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ELECTROPHYSIOLOGY

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Since its launch in the early 1980s, the patch clamp method has been extensively used to study ion channels in the plasma membrane, but its application to the study of intracellular ion channels has been limited. Unlike the plasma membrane, intracellular membranes are usually not stable enough to withstand mechanical manipulation by glass electrodes during seal formation and rupturing of the membrane. To circumvent these problems, we developed a method involving the immobilization of isolated organelles on a solid matrix planar glass chip. This glass chip contains a microstructured hole that supports the formation of gigaseals and subsequent electrophysiological recordings despite the high fragility of intracellular membranes. Here, we report the experimental details of this method using lysosomes, which are the smallest cellular organelles, as a model system. We demonstrate that we can record endogenous ionic currents from wild-type lysosomes, as well as from lysosomes overexpressing ion channels, and expect that this method will provide electrophysiological access to a broad range of intracellular ion channels.

Introduction

The development of the patch clamp method by Neher and Sakmann revolutionized the electrophysiological characterization of ion channels in cells (*1*). This technology made possible the resolution of ionic currents even through single channels in the plasma membrane and has become the standard method for the functional characterization of ion channels. However, the patch clamp technology has been mostly limited to the characterization of ion channels in the plasma membrane. In contrast, ion channels localized in intracellular compartments, such as lysosomes, endosomes, mitochondria, and cisternae of the endoplasmic reticulum (ER), are not readily accessible by glass pipettes that are used to perform patch clamp analysis.

The membrane of intracellular organelles makes up more than 95% of the total cell membrane system. Thus, it is not surprising that the group of intracellular ion channels is at least as large as that of ion channels in the plasma membrane. Intracellular ion channels are present in various cellular organelles (Table 1). Among these, intracellular Ca^{2+}-release channels have raised particular interest, because these channels are fundamental for the control of numerous physiologic functions, including muscle contraction, secretion, cell motility, and immune response (*2*). Traditionally, intracellular Ca^{2+} release has been attributed to ryanodine and inositol trisphosphate (IP_3) receptors in the sarcoplasmic and endoplasmic reticulum (SR/ER) and the nuclear envelope (*2*–*6*). Ca^{2+} flux through these channels has been functionally investigated with bilayer studies and Ca^{2+}-imaging experiments. There is growing evidence that lysosomes are also critically involved in Ca^{2+} signaling (*7*–*10*). Furthermore, specific Ca^{2+}-release channels and numerous other ion channels have been identified in the lysosomal-endosomal compartment (Table 1). Loss of function or dysfunction of lysosomal ion channels is involved in human lysosomal storage diseases (Table 1) (*11*–*14*), which suggests that lysosomal ion channels are pathophysiologically relevant proteins that have potential to become drug targets.

So far, a limited number of methods are available for the characterization of intracellular ion channels. The standard method is the reconstitution of purified ion channel proteins or membranes of isolated cell organelles into synthetic phospholipid bilayers (*15*–*17*). Alternatively, purified channel proteins or membrane vesicles can be reconstituted into liposomes (*18*) that are quite large and can be analyzed by means of conventional patch clamp. The main conceptual drawback of these methods is that ion channel proteins are extracted from their physiological environment. This procedure bears the risk that important factors, such as crucial components of the lipid membrane as well as specific modulators and accessory subunits, that are associated in vivo with the ion channel protein are lost. Additionally, copurification of proteins that nonspecifically interfere with the activity of the ion channel of interest or that form additional conductances can potentially lead to wrong interpretations of currents obtained from bilayer experiments. As a consequence, ionic current measurements in lipid bilayers are intrinsically difficult to standardize and validate. Other approaches to investigate intracellular ion channels in organelles included Ca^{2+} imaging (*19*) and flux measurements (*20*, *21*). However, these methods lack some advantages of patch clamp analysis, including the ability to directly access ion channels.

Technically, the major problem preventing the characterization of intracellular ion channels by glass pipette-based patch clamp measurements has been the maintenance of organelle integrity. Here, we describe a method that is well suited to characterize ionic currents even from lysosomes, which are the smallest cellular organelles. We use solid-matrix planar glass chips that contain a small hole to immobilize and support the lysosomes (Fig. 1). With this approach, we efficiently obtain gigaseals and electrophysiological recordings. We provide detailed instructions for the recording of endogenous ionic currents from wild-type lysosomes, as well as from lysosomes overexpressing ion channels. This Protocol assumes basic knowledge of patch clamp methods. Compared with conventional patch clamp methods, the planar patch clamp method allows reliable characterization of currents from a large number of isolated lysosomes (up to eight lysosomal recordings per preparation). Moreover, this method does not require extensive and time-consuming protein purification steps commonly required for ion channel reconstitution into lipid bilayers or liposomes. The planar patch clamp method will provide electrophysiological access not only to lysosomal ion channels, but also to a broad range of other intracellular ion channels.

Table 1. Localization, function, and channelopathies associate with intracellular ion channels. Some of the ion channels are present in intracellular organelles as well as in the plasma membrane. "Function" relates to general function of the ion channels. *, mouse model; #, human channelopathy; nd, not determined; TRP, transient receptor potential channel; ClC, chloride channel; IP_3, inositol 1,4,5-triphosphate; K_{Ca}, Ca^{2+}–dependent K^+ channel; K_{ATP}, ATP-dependent K^+ channel; K_V, voltage-dependent K^+ channel; IMAC, mitochondrial inner membrane anion channel; UCP, uncoupling protein.

