Measurement of Extracellular Ca^{2+} Influx and Intracellular H^+ Efflux in Response to Glycerol and PEG6000 Treatments

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[Abstract] The characteristics of Ca^{2+} and H^+ fluxes may reflect the activities of aquaporins, as the up-regulation of aquaporin activities is directly associated with the decrease in cytoplasmic H^+ concentration and increase in cytoplasmic Ca^{2+} concentration. The higher aquaporin activities can protect cells against osmotic stresses by altering water flow into and out of the cells. In order to confirm the contribution of aquaporins to the cell tolerance to different osmotic stresses, net Ca^{2+} and H^+ fluxes are measured using the noninvasive micro-test technique (NMT). NMT provides the real-time *in situ* detection of net ion transport across membranes. Here, we describe the protocol of *in situ* detection of net Ca^{2+} and H^+ fluxes across transformed *Pichia pastoris* cells in response to glycerol and polyethylene glycol 6000 (PEG6000) treatments. The transformed yeast cells are loaded onto a coverslide pre-processed in the poly-L-lysine solution (0.1% w/v aqueous solution). After cell immobilization, microelectrodes are positioned above a monolayer of attached cell population. Micro-volts differences are measured at two excursion points manipulated by a computer. Micro-volts differences could be converted into ion fluxes using the ASET 2.0 and iFluxes 1.0 Software. The method is expected to promote the application of NMT in microbiology. We are very grateful to Younger USA (Xuyue Beijing) NMT Service Center for their critical reading of the manuscript.

Materials and Reagents

1. Transformed *Pichia pastoris* cells (Invitrogen, catalog number: V200-20)
2. Poly-L-lysine solution (0.1% w/v aqueous solution) (Sigma-Aldrich, catalog number: P4707)
3. Polyethylene glycol 6000 (PEG6000) (Merck KGaA, catalog number: 807491)
4. Glycerol (Sinopharm Chemical Reagent, catalog number: 10010692)
5. Yeast extract (Oxoid, catalog number: LP0021)
6. Peptone (Oxoid, catalog number: LP0037)
7. D-glucose (Sinopharm Chemical Reagent, catalog number: 10010592)

8. MES
9. Standard medium buffer (pH 6.0) (see Recipes)
10. Yeast extract peptone dextrose (YPD) medium (see Recipes)
11. Calibration medium buffer (pH 7.0) (see Recipes)
12. Calibration medium buffer (pH 5.0) (see Recipes)

Equipment

1. Non-invasive Micro-test System (YoungerUSA, model: NMT100 series)
2. Shaking incubator (Shanghai Anting Scientific Instrument Factory, model: HZQ-F160)
3. Centrifuge (Thermo Fisher Scientific, model: Fresco 21)
4. Microplate Reader Spectra (Molecular Devices, model: SpectraMax 190)
5. Glass coverslide (20 mm x 20 mm)
6. Petri dish (35 mm in diameter)
7. Micropipettor (Eppendorf, 100-1,000 µl and 10-100 µl)

Software

1. JCal V3.2.1 (a free MS Excel spreadsheet, available at http://www.youngerusa.com or http://www.ifluxes.com)
2. ASET 2.0 software (available at http://www.youngerusa.com)
3. iFluxes 1.0 software (available at http://www.youngerusa.com)

Procedure

1. The transformed cells are incubated in 10 ml YPD at 30 °C in a shaking incubator (200 rpm) for 12 h.
2. Overnight cultures of different transformed cells are adjusted to an OD_{600nm} of 0.2. OD_{600nm} is monitored using microplate reader spectra.
3. Fifty microliters of each are taken and added to 10 ml YPD containing a final concentration of 25% PEG6000 or 1 M glycerol or no exogenous osmolytes. The transformed yeast cells are grown to an OD_{600nm} of 1.0 in YPD containing different exogenous osmolytes at 30 °C in a shaking incubator (200 rpm). OD_{600nm} is monitored using microplate reader spectra.
4. One milliliter of each is taken and pelleted at 2,000 rpm for 5 min at room temperature.
5. Nine hundred microliters of supernatant are removed, and the cells are resuspended in the rest of YPD media.

