressure to break through the cell membrane. Allow 5 min to obtain a seal resistance greater than 100 MΩ.
11. Photoswitching can be accomplished by changing illumination wavelengths while recording from the cell in voltage-clamp or current-clamp mode (Fig. 2). Illumination at a wavelength of 380 nm coverts MAG1 into the cis-isomer that opens GluK2 and produces and inward (negative) current in voltage-clamp or depolarization in current-clamp. Illumination at a wavelength of 500 nm light coverts MAG1 into the trans-isomer allowing GluK2 to close.

4. Notes

1. In our experience, all mammalian expression vectors containing the cytomegalovirus (CMV) promoter performed well in HEK293 cells and neurons with robust protein expression. Increased expression of ion channels was achieved using the hybrid CMV enhancer/chicken β-actin promoter along with woodchuck hepatitis virus posttranscriptional regulatory element (16). Most ion channel genes can be obtained in expression vectors from Addgene (http://www.addgene.org/).

2. Co-transfection of fluorescent proteins allows selecting transfected cells in patch clamp experiments. Furthermore, fluorescent proteins under the control of cell type specific promoters can be used to distinguish cell types. To target neuronal cells in hippocampal cell cultures, a modified pcDNA3.1(-) expression vector containing the neuron-specific human synapsin 1 promoter is available from the authors.

3. Mutagenesis oligonucleotide primers can be reliably designed using the PRIMERX website (http://www.bioinformatics.org/primerx/) following the QuickChange parameters. Sequencing oligonucleotide primers should be designed according to the recommendations of the sequencing provider, which also may offer universal oligonucleotide primers that bind to many vectors.

4. In our experience, cover glasses from some other manufacturers require additional cleaning in 70% EtOH solution followed by washing in water.
5. In the past years, the polychrome has become a standard light source for optogenetic experiments as it offers bright illumination with maximum wavelength flexibility. A good alternative are filter-based light sources capable of producing high-intensity illumination (> 10 mW/mm²) with fast switching time between filters (< 2 ms) (DG4, Sutter Instruments, Science Products, Hofheim, Germany).

6. For accurate optogenetic experiments, it is required to measure the light intensity produced in every individual setup. This is commonly done at the very end of the light path by measuring the light exciting the objective. The total intensity is measured using the power meter, while the illuminated area can be estimated to be the field of view. Latter can be determined using a micrometer-sized grid. For a more elaborate measurement, the illuminated area is controlled using an aperture or pinhole. Most optogenetic applications work well with intensities of > 1 mW/mm², and MAG1 can be reliably converted with as little intensity as 0.1 mW/mm².

7. In the simplest patch clamp setup, the micromanipulator with mounted patch clamp electrode is attached on the optical table using a tower stand, while the cells are placed on the X-Y-table of the microscope. The major advantage of this setup is that localizing fluorescent cells is facilitated as the cells are moved relative to the objective. The major disadvantage is that the field of view or focus cannot be changed after a cell has been patched.

8. In a modified extracellular solution, Na⁺ ions can be replaced by N-Methyl-D-glucamine to reduce cell toxicity caused by PTLs acting as a very weak agonist during labeling.

9. Glass bottom dishes proved to be an easy and affordable alternative to imaging chambers typically used in patch clamp experiments. They can be reused many times or disposed after single use.

10. We have obtained good results when using 20 ng of template vector and following the manufacturer’s protocol, especially for the number of cycles. For the mutagenesis of genes in long or problematic vectors, we recommend supplementing the reaction with additional 40 ng template vector and 3 μL DMSO.
11. Place plates upside down with the base sitting on the ridge of the lid on one side. In this way, condensation will not accumulate on the agar surface and humidity is reduced.

12. The maleimide group of a PTL is sensitive to hydrolysis and precautions are necessary to avoid water contact until the labeling reaction. Store PTL solids in an eppendorf tube that is placed in a 50 mL conical tube containing ~1 cm of desiccant at the bottom. Place the conical tube in the dark and at -20°C or -80°C. Parafilm wrapping of the eppendorf tube is neither required nor desired. When resuspending PTLs, use DMSO that was aliquoted from a new stock and stored in individual tubes in a container with desiccant. PTL stock solutions in DMSO are stored as described above for the solid and can be stored for one year. Stock solutions should be sufficiently concentrated such that DMSO concentration during labeling does not exceed 1% (at a PTL concentration of up to 50 μM). While it has not shown to be a problem, the number of freeze-thaw cycles should be kept to a minimum.