Organelle	Ion channel	Ion selectivity	Function	Channelopathy	References
Lysosomes and Endosomes	TRPM1	nd	Tumor suppressor, potential role in mediating synaptic transmission in bipolar cells	Autosomal recessive congenital stationary night blindness # Metastasis and poor prognosis in melanoma#	(*30*, *31*)
	TRPM2	$Na^+ > Ca^{2+} > Mg^{2+} > Cs^+$	Oxidant stress sensor, mediates H_2O_2 dependent cell death	Guamanian amyotrophic lateral sclerosis/parkinsonism dementia complex	
	TRPM7	$Zn^{2+} > Ni^{2+} > Ba^{2+} > Mg^{2+} > Mn^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+}$	Synaptic vesicle function Anoxia-induced cell death	Guamanian amyotrophic lateral sclerosis/parkinsonism dementia complex#	
	TRPML1	$K^+ > Na^+ > Ba^{2+} > Sr^{2+} > Ca^{2+}$	Membrane sorting, late steps of endocytosis, or both	Mucolipidosis 4#	(*30*, *31*)
	TRPML2	Cation nonselective	nd	nd	
	TRPML3	Cation nonselective	nd	Varitint-Waddler mouse*	
	TRPV1	$Ca^{2+} > Mg^{2+} > Cs^+ = K^+ = Na^+$	Thermosensation and nociception	nd	(*30*, *31*)
	TRPV2	$Ca^{2+} > Mg^{2+} > Cs^+ = K^+ = Na^+$	Thermosensation and nociception	nd	
	TRPV5	Ca^{2+}	Ca^{2+} reabsorption	Osteoporosis, hypercalciuria*	
	TRPV6	$Ca^{2+} > Sr^{2+} = Ba^{2+} > Mg^{2+}$	Ca^{2+} reabsorption	Alopecia, dermatitis, decreased intestinal Ca^{2+} reabsorption*	
	TRPC3	$Ca^{2+} > Na^+$	BDNF-induced chemoattractive turning	Cerebellar ataxia (moonwalker mouse)*	(*30*, *31*)
	TRPC5	$Ca^{2+} > K^+ = Cs^+ = Na^+$	Regulation of growth cone extension	Susceptibility to pyloric stenosis#	
	TPCN 2	Ca^{2+}	NAADP-dependent Ca^{2+} release	Polymorphic TPCN2 variants are associated with blond hair color	(*22, 23, 26*)
	TPCN1/3	nd	Possible NAADP-dependent Ca^{2+} release	nd	
	ClC3	Cl^-	Acidification of endosomes and synaptic vesicles	Loss of hippocampus, blindness*	(*32*)
	ClC5	Cl^-	Acidification of endosomes	Dent's disease characterized by proteinuria and kidney stones#	
	ClC7	Cl^-	Acidification of lysosomes, resorption lacuna of osteoclasts	Osteopetrosis and lysosomal storage disease#	
	ClC4 and ClC6	Cl^-	nd	nd	

Continued on next page

Organelle	Ion channel	Ion selectivity	Function	Channelopathy	References
Melanosomes	TRPM1	See text	See text	See text	
	TRPML3	See text	See text	See text	
Synaptic vesicles	TRPM7	See text	See text	See text	
	ClC3	See text	See text	See text	
Endo/ sarcoplasmic reticulum	IP_3 receptor	Ca^{2+}	IP_3 dependent Ca^{2+} release	Cerebellar ataxia#	(*30, 33*)
	Ryanodine receptor	Ca^{2+}	Ca^{2+} induced Ca^{2+} release	Susceptibility to malignant hyperthermia#, central core disease#, arrhythmia#	(*3, 30, 34*)
	TRPV1	See text	See text	See text	
	TRPM8	$Cs^+ > K^+ > Na^+$	A cold and menthol receptor Integration of thermal and chemical stimuli	nd	(*30*, *31*)
	TRPP1	K^+	nd	Polycystic kidney disease#	(*30*, *31*)
	TRPP2	$Na^+ > Ca^{2+} = Sr^{2+} = Ba^{2+}$	Acid sensing in sour taste and cerebrospinal fluid	nd	
trans-Golgi	TRPV1	See text	See text	See text	
	IP_3 receptor	See text	See text	See text	
Mitochondria	K_{Ca}	K^+	Volume regulation	SNP associated with hypertension, myocardial infarction, and stroke#	(*35, 36*)
	K_{ATP}	K^+	Volume regulation, protection against apoptosis/ischemic injury	Diabetes, hyperinsulinism, dilated cardiomyopathy, adrenergic atrial fibrillation#	(*35, 37, 38*)
	$Kv_{1.3}$	K^+	Cell death	Knockout mice were protected from diet-induced obesity	(*30, 35*)
	IMACs	Anions	Volume regulation	nd	(*35*)
	UCP1-3	H^+	Thermogenesis	nd	(*35*)
	VDAC	Ca^{2+}, K^+, Na^+, ATP, ADP, P_i	Metabolite transport, apoptosis	nd	(*35*)

Materials

5-ml microfuge test tube

175-cm^2 dishes for cell culture (Sarstedt, #831803)

2-, 10-, 200- and 1000-μl pipettes

25-cm cell scrapers (BDFalcon, #353086)

Agar (Applicam, #A0949)

Bleach solution (Nanion) Sodium hypochlorite solution (NaClO), 12% Cl

$CaCl_2$-$2H_2O$

CaMSA

Cell culture flasks (75 cm^2; Greiner Bio one, #658175)

Complete protease inhibitor cocktail, EDTA-free (Roche, #04693132)

DMSO

Dulbecco's modified Eagle medium containing 25 mM glucose (DMEM supplemented with 4.5 g/l glucose and pyruvate and Glutamax; Invitrogen, #31966-021).

EGTA

Fetal bovine serum (FBS; Biochrom, #S0615)

HCl

Human embryonic kidney (HEK) 293 cells stably overexpressing ion channels under investigation; here, mTPCN2

HEPES

Hygromycin B, 50 mg/ml solution (Carl Roth, #CP12.2)

KCl

KF

KH_2PO_4

KMSA

KOH

Mannitol

Methanesulfonic acid (Fluka, #64280)

$MgCl_2$

Micropipettors

Na_2HPO_4-$2H_2O$

NaCl

Pen-strep (penicillin 10,000 units/ml; streptomycin 10,000 μg/ml; Biochrom)

Sterile syringe filters 0.2 μm (VWR, #5140061)

Sterile syringes (VWR, #612-0120)

Sucrose (Sigma, #84100)

Tris(hydroxymethyl)aminomethane (Tris, Prolabo, #103156x)

Vacuolin-1 (Sigma, #V7139)

Equipment

Cell Culture

Humidified, cell culture incubator at 37°C, 10% CO_2

Laminar flow hood

Water bath, 37°C

Lysosomal Preparation

10-ml, round-bottomed, open top, thick-walled polycarbonate tube (Beckman, #355630)

Beckman 45 Ti fixed-angle rotor

Motor-driven tightly fitting glass/Teflon Potter homogenizer (Potter S, B. Braun)

Refrigerated centrifuge for 1.5 microfuge tubes (Eppendorf centrifuge 5415R)

Rotamax 120 rotary shaker (Heidolph)

Rubber adapter sleeve for centrifuge tube 16-mm delrin adaptor tube (Beckman, #303448)

Ultracentrifuge (Sorvall discovery 90)

Electrophysiology

Computer with 21-inch TFT monitor (Dell)

Freezing-point osmometer OM802 (Vogel)

NPC-1 chips (single use, disposable) microstructured glass chip containing an aperture of ~1 μm diameter (Nanion)

Patch-clamp amplifier (EPC-10, HEKA Instruments Inc.)

