Whitepaper

LYSO-Preps for organellar electrophysiology: A new tool for high throughput measurement of lysosomal TRPML1 channels using the SyncroPatch 384, SURFE²R N1 and SURFE²R 96SE

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Significance

- Lysosomes are essential for cellular homeostasis and their dysfunction is linked to a wide range of diseases. Given the immediate medical relevance, there is a current need for high throughput electrophysiology on lysosomes.
- Oria Bioscience have pioneered a novel approach for lysosomal isolation resulting in large batches of ready-to-use "LYSO-Preps" of high purity.
- We have successfully characterized "LYSO-Preps" overexpressing TRPML1 channels through pharmacology of activators, inhibitors and intraluminal pH sensitivity using the SyncroPatch 384, the SURFE²R N1 and the SURFE²R 96SE.

Introduction

The gold standard to functionally characterize lysosomal ion channels remains manual patch clamp recordings of enlarged individual lysosomes isolated from individual cells[1]. This technique is time-consuming and technically challenging, but other electrophysiological techniques are beginning to overcome these challenges, resulting in higher throughput characterization of isolated lysosomes. One of these techniques is automated patch clamp (APC), which uses a planar glass substrate for whole lysosome patch clamp recordings. When using lysosomes with APC, it is still necessary to isolate the lysosomes from the cell, which can be a lengthy process and requires precise timing in order to be used effectively with APC. Another technique to assess lysosomal ion channels is solid-supported membrane-based electrophysiology (SSME). This emergent method has been successfully used to record native lysosomes isolated using sequential centrifugation steps[2,3,4].

Recently, a novel approach for organelle isolation has been reported by Santinho et al[5]. Through the use of high-throughput, fluidic hardware isolation processes, this technique produces highly pure populations of large individual organelles. This breakthrough invention led to the foundation of Oria Bioscience, a company that provides highly pure preparations of organelles such as lysosomes, endosomes, endoplasmic reticulum, and mitochondria to the scientific community.

Oria Bioscience and Nanion Technologies have now developed assays to characterize isolated lysosomes with overexpression of the mucolipin TRP channel 1 (TRPML1) which is the principle Ca^{2+} channel in the lysosome[6,7]. Dysfunction of the TRPML1 gene has been associated with numerous diseases, including Mucolipidosis type IV (MLIV) or Niemann-Pick disease, which underlines the medical importance of TRPML1 and other lysosomal membrane proteins that could represent potential therapeutic targets for new medical strategies[7,8,9].

Consequently, there is a large interest in exploring intracellular ion channels and their pharmacology in their native environment using high-throughput electrophysiology. To this end, APC and SSME are increasingly used to record ion channels and transporters in intact lysosomal membranes. In this report, we functionally characterize LYSO-Preps provided from Oria Bioscience using the SyncroPatch 384 and the SURFE²R N1 and SURFE²R 96SE instruments.

Experimental setup

LYSO-Preps (Ready-to-use enlarged Lysosomes) isolated from HEK cells were provided by Oria Bioscience, either containing endogenous channels or overexpressed TRPML1 channels. For recordings using the SyncroPatch 384, the use of NPC-384T nanoS-type (1x; PN: 22 2111) consumables were critical for the success of these experiments and were specifically designed to support and maintain lysosomal integrity during the recording process. LYSO-preps overexpressing lysosomal marker TMEM192 are shown Figure 1A. The lysosomal diameters in a typical LYSO-Prep are shown in Figure 1B with lysosomal diameters' in the range of a few micrometers. This distribution indicates that the majority of lysosomes used for the APC recordings were larger than 2 µm. Given the highly efficient isolation procedure, the samples from Oria Bioscience for APC recordings with the SyncroPatch 384 recordings usually contained $2x10^7$ lysosomes that were diluted to a density of 200k lysosomes per ml which was sufficient to execute several SyncroPatch 384 chips (> 5). Our studies using the SURFE^{2}R instruments do not require pre-enlargement by vacuolin (or other agents) upon application to the SURFE^{2}R sensors (diameter 3 mm; N1 PN: 161001, 96SE PN: 181001). Native LYSO-Preps (unenlarged lysosomes) had a total protein concentration of ~2 mg/ml as determined via Bradford assay (Data not shown) and were stored frozen at -80°C until usage. As shown in Figure 1C, Western blot analysis confirms that the Oria LYSO-Preps are highly pure and dense, without contamination from other organelles or plasma membranes, a common issue with centrifugation-based isolation methods[10]. For the SyncroPatch 384 recordings, we also implemented a low cell (lysosome) density measurement approach, using