13. This procedure can also be used to determine MAG1 concentration if no scale with mg sensitivity is available.

14. While 2-8 h are recommended for treatment, cover glasses can be left in PLL solution for several weeks, and PLL solution can be reused at least six times if kept sterile.

15. Warming media in the incubator is preferred over the water bath for equilibration with CO₂ and consequently proper pH.

16. Use of serological pipets is recommended as 1 mL pipette tips can damage cells.

17. For transfection of most glutamate receptor vectors, the amount of lipofectamine and vector can be scaled down substantially (up to 5-fold) compared to the recommendation in the manufacturer’s protocol. It is critical to use miniscule amounts of fluorescent protein vector compared to ion channel vector (typically 10-20 times less fluorescent protein vector than the ion channel vector).

18. For whole-cell patch clamp measurements, HEK293 cells should be sparse at the time of experiment to prevent gap junction formation between adjacent cells. For many other
purposes (e.g. imaging) a confluent layer works well and gives best transfection efficiency and cell health.

19. Meninges will cause microglial growth in the cell culture that is harmful to neuronal growth. When removing meninges, discard damaged hippocampal tissue.

20. In a first step, hold the pipette horizontally and heat it 2 cm from the end until the terminal piece drops by gravity to produce a right angle. Repeat to complete the U-shape. Using a glass cutter and appropriate safety equipment, separate the “U”-shape from the rest of the Pasteur pipette.

21. It is not safe to look into the eye pieces when using this modified filter cube as unfiltered stray light may reach to the eye piece.

22. While this procedure describes MAG1 labeling of cells expressing LiGluR, it is generally applicable to other PTLs and ion channels. Labeling and wash steps can be executed either at room temperature or at 37°C. DTT treatment and UV preillumination are optional steps that yield the greatest efficiency of MAG1 labeling and thus photoswitching.

23. Concanavalin A blocks desensitization of GluK2 and is required for experiments in HEK293 cells but not neurons.

24. Efficiency of MAG1 labeling is increased if MAG1 is converted to the cis-isooform in UV light before the labeling. This can be achieved with any handheld UV source, e.g. a UV LED pointer, and at any light intensity. Indicative values: At an intensity of 0.04 mW/mm² ($\lambda_{\text{max}}$ 365 nm) allow 15 min, at an intensity of 5.5 mW/mm² ($\lambda_{\text{max}}$ 374 nm) allow 30 s.

25. It is essential to pump mix the solution to fully dissolve MAG1 as DMSO solutions tend to “sink” to the bottom of the well. Prepare the MAG1 dilution fresh for every cover glass to be labeled.

26. Cells with medium bright fluorescence often allow the formation of high quality whole-cell seals compared to very bright cells.

27. Micropipette positioning can be achieved in the following steps. After focusing on the cell, move the focal plane above the cell by turning the focus wheel on the side of the
microscope by ~ two turns. In a next step, lower the micropipette until it is in this new
focal plane and move it to be centered on the field of view. In the final step, refocus on
the cell lower the pipette slowly.

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References


Fig. 1. Synthetic strategies for manipulating ligand-gated ion channels with light. (a) In the example of the ionotropic glutamate receptor GluK2, glutamate binding in the extracellular ligand binding domain triggers domain closure and gate opening. (b) Gating after ligand uncaging. (c) Gating after reversible conversion of a photochromic ligand to a binding-competent conformation. (d) Reversible light-control by a PTL. (e) Chemical structure of MAG1, the prototypical maleimide-azobenzene-glutamate PTL, and 4-MG. (f) and (g) Crystal structure of the ligand binding domain of GluK2 in complex with 4-MG. 4-MG, residues surrounding the binding site suited for PTL attachment and exit tunnel are highlighted as described in the text.
Fig. 2. Optical control of HEK293 cell currents and action potentials with LiGluR. (a) Whole-cell current in a voltage-clamped HEK293 cells expressing LiGluR (holding potential -60 mV). Current can be photo-controlled with maximum activation at ~380 nm and inactivation at ~500 nm. As a positive control for ion channel expression, glutamate as a free ligand can be added using gravity flow. (b) LiGluR activation depolarizes and triggers action potential firing in a current-clamped hippocampal neuron (top trace) After a brief pulse, depolarization is stable in the dark (bottom trace). Part a is reprinted by permission from Macmillan Publishers Ltd: Nat. Chem. Biol. (7), copyright 2006. Part b is reprinted from Neuron, Ref. (17) with permission from Elsevier Ltd.