PatchControl Software (Nanion Technologies)

Port-a-Patch (Nanion Technologies)

Software for data acquisition (Patchmaster, HEKA Instruments Inc.)

Software for data analysis (OriginPro7.5, OriginLab Corporation)

Recipes

Recipe 1: Cell Culture Medium

Fetal bovine serum	10%
Pen-strep	100 U/ml
Hygromycin B	100 μg/ml
DMEM	500 ml

Mix components and filter-sterilize. Store at 4°C; warm to 37°C before use.

Recipe 2: Stock Solution Vacuolin 1 mM

Dissolve 1 mg of vacuolin in 1.732 ml of DMSO; mix well and store at 4°C.

Recipe 3: 16 mM $CaCl_2$

Dissolve 0.2352 g of $CaCl_2$ in 100 ml of distilled water; mix well and store at 4°C.

Recipe 4: Complete Protease Inhibitor Cocktail

Dissolve 1 tablet in 2 ml distilled water to prepare a 25× EDTA-free solution and store at 4°C or for long-term storage prepare aliquots of 250 μl and store at –20°C.

Recipe 5: Phosphate-Buffered Saline (PBS)

NaCl	137 mM
$Na_2HPO_4x2H_2O$	8 mM
KH_2PO_4	1.76 mM
KCl	2.7 mM

Adjust pH to 7.4 with HCl, prepare 500-ml aliquots, heat sterilize, and store at room temperature up to several months.

Recipe 6: Homogenization Buffer

Sucrose	0.25 M
Tris	10 mM

Adjust pH to 7.4 with HCl, sterilize by passing through a 0.2-μm filter, prepare 1-ml aliquots, and store at –20°C for several months. Before use, add 40 μl of Complete Protease Inhibitor Cocktail (Recipe 4) (final concentration 1×) per aliquot of homogenization buffer.

Recipe 7: Washing Buffer

KCl	150 mM
Tris	10 mM

Adjust pH to 7.4 with HCl, sterilize by passing through a 0.2-μm filter, prepare 4-ml aliquots, and store at –20°C for several months. Before use, add 160 μl of Complete Protease Inhibitor Cocktail (Recipe 4) (final concentration 1×) per aliquot of Washing Buffer.

Note: Recipes 8 through 14 refer to lysosomal recording solutions. Carefully adjust the osmolarity and the pH. Intralysosomal pH (pH 4.6) regulates several lysosomal ion channels. Measure the osmolarity of recording solutions with a freezing-point osmometer and adjust if necessary with nonpermeating molecules, such as mannitol or sucrose, to 290 mOsM for all internal solutions and between 290 and 310 mOsM for all external solutions, unless otherwise stated. External osmolarity should always be higher than the internal. Solutions should be warmed to room temperature (20 ± 2°C) before use.

Recipe 8: 200 mM CaMSA Stock Solution

Dissolve 0.2303 g CaMSA in 5 ml distilled water, sterilize by passing through a 0.2-μm filter, and store at 4°C.

Recipe 9: 200 mM $CaCl_2$ Stock Solution

Dissolve 0.1470 g $CaCl_2$ in 5 ml distilled water, sterilize by passing through a 0.2-μm filter, and store at 4°C.

Recipe 10: Standard Intralysosomal Solution

Reagent final concentration

KMSA	70 mM
CaMSA	60 mM
$MgCl_2$	2 mM
HEPES	10 mM

Adjust pH to 4.6 with MSA, sterilize by passing through a 0.2-μm filter, prepare 1-ml aliquots, and store at –20°C for several months.

Recipe 11: Standard Extralysosomal Solution

Reagent final concentration

KMSA	60 mM
KF	60 mM
HEPES	10 mM

Adjust pH to 7.2 with KOH, sterilize by passing through a 0.2-μm filter, prepare 4-ml aliquots, and store at –20°C for several months.

CaMSA (Recipe 8)	2 mM

Add CaMSA immediately before starting the measurements to avoid precipitation of CaF_2.

Recipe 12: Extralysosomal High Chloride Solution (mM)

Reagent	final concentration
KCl	60 mM
KF	60 mM
HEPES	10 mM

Adjust pH to 7.2 with KOH, sterilize by passing through a 0.2-μm filter, prepare 4-ml aliquots, and store at –20°C for several months.

$CaCl_2$ (Recipe 9)	2 mM

Add $CaCl_2$ immediately before starting the measurements to avoid precipitation of CaF_2.

Recipe 13: Seal Enhancer Solution

Reagent	final concentration
KMSA	60 mM
KF	60 mM
EGTA	10 mM
HEPES	10 mM

Adjust pH to 7.2 with KOH, sterilize by passing through a 0.2-μm filter, prepare 4-ml aliquots, and store at –20°C for several months.

Recipe 14: Extralysosomal Bath Solution

Reagent	final concentration
KMSA	120 mM
EGTA	10 mM
HEPES	10 mM

Adjust pH to 7.2 with KOH, sterilize by passing through a 0.2-μm filter, prepare 1-ml aliquots, and store at –20°C for several months.

Instructions

Chloridating Electrodes

The electrodes are manufactured from Ag/AgCl-coated steel and need to be regularly chloridated in bleach solution. Generally, electrodes should be replaced every 2 months.

1. Place electrodes requiring rechloridating into a bleach solution.
2. Wait approximately 15 min until a black AgCl-layer is obvious on the silver wire.
3. Rinse in clean water and dry the electrodes.