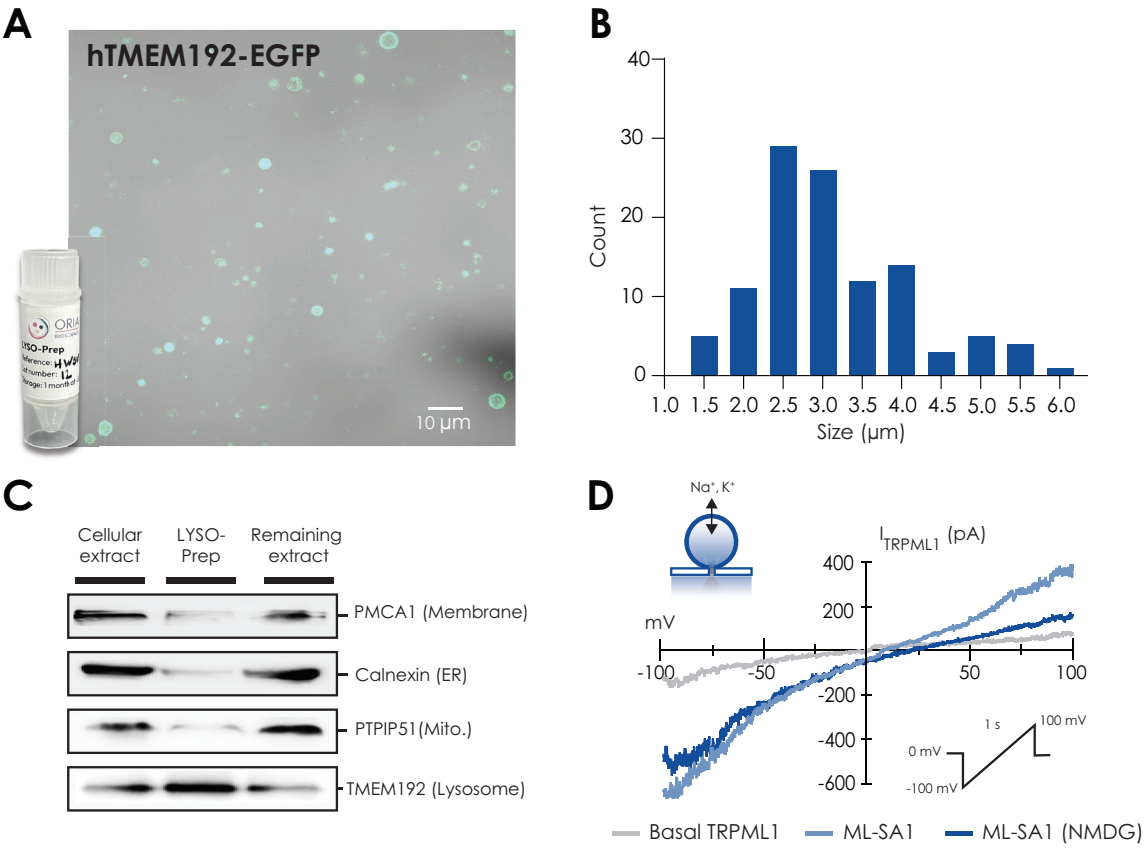

Figure 1 A *Image of lysosomes isolated from HEK293 cells overexpressing hTMEM192-EGFP enlarged with vacuolin which are included in the standard LYSO-Prep package. Image taken with bright field/fluorescence (X63 Oil Objective).* **B** *Example size distribution of isolated lysosomes transiently overexpressing hTMEM192-EGFP after enlargement by 1 µM vacuolin. Only patchable lysosomes (>1 µm) were analyzed using FIJI software.* **C** *Representative immunoblot of protein markers before purification (cellular extract) and after purification (LYSO-Prep and remaining extract) as indicated by the markers for different cellular compartments.* **D** *Example SyncroPatch 384 experiment from a whole-lysosome recording applying a ramp protocol from -100 to 100mV at a holding potential of 0 mV and application of ML-SA1 and NMDG solutions as indicated. Inset: Simplified schematic of the lysosome attracted to the patch clamp aperture.*

fewer lysosomes and a reduced volume while maintaining a high catch rate. This method allowed us to achieve stable lysosomal seals in 'whole-lysosome' mode, with seal resistances consistently exceeding 0.2 GigaΩ throughout the experiment. This stability was crucial for generating cumulative dose-response curves and enabling intraluminal solution exchange (Figures 1D, 2, & 3). This differs to the SURFE^{2}R instruments where the recordings are based on the summary signal (currents) from the sensors' surface where the signal to noise ratio depends on balancing the sample consumption and the desired signal amplitudes.

