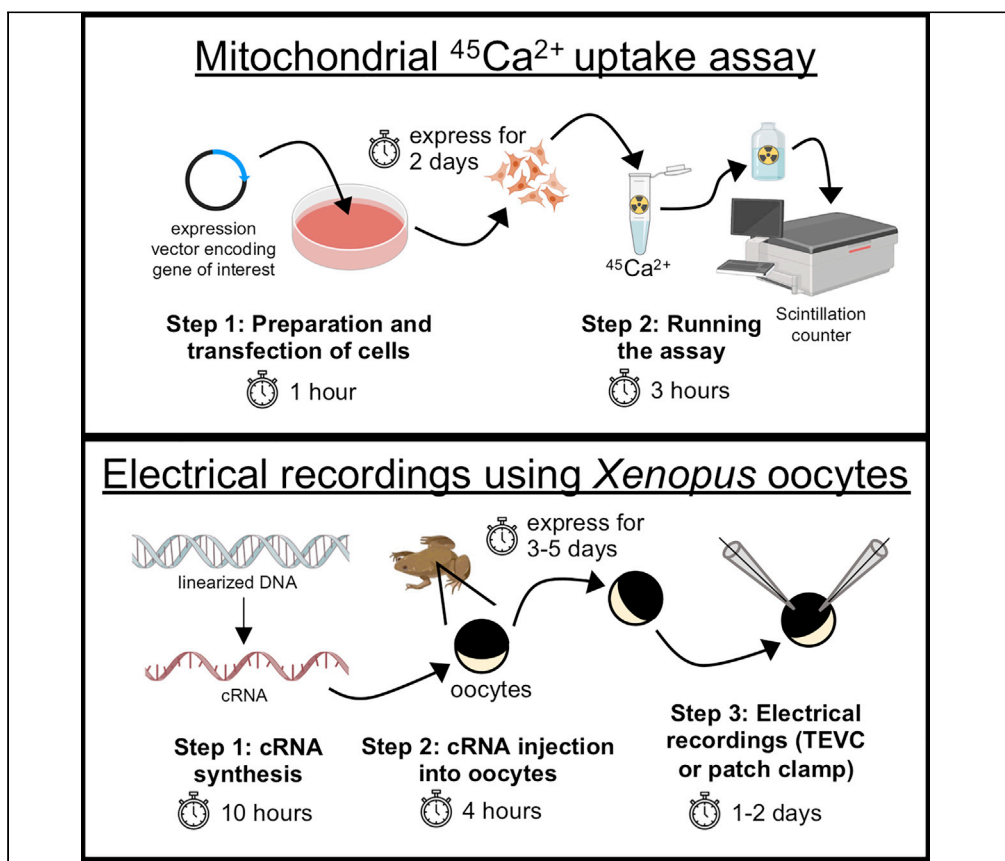


Protocol

Quantitative assays to measure the transport activity of the mitochondrial calcium uniporter in cell lines or *Xenopus* oocytes



The mitochondrial calcium uniporter, which mediates mitochondrial Ca^{2+} uptake, regulates key cellular functions, including intracellular Ca^{2+} signaling, cell-fate determination, and mitochondrial bioenergetics. Here, we describe two complementary strategies to quantify the uniporter's transport activity. First, we detail a mitochondrial Ca^{2+} radionuclide uptake assay in cultured cell lines. Second, we describe electrophysiological recordings of the uniporter expressed in *Xenopus* oocytes. These approaches enable a detailed kinetic analysis of the uniporter to link its molecular properties to physiological functions.

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Highlights

Two quantitative approaches to analyze mitochondrial calcium uptake

Measuring mitochondrial calcium uptake in cell lines using calcium-45 radioisotope

Electrical recordings of the mitochondrial calcium uniporter in *Xenopus* oocytes

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Protocol

Quantitative assays to measure the transport activity of the mitochondrial calcium uniporter in cell lines or *Xenopus* oocytesMadison X. Rodriguez,^{1,2} Anna M. Van Keuren,^{1,2} and Ming-Feng Tsai^{1,2,3,4,*}¹Department of Physiology and Biophysics, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA²These authors contributed equally³Technical contact⁴Lead contact*Correspondence: ming-feng.tsai@cuanschutz.edu
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SUMMARY

The mitochondrial calcium uniporter, which mediates mitochondrial Ca^{2+} uptake, regulates key cellular functions, including intracellular Ca^{2+} signaling, cell-fate determination, and mitochondrial bioenergetics. Here, we describe two complementary strategies to quantify the uniporter's transport activity. First, we detail a mitochondrial Ca^{2+} radionuclide uptake assay in cultured cell lines. Second, we describe electrophysiological recordings of the uniporter expressed in *Xenopus* oocytes. These approaches enable a detailed kinetic analysis of the uniporter to link its molecular properties to physiological functions.