Cell Culture

Creating a cell line stably and abundantly expressing the ion channel in lysosomes is a critical parameter for successfully obtaining lysosomal recordings. We obtain the best results with cells up to passage 15 corresponding to a culture time of 4 to 5 weeks after thawing the cell stocks. We recommend performing pilot experiments designed to establish the lysosomal preparation—for example, by starting with a stable cell line that overexpresses a green fluorescent protein (GFP)–channel fusion protein as a positive control. Once the yield and the quality of the lysosomal preparation have been evaluated with epifluorescence microscopy or Western blotting of the lysosomal preparation from the cells expressing the fusion protein, then the organelle preparation can be performed with cells expressing untagged channels. The use of untagged channels is recommended to rule out interference of the tag with channel properties. We

use HEK293 cells stably expressing TPCN2 channels, which are calcium channels localized to lysosomes (*22*, *23*).

1. Two or three days before performing the experiments, plate 4×10^6 HEK293 cells per dish in two 175-cm^2 tissue-culture dishes with 25 ml of Cell Culture Medium (Recipe 1) for each dish.
2. Grow the cells homogeneously to almost 95% confluence on the day of the experiment. Grow cells at 37°C in a humidified atmosphere of 10% CO_2 in air.
3. Two hours before beginning the lysosomal preparation, add 25 μl Vacuolin Stock (Recipe 2) to each dish.

Preparation of Lysosomes

We describe a method to isolate lysosomes from HEK293 cells stably expressing lysosomal ion channels (Figs. 1 and 2). Before starting the lysosomal preparation, the cells are exposed to vacuolin to enlarge lysosomes (*24*). All steps in this part of the Protocol must be performed on ice. Lysosomes should be used for electrophysiological recordings within 1 to 3 hours of isolation.

The quality of the lysosomal preparation can be monitored by several methods. If HEK293 cells stably expressing a GFP-tagged lysosomal ion channel are available, the lysosome preparation can easily be assessed with an epifluorescence microscope. Alternatively, evaluate the lysosome preparation by performing Western blot experiments to demonstrate that the protein of interest copurifies with lysosomal marker proteins, such as lamp1 or lamp2. A β-hexosaminidase assay can be used to determine the yield of intact lysosomes according to (*25*, *26*).

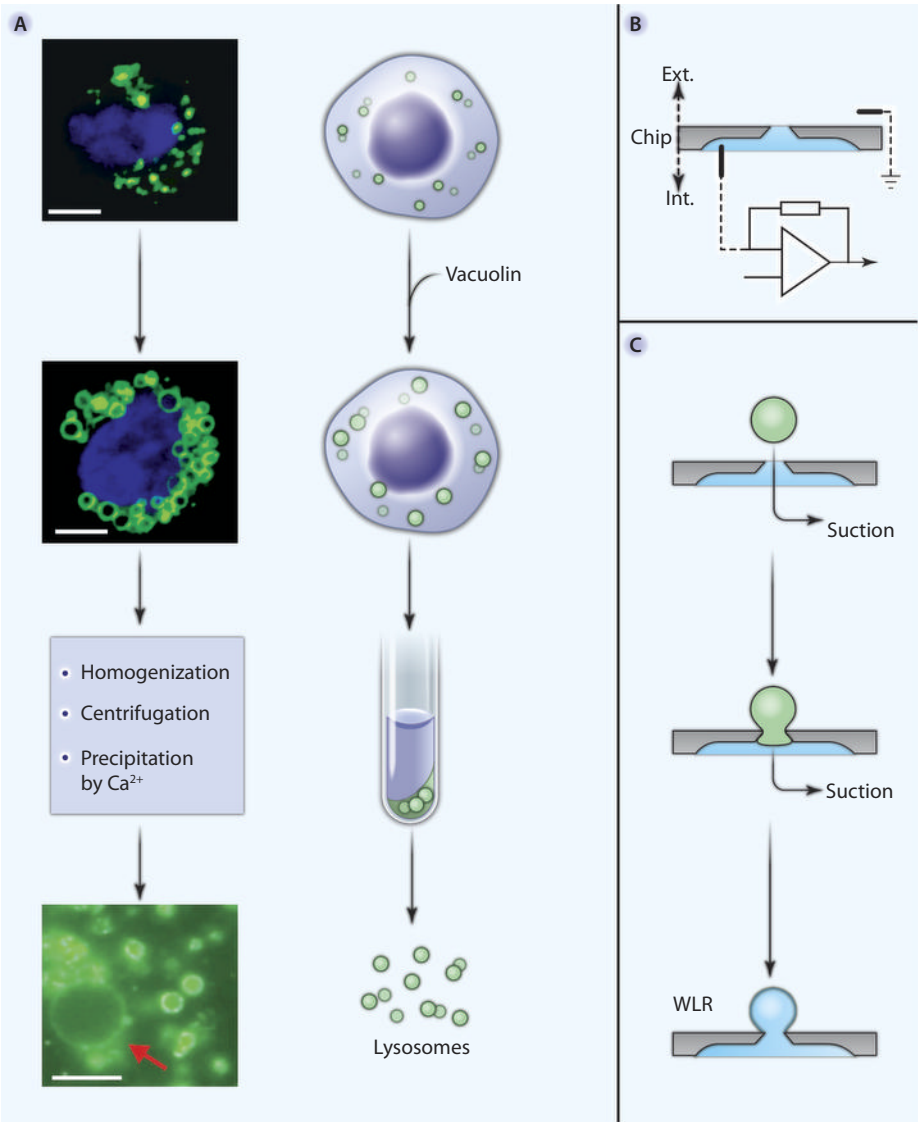


Fig. 1. Lysosomal preparation and the planar patch clamp approach used for lysosomal recordings. (**A**) HEK293 cells stably overexpressing TPCN2 before (top row) and after treatment with vacuolin (second row). After the isolation procedure (third row), highly purified lysosomes are obtained (bottom row). The arrow indicates an enlarged lysosome. Scale bar, 5 μm. (**B**) Schematic of the planar patch clamp method. Both the recording electrode of the amplifier circuitry and the reference electrode are connected to the recording solutions through agar bridges. ext., extralysosomal side of the planar glass chip containing the extralysosomal recording solution; int., intralysosomal side of the chip containing the intralysosomal recording solution (green). (**C**) For whole-cell lysosomal recordings (WLR), suction is applied to the chip in order to attach a single lysosome to the chip (top). After a high seal is obtained, a suction pulse (middle) ruptures the lysosomal membrane to obtain the whole lysosomal configuration (bottom).

1. Remove the medium from the cells and wash the cells once with 15 ml PBS (Recipe 5).
2. Remove PBS and add 250 μl pre-cooled Homogenization Buffer (Recipe 6).
3. Detach the cells with a cell scraper.
4. Transfer cell suspension to the glass-grinding vessel of the potter homogenizer.