Results: APC

Figure 1D shows a representative recording after reaching whole-lysosome configuration with intraluminal pH 7.2 where we activated TRPML1 currents by adding the specific activator ML-SA1 to the cytoplasmic solution[11]. Subsequently, the application of the non-permeable cation NMDG resulted in blockage of the inward current fraction leading to a shift in the reversal potential of approximately 30 mV (data not shown), highlighting TRPML1 as a cation-selective channel.

Given the known pH dependence of lysosomal ion channels, as well as the proton-permeation of some channels under lysosomal acidic pH, we employed intraluminal solution exchange to determine pH sensitivity of both WT - and TRPML1 overexpressing lysosomes Figure 2A). As highlighted in Figure 2B, application of intraluminal solutions with more acidic pH values increased outward and inward currents from WT- as well as TRPML1 overexpressing lysosomes. Normalizing the observed current increases to the maximum response and fitting the data with the Hill-equation yielded an EC_{50}[pH] (± SD) at +100 and -100mV with 5.0 ± 0.7 and 5.1 ± 0.9 for WT- as well as 6.1 ± 0.7 and 5.8 ± 0.9 for TRPML1 overexpressing lysosomes, respectively. We have also determined an exemplary success rate (similar for WT and TRPML1 overexpressing lysosomes) where we observed a final success rate of 43.2 % corresponding to 166 lysosomal recordings in one run where we applied quality filters of RSeal of > 200 MΩ and current at -100 mV < -150 pA.

Next, we employed pharmacological experiments at intraluminal pH 7.2 with known activators (ML-SA1, MK6-83) and inhibitors (ML-SI3) as highlighted in the traces recorded with the SyncroPatch 384 in Figure 3A. To determine the EC_{50} for the compounds, we executed cumulative dose-response experiments with 4 increasing ML-SA1 concentrations and used ML-SI3 to inhibit the currents at the end of the

