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Protocol

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Real-time imaging of axonal membrane protein life cycles

In the format provided by the authors and unedited

Supplementary Methods: Molecular biology protocols for generation of enzymatically-tagged full length proteins for use in imaging assays

Molecular Biology

All insertions and deletions were done by mega mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA).

For insertions, create megaprimers with a minimum of 20-30bp left upstream and downstream of the insertion sequence. The forward primer should therefore have a 20-30bp overhang on the 5' end. The 3' end of the primer will be complementary to the insertion sequence.

1. Amplify the desired insertion sequence. We use the AccuPrime Taq DNA Polymerase High Fidelity, a high-fidelity polymerase (ThermoFisher Scientific, Waltham, MA).
2. Purify and quantitate the PCR product. The PCR product will serve as the mega primer for mutagenesis with the QuikChange Lightning Site-Directed Mutagenesis Kit.

For deletions, create primers with a minimum of 20-30bp upstream and downstream of the region that need to be deleted. The forward primer should have 4-6bp upstream of the region that needs to be deleted and 20-30bp downstream of the region that needs to be deleted. The reverse primer should have 4-6bp downstream of the region that needs to be deleted and 20-30bp upstream of the region that needs to be deleted. The forward and the reverse primers and the template will be used in the QuikChange Lightning Site-Directed Mutagenesis reaction.

Use DpnI digestion to degrade the original template.

Transform plasmids with the insertion sequence into competent E. coli cells according to the QuikChange Lightning Site-Directed Mutagenesis manual.

As an example, we attached HaloTag extracellularly to $Na_V1.7$ with an additional transmembrane domain in 2 steps:

1. Modification of the human β4 subunit sequence to insert HaloTag.
2. Construction of tagged $Na_V1.7$ channels: The β4-HaloTag-chimera was fused in-frame to the N-terminus of $Na_V1.7$ to produce a channel with an extracellular N-terminus and 25 transmembrane segments.

The final topology of our Halo-$Na_V1.7$ construct is, in order from the N-terminus: β4 signal peptide (aa 1-30), 3× myc-tag (EQKLISEEDL), Halo Tag (297 amino acids), 3× HA-tag (YPYDVPDYA), 35 aa β4 stalk, 21 aa β4 transmembrane segments (163 to 183), 7 aa linker (SGSGGAV), full-length codon-optimized human $Na_V1.7$[26,40,43,44].

OPTIONAL: Epitopes for commonly used antibodies can be added to these constructs. For example, we added 3 copies of each of the myc epitope tag and hemagglutinin (HA) tag flanking either side of the Halo-Tag sequence.

Fragment insertion reaction set-up

Combine:

5 µl of 10× reaction buffer

25–100 ng of dsDNA template

300-500 ng of megaprimer

1 µl of dNTP mix

1.5 µl of QuikSolution reagent

Add ddH2O to a final volume of 50 µl

Then add:

1 µl of QuikChange Lightning Enzyme

Fragment insertion thermocycler protocol

1 cycle at 95°C for 30 seconds

5 cycles: 95°C for 30 seconds, 52°C for 1 minute, 68°C for 30 seconds/kb of plasmid length

13 cycles: 95°C for 30 seconds, 55°C for 1 minute, 68°C for 30 seconds/kb of plasmid length

1 cycle at 68°C for 7 minutes

Fragment deletion reaction set-up

Combine:

5 µl of 10× reaction buffer

25–100 ng of dsDNA template

125 ng of forward primer

125 ng of reverse primer

1 µl of dNTP mix

1.5 µl of QuikSolution reagent

Add ddH2O to a final volume of 50 µl

Then add:

1 µl of QuikChange Lightning Enzyme

Fragment deletion thermocycler protocol

1 cycle at 95°C for 2 minutes

18 cycles: 95°C for 20 seconds, 60°C for 10 seconds, 68°C for 30 seconds/kb of plasmid length

1 cycle at 68°C for 5 minutes