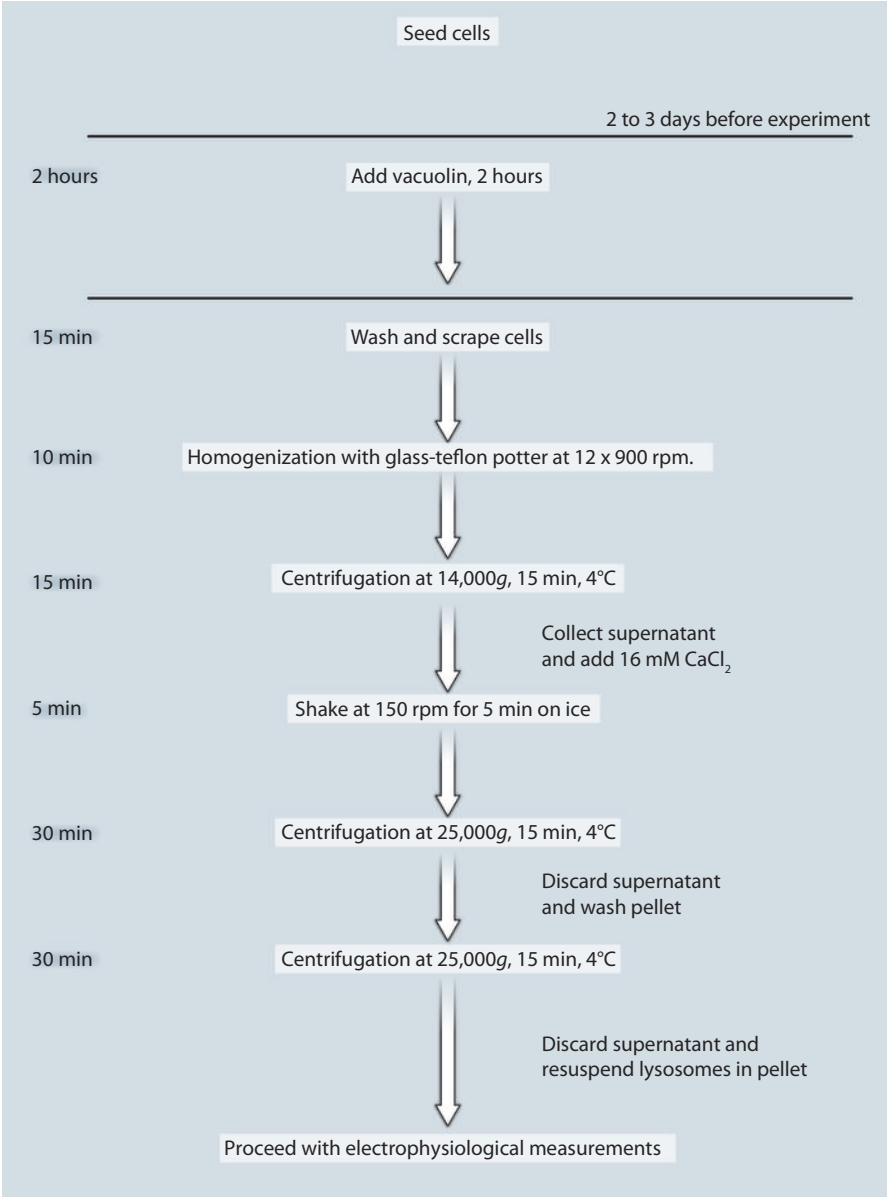
Note: Pre-cool the glassware in an ice bath for 5 min before starting the procedure. Homogenization, as well as the following steps, must be performed at 4°C to minimize the activation of damaging phospholipases and proteases.

5. Wash the plate once with 250 μl Homogenization Buffer (Recipe 6) to detach the remaining cells.
6. Transfer the cells to the same glass-grinding vessel containing the rest of the cells.
7. Assemble the potter homogenizer and homogenize the cells using a Teflon pestle operated at 900 rotations per minute (rpm). Stroke the cell suspension placed in the glass grinding vessel 12 times.

   Note: The Teflon–glass coupling represents the best compromise between homogenization of the cells and the preservation of lysosomal integrity. Harsher techniques, including glass pestle in a glass potter, can easily damage lysosomes.
8. Transfer the homogenate to a 1.5-ml microfuge test tube and centrifuge at 14,000*g* for 15 min at 4°C.
9. Collect the supernatant and transfer it to a 10-ml polycarbonate centrifuge tube.

   Note: If desired, collect 5 μl of the supernatant for the β-hexosaminidase assay or 50 μl for Western blot analysis. This is optional, but provides a sample for establishing that the preparation contains lysosomes.
10. Add an equal volume (typically 1.6 ml) of 16 mM $CaCl_2$ (Recipe 3; final concentration 8 mM) to precipitate lysosomes.
11. Transfer the tube to a rotary shaker and shake at 150 rpm for 5 min at 4°C.

Fig. 2. Workflow of lysosomal preparation from HEK293 cells.



12. Centrifuge at 25,000*g* for 15 min at 4°C in an ultracentrifuge.
13. Discard the supernatant and resuspend the pellet in one volume (typically 3.2 ml) of ice cold Washing Buffer (Recipe 7).
14. Centrifuge at 25,000*g* for 15 min at 4°C in an ultracentrifuge.
15. Discard the supernatant, resuspend pellet containing lysosomes in 20 μl of Washing Buffer (Recipe 7), and transfer the suspension to a 1.5-ml microfuge tube.

    Note: Resuspend the pellet using a 200-μl pipette tip. If resuspension is poor and sample contains large aggregates, then resuspend through a smaller pipette tip more vigorously.
16. Add 20 μl of Washing Buffer (Recipe 7) to the tube that contained the pellet to resuspend, with a 200-μl pipette tip, any remaining pellet. Transfer the suspension to the same microfuge tube from the previous step.

Fig. 3. Configuration of suction and voltage parameters required for seal formation and membrane rupture in planar lysosomal patch clamp. The parameter window of the PatchControl software contains eight tabs. Exact parameters are shown in the screen shots. The "improve seal 1" tab and the "wait for whole cell" tab are deactivated and are not shown.