For complete details on the use and execution of this protocol, please refer to Tsai and Tsai (2018) and Phillips et al. (2019).

BEFORE YOU BEGIN

The mitochondrial Ca^{2+} uniporter is a multi-subunit Ca^{2+} channel complex that transports cytoplasmic Ca^{2+} into the mitochondrial matrix (Kamer and Mootha, 2015; Rizzuto et al., 2012). Half a century of studies have strongly advanced our knowledge about the uniporter's molecular mechanisms and pathophysiological roles. However, many critical mechanistic problems still remain poorly understood, such as the molecular basis of the uniporter's Ca^{2+} selectivity, inward rectification of Ca^{2+} currents, voltage-dependent gating, etc. There are two established methods to study the uniporter's transport properties: first, using Ca^{2+} sensors to detect mitochondrial Ca^{2+} uptake; and second, mitoplast patch-clamp. The former is easy to perform but qualitative in nature (might be semi-quantitative with careful calibration). The latter is quantitative, but the technical barrier is high, so only a few labs worldwide have been able to obtain data using this method. Below we describe two approaches that are highly quantitative but with a much lower technical difficulty compared with mitoplast patch-clamp. We anticipate that adopting these methods would strongly facilitate mechanistic studies of the physiologically crucial uniporter transport process. A comparison of methods to study the uniporter's Ca^{2+} transport is provided below (Table 1):

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
<i>Xenopus laevis</i> frogs	XENOPUS1	#4270
<i>Xenopus laevis</i> ovary	XENOPUS1	#10004

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Lipofectamine 3000	Thermo Fisher Scientific	#L3000015
Ru360	Synthesized in house (also available from Sigma-Aldrich)	(Ying et al., 1991) (557440-500UG)
Digitonin	Sigma-Aldrich	D141
Thapsigargin	Cayman Chemical	10522
Ca-45 radionuclide	PerkinElmer	NEX013001MC
Scintillation cocktail	Atlantic Nuclear Corporation	#111175
Type II collagenase	Worthington Biochemical Corporation	#C6885
NotI-HF	New England Biolabs	#R3189
EGTA	Sigma-Aldrich	#03777
Critical commercial assays		
mMESSAGE mMACHINE T3 Transcription kit	Thermo Fisher Scientific	#AM1348
QIAquick PCR Purification kit	QIAGEN	#28104
RNeasy MinElute Cleanup kit	QIAGEN	#74204
Experimental models: Cell lines		
HEK 293T	American Type Culture Collection	#CRL-3216
Recombinant DNA		
hME-pOX	Addgene	#178181
Other		
Scintillation vials	Thermo Fisher Scientific	#333715
Round nitrocellulose membranes	Thermo Fisher Scientific	#097192D
Vacuum filtration manifold	Merck Millipore	Model 1225
Scintillation counter	Beckman Coulter	LS 6500
Oocyte incubator	Revolutionary Science	RS-IF-233
Pipette puller	SUTTER INSTRUMENT	P-1000
Microinjector	DRUMMOND SCIENTIFIC COMPANY	Nanoject II
Air table with Faraday cage	SUTTER INSTRUMENT	AT-3648
Two electrode voltage clamp station	WARNER INSTRUMENTS	TEV-700
Patch-clamp amplifier	Molecular Devices	Axopatch 200B
Digitizer	Molecular Devices	Digidata 1550B
Inverted microscope	ZEISS	Axiovert 200
Fast solution change system	WARNER INSTRUMENTS ^r	SF-77C
Thermal cycler	Bio-Rad Laboratories	T100
Fluorometer	HITACHI	F-7100

MATERIALS AND EQUIPMENT

Buffer recipes

Wash buffer (WB)

Reagent	Final concentration
KCl	120 mM
MgCl ₂	1 mM
EGTA	50 μM
HEPES, pH 7.2-KOH	25 mM

Store in 4°C for up to a year. Warm up to 25°C before use.

Recording buffer (RB)	
Reagent	Final concentration
KCl	120 mM
MgCl ₂	1 mM
EGTA	690 μM
CaCl ₂	500 μM
HEPES, pH 7.0-KOH	25 mM

Store in 4°C for up to a year. Warm up to 25°C before use.

