# Development and validation of a TRPML1 assay on an automated patch clamp platform

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## Introduction

TRPML1 is a cation-selective ion channel ubiquitously expressed in lysosomes and late endosomes. TRPML1 is intimately involved in the regulation of lysosomal $Ca^{2+}$ homeostasis and is consequently a critical component of autophagy and lysosomal biogenesis. Malfunctions of these processes are linked to various neurodegenerative disorders, including Alzheimer`s and Parkinson`s disease. The relevance of TRPML1 in neurodegenerative disorders is further validated by a loss-of-function mutation that was shown to cause the neurodegenerative lysosomal storage disease mucolipidosis type IV (MLIV).

The patch clamp technique remains the gold standard for studying ion channels as recordings directly measure the protein's activity. Whilst powerful, the conventional patch clamp method requires highly trained scientists and allows only a low throughput. Automated patch clamp systems have evolved to overcome these limitations. These platforms are, therefore, well-suited to test large numbers of compounds quickly to support ion channel drug development programs.

## Summary

- **A robust TRPML1 high throughput patch clamp assay is presented that is well-suited to support drug development processes.**
- **Advanced filter criteria allow fast analysis of the large data set generated form one experiment.**
- **71% successful recordings (273 cells) were obtained during one experiment.**
- **Pharmacology of ML-SA5 was highly reproducible with $EC_{50}$(Max/Min) = 2.**

## Results

### TRPML1 current characterization - ML-SA5 resultat in a pronounced inward rectification, characteristic for TRPML1. Currents reached a steady state after a 9-minute exposure time.

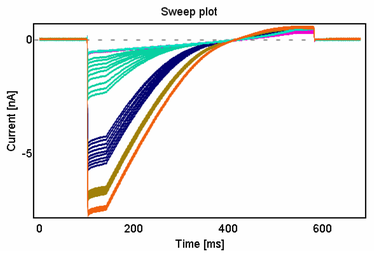


Fig. 1: Representative current traces from an entire experimental course. Small, ohmic current amplitudes were recorded during the baseline and test compound (here, vehicle control) period. Application of 25 µM ML-SA5 resulted in the evolution of a large current with pronounced inward rectification as characteristic for TRPML1.

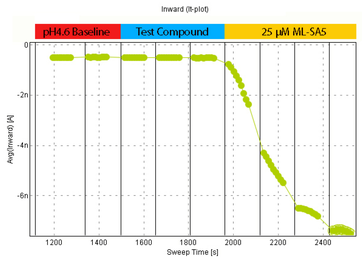


Fig. 2: Current over-time plot at V=-140 mV from the data shown in figure 4. ML-SA5 exhibited a relatively slow on rate. Currents reached a steady state after a 9-minute exposure time.

### Pharmacology and Reproducibility - Pharmacology of ML-SA5 was highly reproducible with $EC_{50}$(max/min) = 2. The EC50 value was calculated to be 3.4 µM

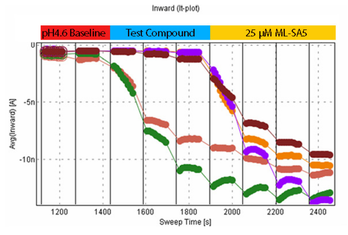


Fig. 5: Representative current over time plot from experiments with different concentrations of ML-SA5. Green = 30 µM; Red = 10 µM; Brown = 1 µM; Orange = 0.3 µM; Blue = 0.1 µM. Test compound was exposed to the cell for 7.5 min.

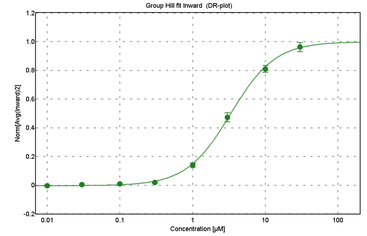


Fig. 6: Average concentration-response curve for ML-SA5. Data shown represents mean ±s.e.m. The $EC_{50}$ value was calculated to be 3.4 µM.

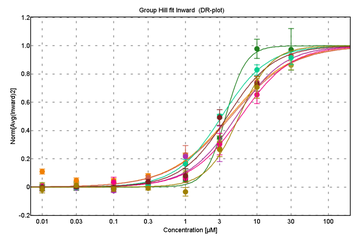


Fig. 7: TRPML1 assay reproducibility. ML-SA5 concentration-response curves from nine individual plate runs. The respective $EC_{50}$ values are shown in Table 1.

Table 1: ML-SA5 EC50 values as shown in Figure 10

| Plate Run | $EC_{50}$ [µM] |
|---|---|
| Run1 | 3.8 |
| Run2 | 5.5 |
| Run3 | 5.2 |
| Run4 | 3.0 |
| Run5 | 6.1 |
| Run6 | 3.0 |
| Run7 | 3.1 |
| Run8 | 3.7 |
| Run9 | 4.6 |
| **Max/Min** | **2.0** |

### Assay Performance - How to determine experiment quality when channel kinetics hamper seal resistance measurements? Inward rectification. This advanced filter allows for fast analysis of the large data set generated from one experiment.

Automated high throughput patch clamp systems such as the Qube 384 platform are capable of generating large datasets in a very short time. The presented TRPML1 assay had a duration of 42 min from start to finish and produced data from 384 cells. To allow fast processing of this data, it is imperative to implement automated, objective analysis algorithms that exclude unsuccessful experiments from the downstream analysis. Using the Analyzer software of Qube384, it is possible to include advanced filter to exclude unwanted experiments objectively.