6. The coverslips are immersed in the poly-L-lysine solution (0.1% w/v aqueous solution) for 24 h.
7. Prior to each flux measurement, the microelectrodes must be calibrated in calibration medium (pH 7.0 and pH 5.0), respectively, following to the same procedure and standards. Only Ca^{2+} electrodes with Nernstian slope > 26 mV/decade and H^+ electrodes with Nernstian slope > 53 mV/decade are used in the protocol. Data are discarded if the post-test calibrations fail.
8. Ten microliters of transformed cells are loaded on the coverslip for 5 min, washed off with standard medium to ensure a monolayer of attached cells and incubated in the standard medium for 5 min at room temperature.
9. Microelectrodes are positioned 10 μm above the attached cell population consisting of 15 cells with equal size. Micro-volts differences are measured at two excursion points, one 10 μm above the cell population and the other 20 μm away, at a frequency of 0.05 Hz manipulated by a computer. The kinetics of net Ca^{2+} and H^+ fluxes near each cell population are monitored for 10 min.
10. For each sample, four clones are incubated in 10 ml YPD, and the resulting four cell populations are measured (see steps 1-9).
11. Micro-volts differences are exported as raw data before they are converted into net Ca^{2+} and H^+ fluxes by using the JCal V3.2.1. The ion flux assay around each type of transformed cells is replicated independently three times.

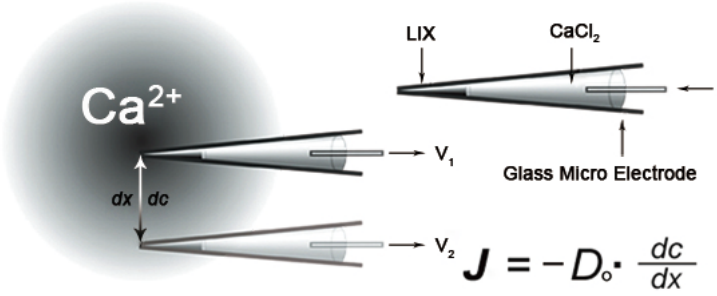

Figure 1. Schematic diagram of ion flux detection (www.xuyue.net). The microelectrode tip is filled with liquid ion exchanger (LIX). A voltage gradient (*dV*) is measured by the electrometer between two positions over the travel range *dx*. A concentration gradient (*dc*) is calculated based on *dV*. D_o, ion diffusion constant; *J*, net ion flux.

Recipes

1. Standard medium (aqueous solution) buffer (pH 6.0)
   0.1 mM $CaCl_2$
   0.1 mM KCl
   0.3 mM MES
   10 mM glucose
   pH is adjusted to 6.0 with HCl
   Stored at 4 °C
2. Yeast extract peptone dextrose (YPD) medium (1 L)
   1% yeast extract
   2% peptone
   2% D-glucose (added to the medium after autoclave)
   10 g yeast extract
   20 g peptone are dissolved in 900 ml of water
   The medium is autoclaved for 20 minutes on liquid cycle, cooled to ~55 °C
   Mixed with 100 ml of 20% D-glucose
   The liquid medium is stored at room temperature
3. Calibration medium (aqueous solution) buffer (pH 7.0)
   0.01 mM $CaCl_2$
   0.1 mM KCl
   0.3 mM MES
   10 mM glucose
   pH is adjusted to 7.0 with HCl
   The medium is stored at 4 °C
4. Calibration medium (aqueous solution) buffer, pH 5.0
   0.1 mM $CaCl_2$
   0.1 mM KCl
   0.3 mM MES
   10 mM glucose
   pH is adjusted to 5.0 with HCl
   The medium is stored at 4 °C

Acknowledgments

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