Note: This recipe will produce an RB solution with 300 nM of free [Ca²⁺]. One can adjust EGTA and CaCl₂ concentrations to obtain various levels of free [Ca²⁺]. An online free-Ca²⁺ calculator can be found via this link: <https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaEGTA-TS.htm>

We recommend using a Ca²⁺ indicator, such as Calcium Green 5N or Fluo-4 to confirm free [Ca²⁺] before flux experiments. In brief, a Ca²⁺ indicator can be added to the solution to obtain the fluorescence reading (F) using a fluorometer. Saturating concentrations of Ca²⁺ can then be added to obtain the maximal signal, F_{max}, followed by adding EGTA to obtain the minimal signal, F_{min}. Free Ca²⁺ can be calculated using the equation $[Ca^{2+}] = K_D * (F - F_{min}) / (F_{max} - F_{min})$, where K_D is the Ca²⁺ dissociation constant for the indicators.

ND96	
Reagent	Final concentration
KCl	2 mM
NaCl	96 mM
CaCl ₂	1.8 mM
MgCl ₂	1 mM
HEPES, pH 7.4-NaOH	5 mM

Store in 4°C for up to a year. Warm up to 25°C before use.

Table 1. A comparison of methods to study the mitochondrial calcium uniporter

Methods	Measuring mitochondrial Ca ²⁺ uptake using Ca ²⁺ indicators	Mitoplast patch-clamp	Mitochondrial ⁴⁵ Ca ²⁺ uptake (1 st section below)	Electrophysiological recordings using <i>Xenopus</i> oocytes (2 nd section below).
Quantitative/qualitative	Qualitative to semi-quantitative	Quantitative	Quantitative	Quantitative
Difficulty	Low	Very high	Medium	Medium to high
Advantages	Quick and easy. Excellent for “yes-or-no” questions.	The use of voltage clamp enables accurate control of the driving force for Ca ²⁺ flux.	The only quantitative method to measure the transport of physiological levels of Ca ²⁺ (0.1–10 μM)	All advantages of mitoplast patch-clamp, but much easier to do.
Limitations	Not suitable for absolute quantification.	Years of experience are required to do this properly. Very low throughput.	Institutional support is needed to properly handle ⁴⁵ Ca ²⁺ waste.	Regulatory MICU subunits of the uniporter are absent.
Suggested references	(Rizzuto et al., 1992; Sancak et al., 2013; Tsai et al., 2016)	(Garg and Kirichok, 2019; Kirichok et al., 2004)	(Csordas et al., 2013; Phillips et al., 2019)	(Tsai and Tsai, 2018; Van Keuren et al., 2020)

Digestion buffer (DB)

Reagent	Final concentration
NaCl	96 mM
KCl	2 mM
MgCl ₂	2 mM
HEPES, pH 7.4- NaOH	5 mM

Store in 4°C for up to a year. Warm up to 25°C before use.

Ca20

Reagent	Final concentration
KCl	2 mM
NaCl	96 mM
CaCl ₂	1.8 mM
MgCl ₂	1 mM
HEPES, pH 7.4-NaOH	5 mM

Store in 4°C for up to a year. Warm up to 25°C before use.

Ca100

Reagent	Final concentration
CaCl ₂	100mM
HEPES, pH 7.6-NaOH	20 mM

Store in 4°C for up to a year. Warm up to 25°C before use.

STEP-BY-STEP METHOD DETAILS

Mitochondrial ⁴⁵Ca²⁺ uptake assay—Preparing cells

⌚ Timing: 1 h, 2–3 days prior to the experiment day

These steps prepare cells for the ⁴⁵Ca²⁺ uptake assay. The protocol below calls for the use of HEK293T cells, but the same procedure can be readily applied to other types of cells, such as HeLa or CHO cells.

1. If transfection is needed, seed HEK293T cells at 25% density (~1 × 10⁶ cells/well) in 4 wells of a 6-well plate 3 days before the experiment, and transfect the cells the following day using Lipofectamine 3000 according to the manufacturer's manual.
2. If transfection is not needed, seed HEK293T cells at 25% density in 4 wells of a 6-well plate 2 days before the flux experiment.

Mitochondrial ⁴⁵Ca²⁺ uptake assay—Performing the assay

⌚ Timing: 3 h

The steps below describe the detailed procedures to perform the mitochondrial ⁴⁵Ca²⁺ uptake assay.