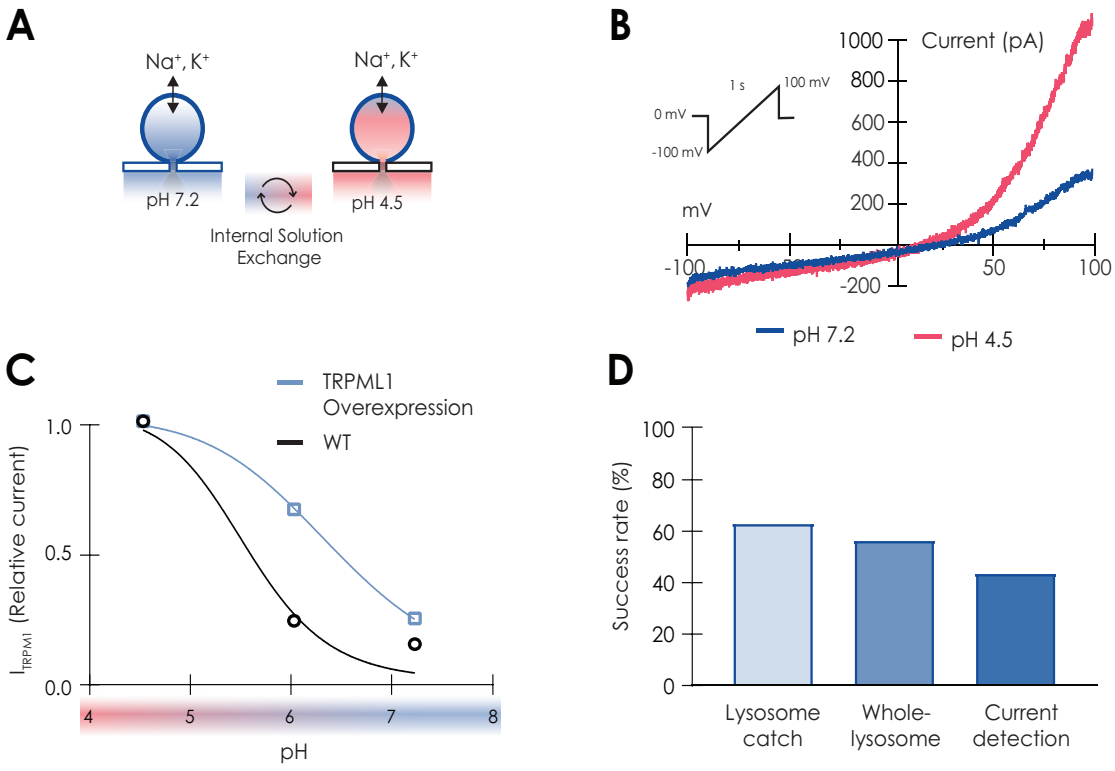

Figure 2 A *We employed repetitive intraluminal solution exchange during continuous experiments to assess pH sensitivity.* **B** *Example SyncroPatch 384 recording from enlarged (vacuolin) isolated WT lysosomes where intraluminal solution was exchanged from pH 7.2 to 4.5.* **C** *Intraluminal pH sensitivity (100 mV) comparing WT- and TRPML1 overexpressing lysosomes with an EC_{50} [pH] (± SD) of 5.0 ± 0.7 (n = 142) and 6.1 ± 0.7 (n = 180), respectively.* **D** *Time course and success rate of an example experiment yielding a success rate of 43.2 % (=166/384) at the end of recording. Quality control filters include: RSeal of > 200 MΩ throughout the entire experiment and current at -100mV < -150pA from the start.*

experiment (Figure 3B). Normalized responses from similar recordings with the data fitted with Hill-equation yielded an EC_{50} (± SD) of 5.5 ± 2.9 µM and 4.8 ± 2.9 µM for +100 and -100mV for ML-SA1 as well as 1.8 ± 1.2 and 2.5 ± 1.3 for MK6-83, respectively (Figure 3C). The data for both compounds shows no significant difference at either voltage for ML-SA1 as well as MK6-83 similar to reported values in the literature[11,12,13]. Figure 3D shows a comparison of the current distributions at -100 mV from TRPML1 overexpressing lysosomes stimulated by the highest concentration of ML-SA1 (30.69 µM) at intraluminal pH of 7.2 yielding a mean current of -312 pA (n = 96) vs activation by luminal acidic pH (4.5) yielding a mean current of -317 pA (n = 214). The data observed was not significantly different (t-test) suggesting similar stimulatory efficacy by either luminal acidification or pharmacological activation by ML-SA1.

Results: SSME

To further analyze and validate the versatility of the LYSO-Preps provided by Oria Bioscience, we employed the SURFE^{2}R instruments, which utilize solid-supported membrane based electrophysiology. SSME is compatible with various membrane preparations, including lysosomes or other organelles, that contain the protein of interest (in this case, TRPML1). These membrane vesicles are attached to a gold sensor, 3 mm in diameter, via a lipid layer (Figure 4). This technology has been widely used to study lysosomal channels, such as TMEM175, in their native environment[4] and, in contrast to patch clamp, is not dependent on lysosomal enlargement. Furthermore, the samples can be stored for months without reduction in recording quality or the necessity of a running cell culture. SSME relies on the exchange of solutions to provide the substrate or ligand to activate the transporter/channel. The resulting charge translocation is detected as a transient capacitive current which is subsequently analyzed. We have highlighted the methodological differences between patch clamp and SSME approaches in Table 1.

TRPML1 is the major Ca^{2+} permeable channel in lysosomes[6,7]. Therefore, all SSME experiments started with the application of 2 mM Ca^{2+} to stimulate TRPML1-mediated Ca^{2+} currents.