PatchControl and Patchmaster Software Setup

The PatchControl software controls the Port-a-Patch device. Patchmaster is the acquisition software that controls the HEKA EPC10 amplifier. The handling of Patchmaster is exactly like that for conventional patch clamp. The instructions below provide the general guidelines for setting the parameters for cell positioning, seal formation, and the cell-opening procedure in PatchControl; the exact settings are provided in Fig. 3.

1. Start PatchControl.
2. Select the "experiment window." Make sure that "batch communication" is turned on to enable communication with Patchmaster and to obtain seal parameters and apply command voltages.
3. Load a preprogrammed PatchControl parameter file—for example, "nanion_fragile.ppf."
4. Enter the "edit mode" to access parameter tabs in the parameter window. From this window, edit the parameters for the cell positioning, seal formation, and cell opening procedure (Fig. 3).

5. Select the "wait for contact" tab to monitor the formation of contact between the electrodes and the solution. Patchmaster reads the membrane resistance.
6. Select "adjust VpOffset" to activate the AutoOffset function in Patchmaster.
7. Select the "wait for cell" tab to apply a negative pressure. Set the system to progressively increase the pressure in steps of 10 mB up to a maximum of –40 mB. This will facilitate the attachment of a single lysosome onto the aperture of the glass chip and improve the seal formation during the experiment.

   Note: Setting these parameters correctly is critical, because the lysosomal membrane is very fragile and the applied pressure is considerably lower than that used for cells.
8. Select the "wait for seal" tab and set the system to increase the seal resistance by changing the pressure and membrane potential. Set the system so that you can apply progressively increasing pressure pulses (–20 to –40 mB; step size, 5 mB) every 10 s. When this step is performed with a lysosome, in parallel PatchControl will progressively hyperpolarize the membrane potential every 0.3 s in steps of 2 mV until the final value of –40 mV is reached.
9. Deactivate the "improve seal1" tab by clicking the khaki-colored tab on the bottom of the parameter window (fifth from the left) (Fig. 3).
10. Select "improve seal2" tab to allow PatchControl to switch pressure between a defined pressure and atmospheric pressure depending on the development of the seal formation over time (dR/dt, time dependent change in seal resistance) (Fig. 3). If the seal improves with a rate greater than the threshold defined, PatchControl will not change the parameters. If not, PatchControl will switch the pressure from defined to atmospheric or vice versa and repeat the routine automatically.
11. Deactivate the "wait for whole cell" tab by clicking the light blue colored tab on the bottom of the parameter window (second from the right; Fig. 3).
12. Select the "maintain whole cell" tab. For lysosomes, set this to apply a constant pressure of –20 mB to maintain the seal during electrophysiological measurements. The holding potential is –80 mV.

    Note: The values required for other intracellular organelles will have to be empirically determined.
13. Save the parameter settings as a parameter file in a user-defined folder.

    Note: Once a parameter file is optimized for a particular organelle, it is suitable for further use with the same organelle type without additional modifications.

Electrophysiological Recordings

The instructions below assume a basic understanding of patch clamp recording. Details of performing the patch clamp recordings and data analysis are not described. Only those steps that are specific to recording from lysosome preparations are described in detail. The appropriate NPC-1 chip for lysosomal recordings, based on our experience, has a chip resistance of 8 to 15 megohms. Of the devices we used, the Port-a-Patch device provided the best results. It is essential to form a high-resistance seal (gigaseal) with the membrane of the lysosome or organelle of interest. Gigaseals are formed usually with the aid of Seal Enhancer Solution (Recipe 13). Seal Enhancer Solution (Recipe 13) contains a high concentration of fluoride, whereas the Standard Intralysosomal Solution (Recipe 10) contains a high concentration of Ca^{2+}. For tight-seal lysosomal patch clamp recordings, it is crucial to have solutions containing high Ca^{2+} on one side of the glass chip and solution containing high fluoride at the other side during seal formation. Omitting either of the ions from the respective solutions after seal formation can cause loss of seal quality and patch clamp stability. Inclusion of fluoride improves patch clamp sealing and stabilizes the cell membrane, resulting in longer, more stable patch clamp recordings (*27*); the mechanism of this effect is unknown. Typically, after seal formation, seal enhancer solution is exchanged for those appropriate to the ion channel under investigation. Both internal and external chip solution can easily be changed. We describe how to change the solutions manually; however, an automated internal and external solution exchanger is available from Nanion.

1. Backfill the NPC-1 chip with 5 μl of the same internal recording solution that will be used during data collection—for example, Standard Intralysosomal Solution (Recipe 10)—and screw the chip onto the chip holder of the Port-a-Patch device.
2. Add 5 μl Extralysosomal Bath Solution (Recipe 14).
3. Put the Faraday top in place and adjust the ground electrode so it is in contact with the external solution.
4. In the "Experiment window" of PatchControl, press "Play," which will establish electrical contact once the resistance drops below the preset threshold (parameter "R" in the "wait for contact" tab).

   Note: Usually, the electrical contact is established within 2 min (the surface tension of the chip is quite high). Check the electrical contact between electrodes and solutions when contact is not established quickly.

5. Click the button "Add cells," which adjusts the voltage offset before the lysosomes are added.
6. Dispense 5 μl of isolated lysosomes into the chamber containing Extralysosomal Bath Solution (Recipe 14) immediately after you hear the suction control unit changing the pressure. PatchControl applies suction pulses from below the chip to bring a lysosome onto the aperture. Contact of a lysosome is recognized by an increase in "Rmembr" during each suction pulse.

   Note: If membrane resistance suddenly jumps to a very high value (above 40 megohms), it is likely that the chip opening is clogged by dirt particles. Use a new chip.
7. Once a lysosome has been "captured," add 30 μl of Seal Enhancer Solution (Recipe 13) to the Extralysosomal Bath Solution to help seal formation.
8. Allow PatchControl to perform the steps predefined in the parameters section (see previous section) to optimize seal formation.

   Note: After completion of the full protocol to optimize seal formation, PatchControl enters measurement mode. If seal fails to form, then changing the seal enhancer solution, adjusting the membrane potential, increasing the suction pulse, or applying a voltage ramp may be helpful (see details in later section).
9. After seal formation, exchange Seal Enhancer Solution for those appropriate to the ion channel under investigation. Exchange the external solutions manually with a pipettor by replacing the external solution with one volume of standard extralysosomal solution (Recipe 11) four to five times.