3. Fill 12 scintillation vials with 5 mL of WB, and place these vials on ice to keep them cold.
4. Set up the vacuum filtration manifold using 0.45 μm pore size round nitrocellulose membranes. Connect the manifold to a vacuum outlet or a vacuum pump. Pass 5 mL of water through each membrane to confirm vacuum power and to wet the membrane.
5. Prepare 1000× digitonin (30 mM in water) and 500× thapsigargin (2.5 mM in DMSO) stock.

△ **CRITICAL:** Digitonin stock solution must be freshly prepared right before the experiment. Thapsigargin stock can be stored in 4°C for up to a month.

6. Add 0.6 μL of concentrated $^{45}\text{Ca}^{2+}$ stock (specific activity: 15–30 mCi/mg) to 0.6 mL of RB. Also, add 30- μM digitonin (to permeabilize cells) and 5- μM thapsigargin (to block the endoplasmic reticulum Ca^{2+} -ATPase pump).
7. Split the solution into 2 microcentrifuge tubes. Add 100 nM Ru360 (a potent uniporter inhibitor) to one of the two tubes, labeled as tube A (no Ru360) and tube B (with Ru360).
8. Trypsinize HEK293T cells in 2 wells of a 6-well plate. Resuspend the cells in 2–4 mL of DMEM (with 10% FBS), and then count cells using a hemocytometer or an automatic cell counter.

△ **CRITICAL:** It is important to apply adequate trypsinization to break large cell clumps, or cell counting would be inaccurate.

9. Transfer 4×10^6 cells to a microcentrifuge tube, spin down at 2000 g for 1 min, resuspend the cell in 1 mL of WB, and then spin down again at 2000 g for 1 min.
10. Discard the supernatant and resuspend the cells in 200 μL of WB, supplemented with 30- μM digitonin and 5- μM thapsigargin. Allow the cells to be permeabilized for at least 2 min before proceeding to step 11.
11. Add 100 μL of the cell suspension to tube A and mix well to start mitochondrial $^{45}\text{Ca}^{2+}$ uptake. 1 min later, add 100 μL of the remaining cell suspension to tube B to start $^{45}\text{Ca}^{2+}$ uptake in this tube.
12. 2 min, 4 min, and 6 min after $^{45}\text{Ca}^{2+}$ uptake started, transfer 100 μL of each sample into the respective scintillation vials with ice-cold WB. Immediately filter the mix through the vacuum manifold.
13. Once the solution is filtered through, immediately filter through 5 mL of WB to remove any remaining extramitochondrial $^{45}\text{Ca}^{2+}$. 6 membranes would be used in steps 6–13 (3-time points for both tubes A and B).
14. Transfer the membranes into empty scintillation vials and add 15 mL scintillation cocktail for counting. Also, transfer 10 μL of remaining solutions from tube A and tube B into different scintillation vials and add 15-mL scintillation cocktail for counting.

△ **CRITICAL:** Shake those scintillation vials with membranes vigorously for at least 30 s to fully extract $^{45}\text{Ca}^{2+}$ into the scintillation cocktail.

15. Repeat steps 6–14 for technical replicates. There should be two membranes for each time point for both tubes A and B.
16. Measure the counts (CPM) in each vial using a scintillation counter.
17. Calculate the average of the two readings for each time point to generate a single data point. Multiply the signal from 10 μL of the remaining solution by 10 to obtain the total signal in 100 μL solution.
18. Follow the instructions in the Expected Outcomes section below to analyze the data.

Electrical recordings using *Xenopus* oocytes—Preparing cRNA

⌚ Timing: 10 h

These steps provide instructions about how to prepare cRNA to express the uniporter's transmembrane subunits in *Xenopus* oocytes.

19. Linearize 10 μg of a pOX vector containing a human MCU-EMRE (hME, Addgene 178181) fusion construct (MCU and EMRE form the uniporter's transmembrane region) using 20 U of the NotI-HF restriction enzyme.
20. Digest hME-pOX for 6 h at 37°C, followed by heat inactivation for 20 min at 65°C using a thermal cycler.