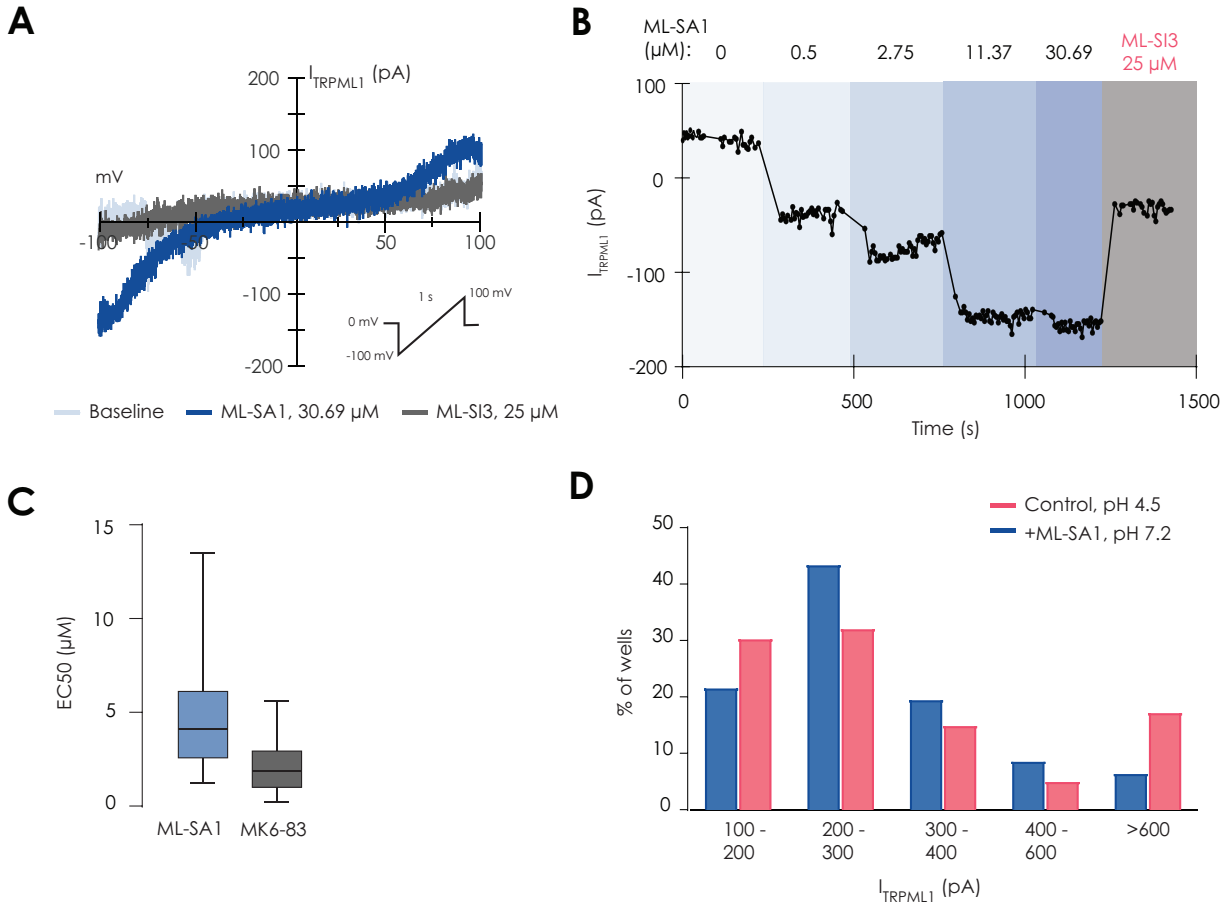

Figure 3 A *Example SyncroPatch 384 recording from enlarged (vacuolin) isolated lysosomes overexpressing TRPML1 activated by ML-SA1 and inhibited by ML-SI3.* **B** *Currents at -100 mV upon application of increasing concentrations of ML-SA1 and inhibition by ML-SI3 over a timecourse of > 23 min.* **C** *Box plot of EC_{50} from the mean current responses at -100 mV (Hill-equation) in the presence of ML-SA1 and MK6-83 in the low micromolar range.* **D** *Current distributions at -100 mV show no significant difference (t-test) upon acidification or stimulation with ML-SA1 at the highest concentration used (30.69 µM).*

	Manual Patch Clamp	APC (SyncroPatch 384)	SSME (SURFE²R N1 & SURFE²R 96SE)
Measurement Principle	Voltage Clamp (Current Clamp)	Voltage Clamp (Current Clamp)	Capacitive Currents induced by concentration jump via fast solution exchange.
Cell Culture & Sample Storage	Fresh lysosomal isolation from running culture.	Fresh lysosomal isolation from running culture.	Fresh lysosomal isolation from running culture & sample freezing possible.
Target Range	Voltage gated, ligand gated & leak channels.	Voltage gated, ligand gated & leak channels.	Transporters, pumps, ligand gated & leak channels.
Sample Treatment	Artificial enlargement (e.g. Vacuolin, Apilimod).	Artificial enlargement (e.g. Vacuolin, Apilimod).	No lysosomal enlargement needed.
Sample Consumption	One lysosome per cell.	100-200 k lysosomes/ml (1 ml/plate).	Total protein amount 40 µg/plate (SURFE²R 96SE).
Throughput	~4-8 recordings/day.	~200-1000 recordings/day.*	N1: ~15-20 recordings/day.# 96SE: ~250-450 recordings/day.#

*Successful 4 -point concentration response curve. 5 plates per day.
#Successful 4 -point concentration response curve. 8 hour day.