   Note: Solutions containing pharmacological agents or different ionic compositions may also be used to characterize channel properties. Directions for manually exchanging the internal solution are provided in the next section.
10. Perform the electrophysiological recordings with Patchmaster, using the same methods as for conventional patch clamp experiments, with a protocol appropriate to characterize the ion channel of interest using a pulse generator file.
11. After completion of the electrophysiological recording session, close the PatchControl software by selecting "quit." Select "save and exit" when shutting down Patchmaster to ensure that the data files are saved properly.
12. Perform data analysis in the same way as in conventional patch clamp experiments. We usually directly export the data with the standard export functions of the HEKA software and analyze the data in OriginPro 7.5.

Manual Exchange of the Internal Solution

This is optional.

1. Deactivate the suction by clicking "enable" in the "pressure control window" in PatchControl.
2. Remove the Faraday top.
3. Disconnect the NPC-1 chip from the chip holder.
4. Exchange the internal solution with a pipettor by replacing 5 μl of Intralysosomal Solution five times with the new solution.

   Note: Be careful not to lose the external solution (Fig. 4).
5. Screw the NPC-1 chip onto the chip holder.
6. Put the Faraday top in place and adjust the ground electrode.
7. Perform electrophysiologial recording, save and export data, and analyze.

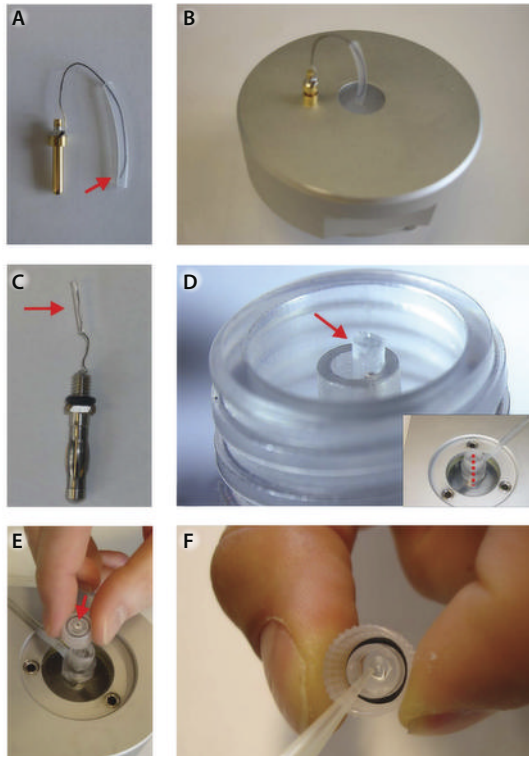


Fig. 4. Preparation and mounting of agar bridges and exchange of intracellular solution. (**A**) Extralysosomal agar bridge. The red arrow marks the position of the electrode tip. (**B**) Extralysosomal agar bridge connected to the Faraday top of the Port-a-Patch. (**C**) Internal recording electrode with an intralysosomal agar bridge. (**D**) The intralysosomal agar bridge (red arrow) is mounted to the chip holder. (Inset) Overview of the Port-a-Patch containing the chip holder. The red dotted line marks the position of the intralysosomal agar bridge. (**E** and **F**) Exchange of the intralysosomal recording solution. (E) The chip is disconnected from the holder and flipped 180° without losing the external recording solution (arrow indicates the droplet of the external recording solution). The internal solution is now changed with a pipettor (F).

Improving Seal Formation

If a seal fails to form, then there are several steps that may improve seal formation.

1. Replace the Seal Enhancer Solution (Recipe 13).
2. Step the membrane potential to 0 mV.
3. Increase suction pulse to a maximum of –80 mB.

   Note: This can improve the seal, but the lysosome might be sucked through the chip.
4. Step the membrane potential to +40 mV
5. Apply a voltage ramp from –100 mV to +100 mV from a holding potential of –80 mV.

   Note: This step is optional and can be avoided if the previous steps result in a seal.

Preparation and Placement of Agar Bridges

To avoid voltage offsets, if internal or external solutions containing low concentrations of chloride are used, then agar bridges need to be used instead of plain Ag/AgCl electrodes. As in conventional patch clamp, agar bridges are used for the reference electrode. In addition, in planar patch clamp it is also possible to use internal agar bridges (Fig. 4).

1. Dissolve 4 mg of agar in 100 ml of 3M KCl solution by boiling and transfer to 10-cm dishes.
2. After the agar has cooled, fill a small silicon tube (1 mm diameter) with agar by sticking the tube into the cooled agar (Fig. 4). This plugs the tip of the tube with agar.
3. Fill the tube with a solution of 3M KCl and push the tip of the reference electrode or the internal electrode into the agar (do not push the electrode completely through the agar) (Fig. 4).

Troubleshooting

Poor or No Lysosome Recovery

If no lysosomal pellet is obtained or if lysosomal recovery is low from the lysosomal preparation, then the density of the cells may be too low, homogenization may be too harsh, or lysosomes may be poorly resuspended. We recommend growing the cells to 90 to 95% confluence. If the confluence is less, decrease the volume of Homogenization Buffer (Recipe 6). Avoid homogenizing with a glass pestle in a glass potter, which can easily damage lysosomes. If the resuspension step was not complete, then the lysosomal preparation will contain large aggregates of lysosomes. For more complete resuspension and separation of lysosomes, use smaller pipette tips and resuspend longer or more vigorously.

Inadequate Seal Formation or Unstable Seal

If the lysosome preparation is not good quality, then it will be difficult to find a lysosome that forms a good seal even in the presence of seal enhancer.

If analysis of lysosomes by means of β-hexosaminidase assay or Western blotting suggests that lysosome preparation is good, then we recommend using a seal enhancer.

If in the presence of seal enhancer, a seal fails to form, then changing the seal enhancer solution, adjusting the membrane potential, increasing the suction pulse, or applying a voltage ramp may be helpful (see Instructions).

The seal is fragile and can be lost when exchanging solutions. It is critical to gently exchange solutions. The exchange of the internal solution is technically demanding, and during this process it is critical not to lose the drop of external solution (Fig. 3, E and F).

During the time course of a patch clamp session, the quality of the lysosomal preparation and the ability to form seals usually gradually decreases with time. In our hands, electrophysiological experiments can be performed for up to 2 to 3 hours after the lysosomes have been prepared. Thereafter, a new lysosomal preparation should be used for electrophysiological recordings.

