Remove 1st loading solution and add 2nd loading solution containing 12.5 microM DiSBAC2(3), 1.25 mM Xylene Fast Yellow and 0.0075 % Pluronic F-127 in assay buffer.

Place the plate under the dark at rt for 25 minutes.

Add compound solutions into the assay plate.

Set the assay plate in FDSS and place an EFS device on the plate.

Measure EFS-induced fluorescent response by FDSS.

[0230] The data are analyzed and reported as normalized ratios of intensities measured at 440 nm. The process of calculating these ratios is performed as follows:

[0231] [Math.1]

$$\%\ \text{Inhibition} = \left\{ 1 - \frac{(\text{FIR of each well} - \text{median FIR in 100\% Inhibition})}{(\text{median FIR in 0\% Inhibition} - \text{median FIR in 100\% Inhibition})} \right\} \times 100$$

"FIR" = Fluorescence Integration Ratio

= the integral of the ratio normalized by baseline (before EFS)

[0232] This analysis is performed using a computerized specific program designed for FDSS generated data. Fluorescence ratio values are plotted using XLfit to determine an IC_{50} value for each compound.

[0233] All tested compounds of Examples show less than about 1 microM of IC_{50} against Nav1.7 in the above assays. Preferable compounds show less than about 0.5 microM of IC_{50} against Nav1.7 in the above assays.

Compounds with IC_{50} against Nav1.7 < 0.5 microM are:

Examples 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 14, 15, 16, 19, 21, 22, 23, 25, 26, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 49, 54, 56, 57, 58, 59, 60, 61, 64, 65, 66, 67, 68, 69, 70, 71, 73, 74, 76, 77, 80, 87, 89, 90, 93, 94, 96, 97, 98, 105, 106, 107, 108, 113, 114, 115, 116, 117, 119, 123, 126, 127, 128, 129, 130, 131, 132, 133, 134, 138, 139, 140, 141, 142, 143, 144, 145, 146, 148, 149, 150, 151, 152, and 154.

[0234] FRET Assay

This screen is used to determine the effects of compounds on human Nav1.8, and human Nav1.5 channels, utilizing the cell imaging technology by Hamamatsu Photonics's Functional Drug Screening System (FDSS). The changes of membrane potential are monitored with fluorescent membrane potential dye pair, DiSBAC2(3) and CC2-DMPE, using FRET technology.

[0235] Cell Maintenance:

HEK293 cells expressing human Nav1.8 channels or human Nav1.5 channels are grown in T225 flasks, in a 5% CO_2 humidified incubator to about 80% confluence. Media composition of HEK293 cells expressing human Nav1.8 channels consists of