Table 1 *Overview of techniques to record lysosomal channels. The 'gold standard' manual patch clamp, automated patch clamp (APC) and solid supported membrane-based electrophysiology (SSME).*

We then repeated Ca^{2+} activation in the presence of inhibitors and enhancers to modulate the Ca^{2+} currents and further study the pharmacological properties of TRPML1. Figure 5C shows the dose dependent activation by increasing concentrations of ML-SA5 leading to increasing Ca^{2+} currents. Figure 5E shows the currents normalized to the Ca^{2+} response in the absence of compound on th SURFE²R N1. The data was fitted with a Hill-equation yielding an EC_{50} of 22 ± 9 nM which is somewhat lower than previously reported values studied in metastatic melanoma tissue[14]. Intriguingly, ML-SA5 did not significantly affect currents recorded with isolated lysosomes without TRPML1 overexpression highlighting the sensitivity of the assay. We then transferred the assay to the high throughput SURFE²R 96SE. This, coupled with a very high success rate (~99%), allows for a large increase in the amount of measured samples (95 recordings for one plate) without an impact on pharmacology (Figure 5F, EC_{50} = 33 ± 22 nM). We also investigated the effect of ML-SI3 with the N1 under the same conditions yielding an IC_{50} of 0.74 ± 0.1 µM, again in good agreement with the literature[15]. This complements the SyncroPatch 384 results and confirms the specificity of these compounds for TRPML1. The results from both APC and SSME techniques were in good agreement with the literature for ML-SA1, MK6-83 as well as ML-SI3[11,12,13,14,15]. To the best of our knowledge, EC_{50} values have not been previously reported from isolated cells treated with ML-SA5.

Conclusions:

We report here for the first time the use of freshly isolated, shipped and ready-to-use (no cell culture needed) lysosomes from Oria Bioscience. The samples were successfully characterized by two different techniques, APC and SSME, confirming their applicability for high throughput investigation. Due to the near 100% success rate associated with the SURFE²R 96SE, it is extremely well-suited for drug development applications in the pharmaceutical industry.

The samples from Oria Bioscience represent a highly pure and reliable source of homogeneous high-quality lysosomes that we could use across both devices delivering EC / IC_{50} values in accordance with the literature. This allows a more detailed understanding of the function of lysosomal channels as well as their role as a therapeutic target. This also circumvents the possible caveats that stem from the use of heterologous expression systems expressing these channels in the plasma membrane or the usage of indirect recording approaches[4]. We have applied our approach to record currents from WT as well as TRPML1 overexpressing lysosomes and could detect the intraluminal pH sensitivity shifted by ~1 pH unit to more alkaline upon TRPML1 overexpression versus WT lysosomes. We used specific pharmacology (ML-SA1/5, MK6-83; ML-SI3) and strict quality control to confirm the validity of our recordings. The same is also true for the experiments performed on the SURFE²R instruments, where we could use unenlarged samples from the same shipment of lysosomes to verify the pharmacological properties of TRPML1, with no signal present in control recordings from lysosomes without overexpression.

Our results clearly indicate that the combination of our NPC-384T nanoS-type chips and low cell density approach for the SyncroPatch 384 as well as usage of the SURFE²R instruments should provide efficient and cost-effective means of electrophysiological access to lysosomal channels.