Notes and Remarks

The experimental conditions may be optimized on the basis of data obtained. There are various aspects to optimize—including lysosome preparation, size of the aperture of the glass chips, ionic solutions during seal formation, and suction and voltage parameters for forming seals—and to breaking through to achieve the whole-lysosome configuration.

We found that lysosomal recordings were most efficiently performed in the whole-lysosome configuration. The lysosomal membrane is very fragile. After breaking through the lysosomal membrane, stable recordings can be obtained for more than 20 min. In contrast, it is much more difficult to obtain stable lysosome-attached recordings for a long period of time. We found that rapidly after obtaining the lysosome-attached mode, the lysosomal membrane spontaneously broke open without losing the seal. Thus, the configuration is converted to whole-lysosome mode.

The current conventions for electrophysiological experiments of lysosomes and other organelles is the same as for conventional patch clamp. An inward current is defined as a current that flows out of the organelle into the cytosol. It is therefore necessary to perform lysosomal recordings using the "inside out" configuration in Patchmaster. Alternatively, the *x* axes and the *y* axes of data recorded by using the "whole cell mode" in Patchmaster need to be inverted.

We briefly outline here the expected results for two examples that illustrate applications of the planar patch clamp method of whole lysosomes. First, we applied our method to native lysosomes isolated from control HEK293 cells. We resolved endogenous lysosomal currents, specifically chloride currents (Fig. 5). The reversal potential of endogenous chloride currents [70.5 ± 1.3 mV (n = 4 different lysosomes)] closely matches the calculated reversal potential for chloride 71.2 mV. We commonly obtained up to eight recordings of native chloride currents per lysosomal preparation. Second, we have detected NAADP (nicotinic acid adenine dinucleotide phosphate)–sensitive current (I_{NAADP}) from lysosomes stably expressing a GFP-tagged TPCN2, which is a member of the two-pore channel family (Fig. 5). Here, the success rate ranged between four to five recordings per preparation. Two-pore channels (TPCN1,2,3) belong to the superfamily of voltage-gated ion channels. Within this superfamily, TPCN channels are most closely related to some members of the TRP (transient receptor potential) cation channels (*28*). TPCNs are localized in endosomal and lysosomal stores and form NAADP-gated Ca^{2+}-release channels (*22*, *23*). NAADP is a second messenger that at low nanomolar concentrations releases Ca^{2+} from endolysosomal stores. NAADP-evoked Ca^{2+}-release has been demonstrated in invertebrates and numerous mammalian cell types, including pancreatic acinar and β cells, cardiac and smooth muscle cells, T lymphocytes, platelets, and neurons (*29*).

These examples illustrate the application of this Protocol to investigate lysosomal ion channels. This glass chip–based method should provide electrophysiological access not only to lysosomal TPCN channels, but also to various ion channels in other types of organelles and intracellular compartments.

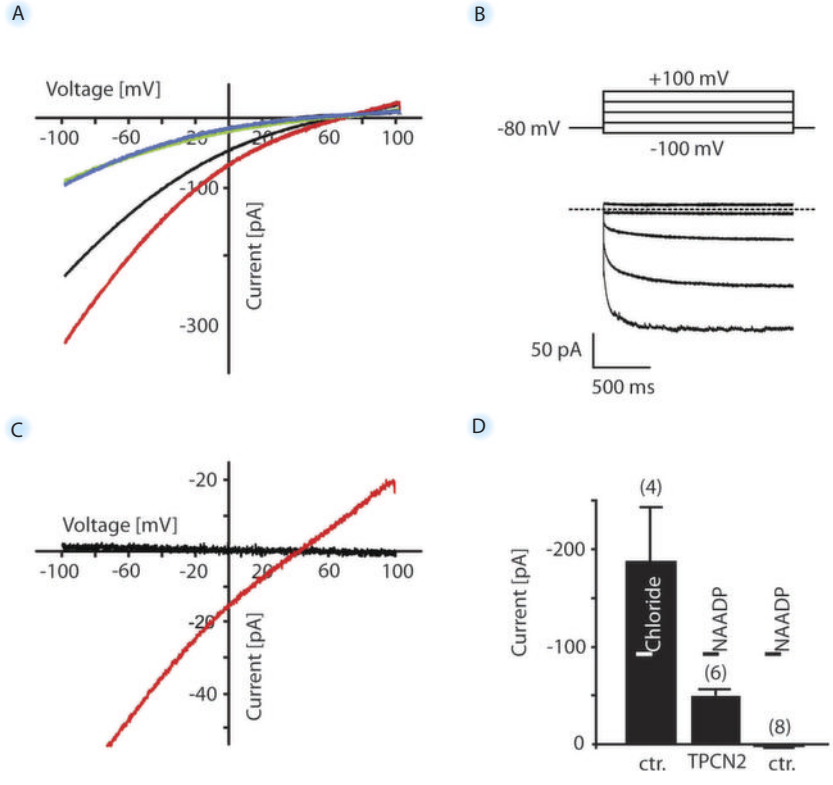


Fig. 5. Example of lysosomal current recordings. (**A**) Endogenous chloride currents recorded from wild-type lysosomes. Current-voltage (*IV*) curves were recorded in the absence [Standard Extralysosomal Solution (Recipe 11)] and presence of external Cl^- [Extralysosomal $_{highCl}$ Solution (Recipe 12)]. The internal solution was Standard Intralysosomal Solution (Recipe 10). Chloride currents were determined as the difference currents between these *IV* curves. The membrane potential was held at –80 mV, and 500 ms voltage ramps from –100 to +100 mV were applied every 5 s. Representative current recordings from four lysosomes are shown in different colors. (**B**) Family of endogenous chloride current traces recorded from wild-type lysosomes. The membrane potential was held at –80 mV, and test pulses were applied from –100 mV to +100 mV (step size, 50 mV). (**C**) Current-voltage relations for Ca^{2+} currents through TPCN2 channels from a single lysosome in the presence of 60 nM NAADP (red). Black trace, NAADP-dependent current of a wild-type lysosome. The protocol was the same as in (A). Currents were recorded with standard extralysosomal and intralysosomal solution (Recipes 10 and 11). (**D**) Population data for current amplitudes at –100 mV obtained from similar experiments as shown in (A) and (C). Inward currents are currents that flow out of the lysosomes into the cytosol. Ctr, control.

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