Supplementary material

Analysis of fluorescence signals

The analysis of the fluorescence images was based on the assumption of a constant, uniform proton flux across a spherical cell (Zifarelli et al, 2008). The focal plane was adjusted to coincide with the equator of the oocyte (see supplementary Fig. 1A). Ratio images were analyzed by defining a conical section (see supplementary Fig. 1A, B) and averaging the signal over each slice (see slices in supplementary Fig. 1B). The size and position of the conical section that was used for the analysis (see supplementary Fig. 1B) was fitted to the oocyte surface in a custom analysis program.

After subtraction of the initial ratio at time zero, the ratio change was converted to a change of total proton concentration employing the linear BCECF calibration (Fig. 1B). This procedure provided the experimental change of the total proton concentration, \( \Delta H_{\text{tot}}^{\text{exp}}(r,t) \), as a function of distance and time. Ideally, for relatively small pH changes, the total proton concentration change at time \( t \) after switching on the proton current and at a distance \( r \) from the oocyte center is given by

\[
\Delta H_{\text{tot}}^{\text{theory}}(r,t) = \frac{I}{4\pi FD}\left\{\text{erfc}\left(\frac{r-a}{2\sqrt{Dt}}\right) - e^{(r-a)^2/4Dt}\text{erfc}\left(\frac{r-a}{2\sqrt{Dt}} + \sqrt{Dt}/a\right)\right\} \quad \text{(Eq. A)}
\]

where \( I \) is the transmembrane proton current, \( F \) Faraday’s constant, \( D \) the diffusion coefficient of the buffer (in our case BCECF), \( a \) the radius of the oocyte, and \( \text{erfc} \) the complementary error function (Zifarelli et al, 2008).

Because of the finite focal depth, however, the measured signal is a superposition of fluorescence from in-focus and from out-of-focus planes. To estimate the contribution of out-of-focus fluorescence we measured the fluorescence of a small BCECF spot on a cover glass as a function of the distance from the focal plane (supplementary Fig. 1...
The signal could be fitted as a function of the distance from the focal plane, \( d \), in a satisfactory manner with a Gaussian function of the form

\[
F(d) = F_{\text{min}} + (F_{\text{max}} - F_{\text{min}}) \exp \left( -\left( \frac{d}{d_0} \right)^2 \right)
\]

(Eq. B)

with a minimal (background) fluorescence \( F_{\text{min}} \), a maximal fluorescence, \( F_{\text{max}} \), and a cut-off distance, \( d_0 \), which was estimated at \( \sim 300 \) µm (see solid line in supplementary Fig. 1 C). Since the estimated \( d_0 \) is of comparable magnitude as the oocyte radius, we corrected for the out-of-focus fluorescence in the following way:

We included the contribution of fluorescence from points above and below the focal plane using the attenuation function (Eq. B). The change in the total proton concentration at a point that has a certain distance, \( d \), from the focal plane will be given by Eq. (A) with an increased distance

\[
r' = \sqrt{r^2 + d^2}
\]

(see supplementary Fig. 1D). However, its influence on the measured fluorescence ratio will decrease in a Gaussian manner according to Eq. (B).

The total \([H^+]\) change is linearly related to the change of the fluorescence ratio, \( F_{490}/F_{440} \)

\[
\Delta H_{\text{tot}} \sim \frac{F_{490}}{F_{440}}
\]

(see calibration above). Furthermore, the fluorescence at 440 nm is relatively insensitive to pH changes. Thus, to a first approximation

\[
\Delta H_{\text{tot}} \sim F_{490}
\]

at any point in time and space. Based on these considerations, we implemented the out-of-focus correction by fixing a maximum distance, \( d_{\text{max}} \), of correction (usually the radius of the oocyte) and a maximum number, \( N_{\text{plane}} \), of equidistant correction
“planes” above and below the plane of focus (10 planes were normally used; using a larger number did not significantly alter the results). We then calculated correction coefficients, $c_i$, ($i=-N_{\text{plane}}, ..., N_{\text{plane}}$) according to

$$c_i = A \exp\left(-\frac{i d_{\text{max}}/N_{\text{plane}}}{d_0}\right)$$

where $d_0$ was chosen from the fit in supplementary Fig. 1C ($d_0 = 300 \mu m$), and $A$ was calculated such that the coefficients sum up to 1:

$$A = \frac{1}{\sum_{i=-N_{\text{plane}}}^{N_{\text{plane}}} \exp\left(-\frac{i d_{\text{max}}/N_{\text{plane}}}{d_0}\right)^2}$$

The experimental $\Delta H_{\text{tot}}^{\text{exp}}(r,t)$ values were then fitted to the weighted sum of the theoretical values

$$\text{Fit}(r,t) = \sum_{i=-N_{\text{plane}}}^{N_{\text{plane}}} c_i \Delta H_{\text{tot}}^{\text{theory}} \left(\sqrt{r^2 + \left(i d_{\text{max}}/N_{\text{plane}}\right)^2}, t\right)$$

(Eq. C)

by a least-squares procedure, resulting in estimates of the proton current, $I$, and the diffusion coefficient $D$. 


Supplementary Figure legends

**Supplementary Figure 1.** Illustration of the analysis of the fluorescence experiments. 
A shows a typical false color image as it appears in the custom analysis program. The position and size of the conical section are adapted to the oocyte in a graphical manner. One cursor position determines the starting radius for the gradient analysis (used to reduce the effect of surface irregularities and to avoid buffer saturation effects (Zifarelli et al, 2008)). Two distant cursors determine a region used to track drifts in fluorescence ratios during the measurement (assuming no change in pH distantly from the oocyte). B illustrates the division of the analysis section in concentrical shells. Ratios are averaged through each shell, yielding the ratio as a function of the distance from the oocyte center. C shows the fluorescence intensity of a small BCECF spot on a cover slip as a function of the distance of the spot from the focal plane of the objective. The solid line is a fit of Eq. (B) with $d_0=300 \ \mu m$. D illustrates that points above and below the focal plane have an increased distance, $r'$, from the oocyte center, compared to a point in the focal plane at distance $r$.

**Supplementary Figure 2.** Representative measurement of the extracellular acidification produced by the activation of the mutant S168P with repetitive voltage pulses to 60 mV. Stimulation is indicated by the bar. Initial pH of the solution was 6, imposing an inwardly directed proton gradient. Note that the acidification implies active outward H$^+$ transport.
Supplementary Figure 3. Conductivity properties of mutants S168A (A), S168G (B), and S168T (C). Shown are normalized current-voltage relationships in extracellular solutions containing Cl\(^-\) (filled circles), NO\(_3^-\) (triangles), Br\(^-\) (squares), I\(^-\) (open circles) (n\(\geq\)4 oocytes).

Supplementary Figure 4. Comparison of proton transport efficiency in chloride and nitrate containing solutions of WT ClC-5 (A) and mutants S168A (B), S168G (C), S168T (D) and S168P (E). Shown are representative measurements performed with a pH sensitive microelectrode of the extracellular acidification produced by the activation with repetitive voltage pulses to 100 mV in extracellular Cl\(^-\) (circles) and extracellular NO\(_3^-\) solution (squares), each from the same oocyte.
Supplementary Figure 1

A

B

C

D

Supplementary Figure 1

A

B

C

D
Supplementary Figure 2

![Graph showing pH as a function of time with a stimulus event at time 25 seconds.](image-url)
Supplementary Figure 4

(A) WT CIC-5
(B) S168A
(C) S168G
(D) S168T
(E) S168P

Time (s)