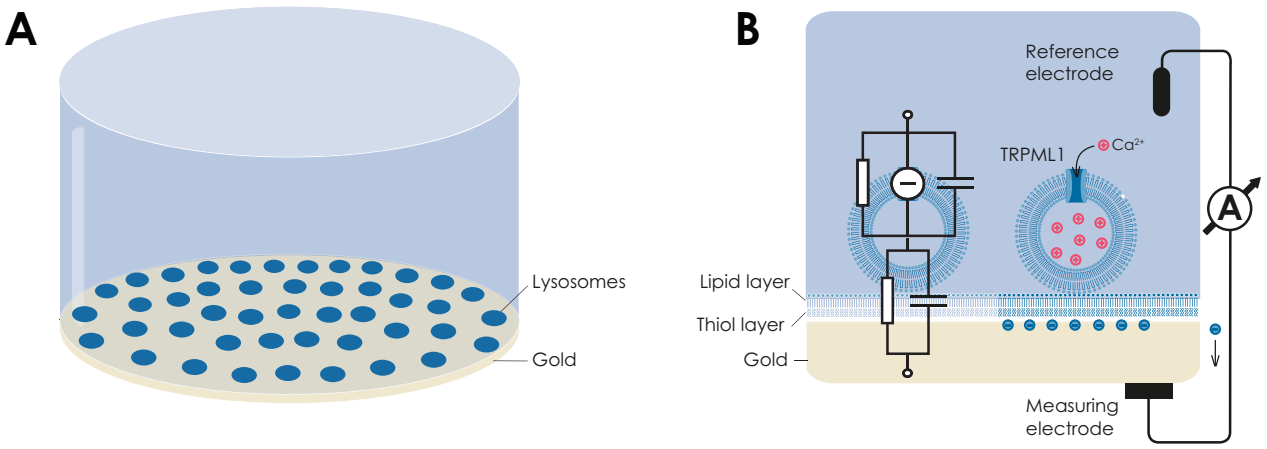

Figure 4 A *Schematic representation of a gold coated 3 mm SURFE²R sensor with multiple lysosomes attached.* **B** *A magnified schematic of the solid supported membrane (SSM) which covers the gold sensor. Ca^{2+} influx through TRPML1 is stimulated by 2 mM Ca^{2+} concentration jump at 0 mV. The resulting transient current is recorded via capacitive coupling of the lysosomal membrane with the SSM and the peak current corresponds to the Ca^{2+} flux through TRPML1 under steady-state conditions.*

We expect this can be expanded to include various ion channels expressed in other types of organelles and intracellular compartments as has been demonstrated in Santinho et al[5] highlighting the broad range of applications possible with Nanion devices.

Methods: Lysosomal isolation

LYSO-Preps contain lysosomes enlarged (1µM vacuolin) or unenlarged at a number of $2x10^7$ for APC or at a protein concentration of ~2mg/ml for SSME. LYSO-Preps were produced & provided by Oria Bioscience (https://www.oriabs.com) and its proprietary platform where lysosomes were isolated from HEK cells as described previously[5].

Methods: Electrophysiology APC

Whole-cell patch clamp recordings were conducted according to Nanion's standard procedures for the SyncroPatch 384. All recordings were performed at room temperature (21°C) using planar borosilicate glass chips and the use of NPC-384T nanoS-type (1x; PN: 22 2111). Lysosomes were held at a holding potential of 0 mV before the application of a voltage ramp protocol from -100 to 100 mV over 1 s. This was applied every 5 s at a sampling frequency of 5 KHz without leak-subtraction. To estimate EC_{50}, currents were normalized to maximum response and data fitted using the Hill- equation. More detailed experimental procedures are available upon request.

Methods: Electrophysiology SSME

SSME recordings were performed under continuous solution flow with the SURFE²R N1 instrument and the SURFE²R 96SE instrument, a high throughput device capable of simultaneously measuring 96 sensors, with a near 100% success rate[4]. For the SURFE²R N1, Each sensor was prepared by adding ~0.5 µg total protein as determined using a Bradford assay. For SURFE²R 96SE, the sensor plate coating and preparation of compound plates was done on the instrument itself according to Nanion standard protocols. Ca^{2+} influx through TRPML1 was stimulated by solution exchange from non-activating solution to the activating solution at 0 mV. Non-activating solution contained an additional 4 mM choline chloride, while activating solution was supplemented with 2 mM $CaCl_2$. Enhancers and blockers were provided by rinsing the sensor with 1 ml of non-activating solution and incubating for 3 minutes before the measurement. They were present in both non-activating and activating solutions during the measurement. More detailed experimental procedures are available upon request.