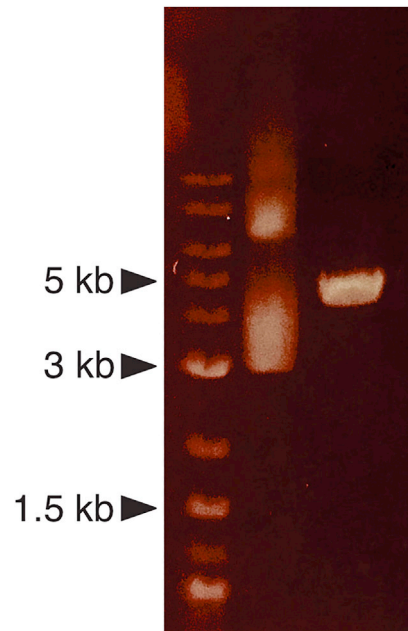


Figure 1. Linearization of hME-pOX

The middle lane is nonlinearized sample, while the right lane shows fully linearized DNA.

▮▮ **Pause point:** The DNA can be left in the thermal cycler at 4°C, with the next steps performed in the following day.

21. Confirm complete linearization of the hME vector by running the sample on a 1% DNA gel. A fully linearized sample should run as a single band, as opposed to linearized samples, which show a smear due to DNA supercoiling (Figure 1).
22. Use a Qiagen PCR purification kit to purify linearized DNA according to manufacturer's instructions.
23. Use a mMessage mMachine T3 transcription kit to produce cRNA following the manufacturer's instructions, with 1 μg of linearized DNA used as the template.

△ **CRITICAL:** After cRNA synthesis, make sure to digest the DNA template using the Turbo DNase provided with the T3 transcription kit.

24. Purify the cRNA using the Qiagen RNeasy MinElute kit according to the manufacturer's protocols. and elute with 20–30 μL of RNase free water. The final yield should be 1–2 μg/μL.
25. Check the quality of RNA by standard RNA gel electrophoresis under denaturation conditions (Rio, 2015).

△ **CRITICAL:** If the result of RNA electrophoresis shows a smear or multiple bands, redo the cRNA prep and be careful applying RNA handling techniques to avoid RNase contamination (Nilsen, 2013).

26. If no smearing is observed, make 2 μg RNA aliquots and store in –80°C freezer.

Electrical recordings using *Xenopus* oocytes—Defolliculating oocytes and injecting cRNA

Ⓞ **Timing:** 10 h totally over 4–6 days

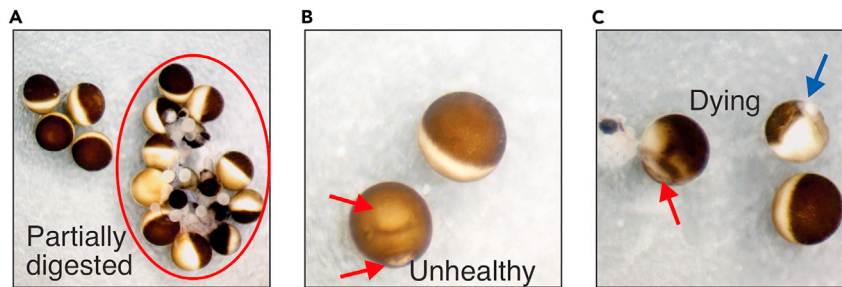


Figure 2. Images of oocytes

(A) Fully digested (left) vs incompletely digested oocytes (right).

(B) Unhealthy oocyte (left) vs healthy oocyte (right). Healthy oocytes are round, smooth, and have a clearly defined light hemisphere and dark hemisphere. The appearance of lighter spots (top arrow) or white colored lesions (bottom arrow) on the dark hemisphere suggests deteriorating health.

(C) Healthy oocyte (bottom) vs dying oocytes (top left and right). Dying oocytes lose the clear definition between the light and dark hemisphere (red arrow) and may begin to leak (blue arrow).

The steps below describe the procedure to prepare *Xenopus* oocytes and to inject cRNA into these cells to induce uniporter expression for subsequent electrical recordings.

27. *Xenopus laevis* ovary lobes can be purchased directly from Xenopus1. Alternatively, frogs can be housed in the institutional vivarium with ovary lobes obtained via laparotomy.
28. Rinse the ovary lobes three times with ND96. Fill a 50 mL conical tube with 20 mL of DB. Use scissors to cut the ovaries into lentil sized chunks into the tube, till the oocytes fill a volume of 5–10 mL.
29. Rinse oocytes with DB multiple times until the buffer runs clear.
30. Dissolve 12.5 mg of type II collagenase in 10 mL of DB. Use all this solution to digest oocytes at 25°C for 30 min. Discard the solution and rinse oocytes multiple times with fresh DB until the buffer runs clear.
31. Repeat step 30, 2–3 more times, until the follicle layer is fully digested (Figure 2).