An attractive way of filtering data is by including a seal resistance filter. Seal resistance typically correlates directly with the quality of the recording. TRPML1, in its activated state, conducts current over the entire voltage spectrum, and because of this, seal resistance cannot be correctly determined. As an alternative, we found that filtering data on parameters that are a measure for inward rectification (I(@V=+60mV)/I(@V-140mV)) and channel expression (I(Ref)/I(Baseline)) successfully excluded poor recordings to generate a robust dataset.

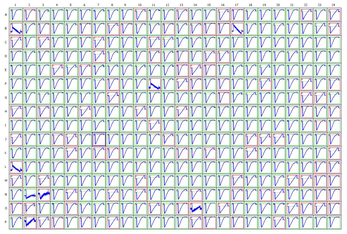

Fig. 3: 384-well plate view showing the last sweep of the experiment in the presence of 25 µM ML-SA5. Wells with a green frame passed the filter criteria described in the method section. In the shown example, a **success rate of 71%** was achieved.

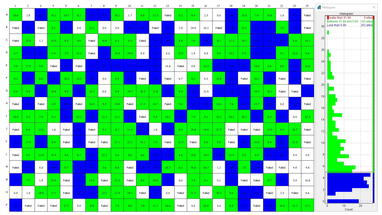

Fig. 4: The inducible TRPML1 cell line showed varying expression levels, with some cells devoid of the channel and others exhibiting pronounced expression. An important step in designing a robust automated assay is to select filter criteria that automatically exclude cells with specific objective parameters. Channel expression can be evaluated by dividing the current amplitude recorded at the end of experiment I(Reference) by the current recorded at the beginning (Baseline). Here shown is a plate view of this parameter. The visual representation helps the user to define the correct criteria. The histogram in the right panel allows moving the threshold for coloring wells. In the shown example, all white wells failed by the filter criteria as described in the method section; the blue wells showed a 1.6 and 5-fold current increase at V=-140 mV from the baseline to the reference period, and all green wells exhibited a larger than 5-fold increase.

## Methods

**Cell Culture:** Recombinant HEK293T-TRPML1 cells were generated by replacement of the two dileucine motifs ($15_{LL}$ and $577_{LL}$) of TRPML1, which are responsible for lysosomal targeting, with alanines to allow trafficking to the plasma membrane. Cells were induced with one µg/mL tetracycline 24h prior to the experiment.

**Solutions:** External solutions (in mM) 140 NaCl, 13 KCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES (only pH7.4 solution), 10 MES (only pH4.6 solution). pH4.6 solution was adjusted with HCl and the pH7.4 solution with NaOH.

Internal solution (in mM): 122 CsMeSO4, 4 NaCl, 10 EGTA, 2 $Na_2$-ATP, 2 $MgCl_2$, 20 HEPES, pH was adjusted with CsOH to pH7.2

**Experimental setup**: Experiments were conducted using single-hole QChips on the Sophion Qube 384 platform equipped with temperature control that was set to 22°C.

Immediately prior to the experiment, cells were prepared using Qube's onboard cell preparation unit. 4 ml of 2-4M cells per ml solution was transferred to the 384 well consumable. During cell addition and seal formation, an external solution with pH 7.4 was used. Following a 300 s-long seal formation period, a pressure pulse, as shown in figure 8, was applied to the cells to obtain the whole cell (WC) configuration. Figure 9 illustrates the experimental workflow. Briefly, once the WC configuration was established, the external solution was exchanged for the pH4.6 solution. In this more acidic environment, test compounds were evaluated in a non-cumulative fashion with three additions of the same concentration and a total of 7.5 min exposure time. The experiment was finished with an application of a reference compound (25 µM ML-SA5)

**Voltage protocol**: Cells were held at 0 mV throughout the experiment. Currents were evoked using 400 ms-long voltage ramps ranging from -140 mV to +60 mV (Figure 10). The inter sweep interval was 10 s, and currents were sampled at 1 kHz, with a cut-off at 300 Hz and Bessel filtering.

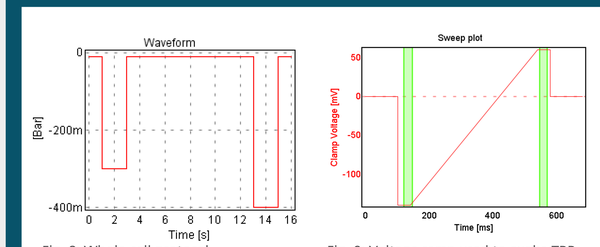


Fig. 8: Whole-cell protocol

Fig. 9: Voltage ramp used to evoke TRPML1 currents. The green curser intervals indicate the range that was used for further analysis.

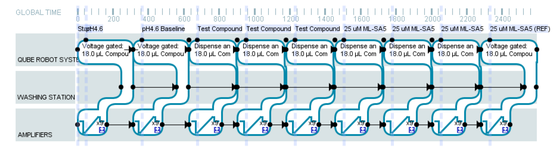


Fig. 10: Experimental set-up. Each block represents liquid addition as defined in the header. The liquid addition was followed by nine consecutive applications of the voltage ramp shown in figure 9.

**Analysis**: Data analysis was performed using Qube Analyzer (Sophion Bioscience). Compound effects were analyzed at V = -140 mV, and data was normalized to the second (Baseline) and the last (Reference) liquid period. 25 µM ML-SA5 was used as reference compound.

Data was filtered using the following filter criteria.

- Leak current: I(@V=+60mV) < 2nA
- Whole-cell formation: $C_{slow}$<2pF
- Ion Channel Expression: I(Ref)/I(Baseline) > 1.6
- Rectification: I(@V=+60mV)/I(@V-140mV) > -0.5
- Stability: 0.7<$C_{slow}$(Baseline/Reference)<1.3