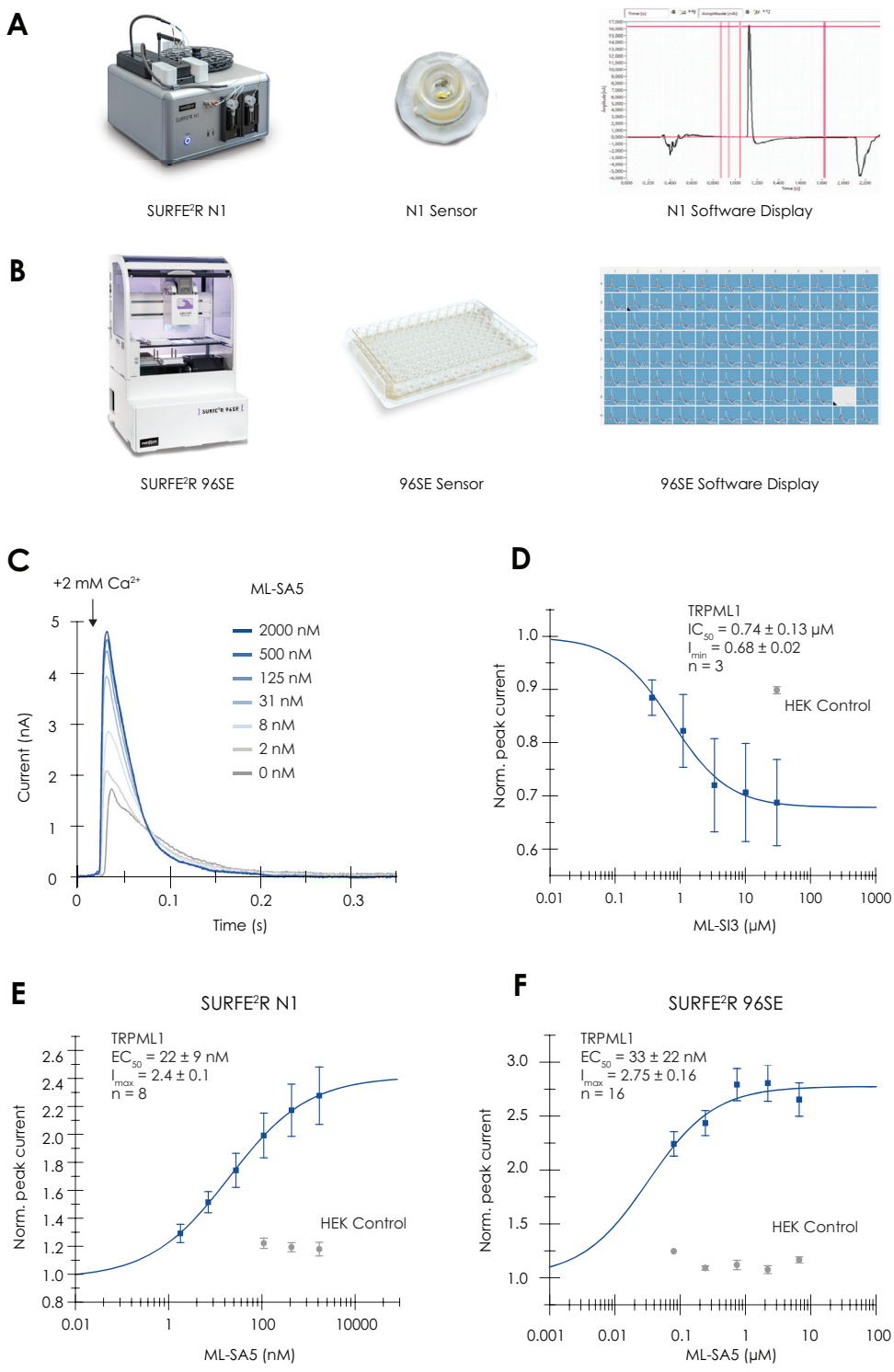

Figure 5 A *SURFE²R N1 platform for SSME recordings in a single-well format (left). Three-millimeter sensor for SURFE²R N1 recordings (middle). Screenshot of the SURFE²R N1 control 1.7.0.2 software (right).* **B** *SURFE²R 96SE platform for SSME recordings in a high throughput format (left). 96-sensor well-plate for SSME measurements with the SURFE²R 96SE (middle). Screenshot of the SURFControl 96 1.7 software (right).* **C** *Representative current traces recorded from the same 3 mm sensor using different concentrations of the TRPML1 enhancer ML-SA5.* **D** *Peak currents recorded from the SURFE²R N1 were normalized to the peak current in absence of ML-SI3 and then averaged to obtain mean and SD. The data was fitted with a standard Hill equation to obtain IC_{50} and I_{max} values. The same experiment and analysis was carried out using lysosomes purified from HEK293 cells that did not overexpress TRPML1. Here, no significant effect was observed at the highest Inhibitor concentrations. The remaining current at the highest blocker concentration (I_{min}) reflects the background current resulting from Ca^{2+} binding to the membrane, which can be subtracted to obtain the net TRPML1 current.* **E** *Peak currents recorded from the SURFE²R N1 were normalized to the peak current in absence of ML-SA5 and then averaged to obtain mean and SD. The data was fitted with a standard Hill equation to obtain EC_{50} and I_{max} values.* **F** *The same experiments as in* **E** *were carried out with the SURFE²R 96SE. Fit algorithm and normalization was the same as* **E**.

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