△ **CRITICAL:** Check under a stereoscope to make sure that the follicle layer is mostly gone.

32. Rinse with DB till the solution is clear. Wash the oocytes 3–4 times with 20 mL of ND96. Transfer digested oocytes to multiple 10-cm tissue-culture dishes containing ND96 with <100 oocytes/dish. Store the dishes at 17°C for 12–24 h before cRNA injection.

△ **CRITICAL:** Ensure that oocytes are completely submerged in ND96. Maintaining a low oocyte density (<100 oocyte/dish) in 10-cm dishes is **CRITICAL:** to keep oocytes healthy.

33. Next day, select healthy stage V–VI oocytes, whose diameter should be ~1 mm. Transfer them to new dishes with ND96.
34. Inject these oocytes with 30–60 ng of cRNA using a microinjector. 30–40 ng of cRNA is suitable for two-electrode voltage clamp, while 50–60 ng of cRNA is often necessary for patch clamp.
35. Transfer injected oocytes into 6-well plates (5 oocytes/well) filled with ND96. Change the buffer every day and remove dead oocytes.

△ **CRITICAL:** It is important to remove dead oocytes (Figure 2) and remove buffers containing oocyte debris. In our hands, the presence of dead oocytes and the substances these cells release seem to make other oocytes sick.

36. The oocytes will be ready for two-electrode voltage clamp or patch-clamp 3–5 days after cRNA injection.

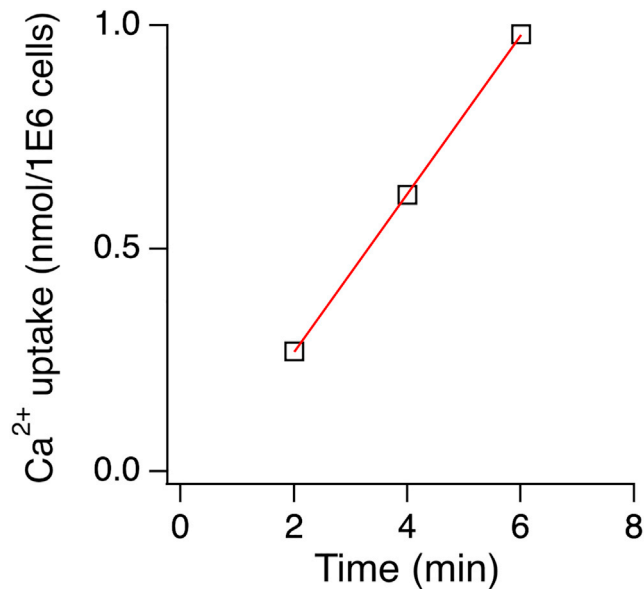


Figure 3. Uniporter-mediated mitochondrial ⁴⁵Ca²⁺ uptake

Data points were fit with a linear equation (red line), yielding a rate of mitochondrial Ca²⁺ uptake of 0.18 nmol/min/1 × 10⁶ cells.

EXPECTED OUTCOMES

Mitochondrial ⁴⁵Ca²⁺ uptake assay

Below is the raw data from a typical mitochondrial ⁴⁵Ca²⁺ uptake experiment using MICU1-knockout (KO) HEK293T cells (Phillips et al., 2019) in the presence of 300 nM free Ca²⁺ (MICU1 is a regulatory subunit that blocks uniporter Ca²⁺ transport in submicromolar Ca²⁺ levels):

The uniporter-specific Ca²⁺ uptake is then plotted against time (Figure 3) [Troubleshooting 1]. The data points are fitted with a linear equation to obtain the rate of uniporter-specific Ca²⁺ uptake (nmol Ca²⁺/1 × 10⁶ cells/min). Repeat this analysis in at least 3 independent replicates to obtain the average rate of uniporter-specific Ca²⁺ uptake for data presentation. The example provided above (Table 2 and Figure 3) shows that MICU1-KO allows the uniporter to take up a significant amount of Ca²⁺ in resting levels of cellular Ca²⁺ as detailed in our previous work (Phillips et al., 2019; Tsai et al., 2016).

Electrical recordings using *Xenopus* oocytes—Two-electrode voltage clamp

We typically perform two-electrode voltage clamp using oocytes injected with 30–40 ng of hME cRNA for 3–4 days of expression. Oocytes are initially perfused in ND96 prior to the impalement of electrodes. Afterwards, currents are recorded in a Ca20 recording solution. Ca²⁺ influx through the uniporter would induce outwardly rectifying, Ca²⁺-activated Cl⁻ currents (CACC, Figure 4) [Troubleshooting 2]. Adding Ru360 inhibits the uniporter, thus causing a significant reduction of CACC (Figure 4). The amplitude of CACC can therefore be used to indirectly assess uniporter transport activity. To directly record uniporter-mediated Ca²⁺ currents, one could inject 10 mM EGTA into oocytes before recording in Ca20 [Troubleshooting 3]. This maneuver will abolish CACC, thus revealing inwardly rectifying uniporter Ca²⁺ currents (Tsai and Tsai, 2018; Van Keuren et al., 2020).

Electrical recordings using *Xenopus* oocytes—Patch clamp

We normally perform patch-clamp recordings using the outside-out mode, with 10 mM EGTA in the pipet solution to eliminate CACC and 100 mM Ca²⁺ in the perfusion solution (Ca100). Outside-out mode is used so that Ru360, which inhibits the uniporter from the intermembrane space, can be

Table 2. Data from a typical $^{45}\text{Ca}^{2+}$ uptake experiment

	Average signals from membranes, 2-min data point	Average signals from membranes, 4-min data point	Average signals from membranes, 6-min data point	Total signals in 100 μL solution
Signals from tube A (no Ru360). Unit: cpm (count per minute)	14435	30545	46954	4545195
Tube B (+ Ru360)	2315	2481	2379	4607190
Uniporter-specific fractional uptake ^a	2.7×10^{-3}	6.2×10^{-3}	9.8×10^{-3}	
Uniporter-specific Ca^{2+} uptake ^b . Unit: nmol Ca^{2+} / 1×10^6 cells	0.27	0.62	0.98	

^aUniporter-specific fractional uptake = (Signals from membranes from tube A/total signals in 100 μL solution in tube A) – (Signals from membranes from tube B/total signals in 100 μL solution in tube B)

^bUniporter-specific Ca^{2+} uptake (nmol Ca^{2+} / 1×10^6 cells) = total calcium concentration in RB (after adding $^{45}\text{Ca}^{2+}$) 100 μL fractional uptake / 5×10^5 cells

perfused to shut the uniporter during recordings. Oocytes injected with 50–60 ng of cRNA are allowed to express proteins for 3–5 days. The vitelline membrane of oocytes is removed right before the oocytes are placed in the recording chamber [Troubleshooting 4]. When using pipets with 3–4 M Ω pipet resistance, we can routinely obtain Ru360-inhibitable, macroscopic MCU Ca^{2+} currents of <5 pA (Figure 5) with the membrane potential clamped at -90 mV [Troubleshooting 5]. The single-channel amplitude of the uniporter is ~ 0.25 pA under this recording condition, as also reported in mitoplast patch-clamp recordings (Kirichok et al., 2004).

LIMITATIONS

The main limitation for the mitochondrial $^{45}\text{Ca}^{2+}$ uptake assay is the inability to control inner membrane potential, which is the driving force for Ca^{2+} influx (Bernardi, 1999). It is possible that Ca^{2+} influx could decrease the inner membrane potential, leading to gradually reduced driving force during the 6-min assay time course. However, we have shown elsewhere (Van Keuren et al., 2020) that this is not a concern as long as the researcher keeps Ca^{2+} concentrations within the physiological range (< 10 μM) when doing such experiments.

The membrane potential can be accurately clamped when doing electrical recordings of the uniporter in *Xenopus* oocyte membranes. The main limitation right now is that we only reconstituted the uniporter's transmembrane subunits, MCU and EMRE, in the oocytes. We are actively working on establishing a procedure to also incorporate the regulatory MICU subunits (MICU1 and MICU2) to the system. However, recording with only the MCU and EMRE subunits will be adequate to study the most fundamental questions regarding the uniporter's ion-conducting and Ca^{2+} -selectivity mechanisms.

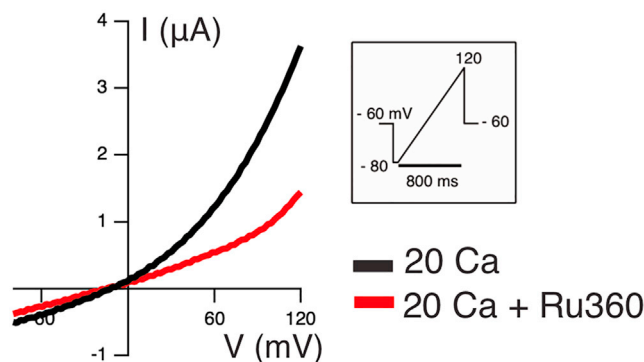


Figure 4. Two-electrode voltage clamp recordings of the uniporter in *Xenopus* oocytes
Adding Ru360 inhibits the uniporter, thus reducing the amplitude of the outwardly rectifying CACC.

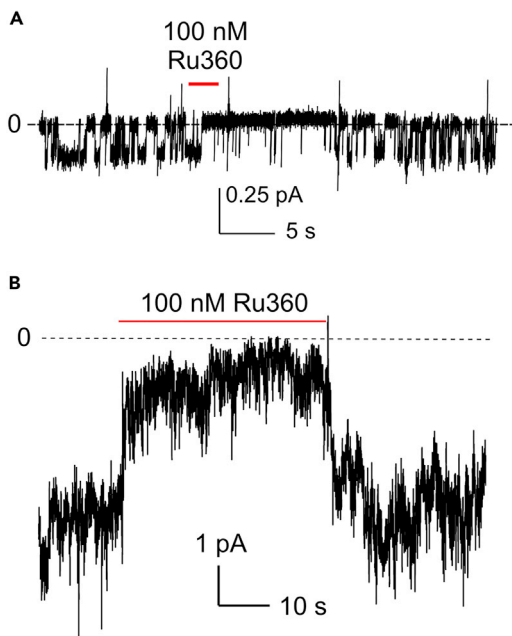


Figure 5. Patch-clamp recordings of the uniporter in *Xenopus* oocyte membranes

(A) A representative single-channel recording.

(B) A representative macroscopic recording. Ru360 was shown to reversibly inhibit the uniporter.

TROUBLESHOOTING

Problem 1

Low signal-to-noise ratio in the mitochondrial $^{45}\text{Ca}^{2+}$ uptake assay.

Potential solution

Make sure to use $^{45}\text{Ca}^{2+}$ with enough specific activity. It's imperative to use the $^{45}\text{Ca}^{2+}$ stock within 6–8 months after delivery (the half-life of $^{45}\text{Ca}^{2+}$ is ~5 months). Incomplete block of the ER Ca^{2+} -ATPase pump can also increase noise levels, so it's important to replace the thapsigargin stock solution at least once per month. Finally, if the uniporter activity is from transiently expressed uniporter proteins, it is important to first confirm successful expression using Western blots.

Problem 2

Small currents in *Xenopus* oocyte two-electrode voltage clamp recordings.

Potential solution

In our hands, <50% of *Xenopus* frogs produce useful oocytes for recordings. If the current is small, try another batch of oocytes from a different frog. It is also helpful to keep the oocytes in the best condition by maintaining a low cell density, removing dead cells, and changing media frequently.

Problem 3

Injecting EGTA into oocytes produces big leak currents in two-electrode voltage clamp.

Potential solution

We normally inject EGTA into oocytes and let the cells recover for at least 30 min before doing two-electrode voltage clamp. The wound caused by EGTA injection usually heals in this amount of time. However, this maneuver could produce big leak currents in sicker oocytes. If the leak issue becomes a big problem, we recommend setting up an injection electrode in the recording station. After the impalement of voltage and current electrodes, penetrate the oocyte with the injection electrode to inject EGTA and leave the electrode there for the entire recording.

Problem 4

Users encountering difficulties to remove the vitelline membrane.

Potential solution

Removing the oocyte vitelline membrane might be a challenge for inexperienced investigators. We find this video to be helpful to show new users how to execute this step. Oocytes become very fragile without the vitelline membrane, so it is very important to transfer the oocytes to recording chambers gently.

Problem 5

No uniporter currents in patch-clamp recordings with excised patches.

Potential solution

This is most likely due to low hME expression. One can increase the size of the pipet to have a resistance of 1–2 M Ω to increase the chance of capturing channels in excised patches. We also recommend the users to check hME expression by measuring whole-cell currents using two-electrode voltage clamp. Without at least 20–30 μ A of CACC (120 mV), it would be very difficult to record currents in patch-clamp with 3–4 M Ω pipets.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ming-Feng Tsai, ming-feng.tsai@cuanschutz.edu

Materials availability

The hME *Xenopus* oocyte expression has been deposited into Addgene (#178181).

Data and code availability

The protocol did not generate datasets or codes.

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AUTHOR CONTRIBUTIONS

All authors worked together to prepare figures, draft the protocol, and complete the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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