Chapter 5

Ionic strength sensing in living cells

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Abstract

Knowledge of the ionic strength in cells is required to understand the in vivo biochemistry of the charged biomacromolecules. Here, we present the first sensors to determine the ionic strength in living cells, by designing protein probes based on Förster resonance energy transfer (FRET). These probes allow observation of spatiotemporal changes in the ionic strength on the single-cell level.
Introduction

The ionic strength influences a wide array of electrostatic interactions in the cell.\textsuperscript{1,2} To understand the role of the ionic strength in cell physiology, a sensor to quantify the ionic strength in vivo is needed. The ionic strength is the effective ion concentration and screens electrostatic interactions of (macro)molecules, which is obviously of crucial importance to the cell. For example, the ionic strength determines the structure of intrinsic disordered proteins,\textsuperscript{3} activity of enzymes,\textsuperscript{4} protein aggregation,\textsuperscript{5} quinary assemblies/phase separations,\textsuperscript{6} protein binding to (poly)nucleic acids,\textsuperscript{7} the catalytic function of riboswitches,\textsuperscript{8} and many other processes. The ionic strength governs the cell volume by activating the channels and transporters involved.\textsuperscript{9,10} The ionic strength can vary however, depending on the extracellular environment, due to for example abrupt changes in medium osmolality, and fluctuations with intracellular events such as metabolite or polynucleotide synthesis.\textsuperscript{11}

To quantify the ionic strength one cannot simply use the concentration of ions, because the effective ion concentration (i.e. thermodynamic activity) is altered due to interaction of the ions with each other and the biomolecules. Indeed, a subset of ions has a higher affinity for macromolecules, such as magnesium, while others are more loosely associated, and their activity coefficient may approach that in the extracellular medium.\textsuperscript{12} Such effects result in a strong dependence of electrostatic screening on the identity of the ions, roughly following the Hofmeister series.\textsuperscript{13,14} Thus, although it is possible to determine the concentration of a particular ion with for example flame photometry of dry cell mass,\textsuperscript{15} this will not immediately reveal the ionic strength; that is, the effective concentration of an ion depends on its environment. In addition, these techniques will not yield information on population heterogeneity or rapid temporal changes in the ionic strength. Probes to infer the ionic strength from the activity of a membrane protein\textsuperscript{9} or the fluorescence of a fluorescein-BSA conjugate\textsuperscript{16} are complicated to use and interpret, and they have only been applied vesicles and isolated mitochondria but not in living cells.

Here, we fill this void by designing the first probes that sense the ionic strength in living cells. The sensors are based on FRET and are genetically encoded, because these properties allow monitoring analytes with high spatiotemporal precision inside living cells. Fluorescent protein probes can be affected by ions nonspecifically,\textsuperscript{17} and FRET sensors selective for a specific ion have been developed, for example zinc, calcium, or chloride.\textsuperscript{18,19,20} We construct our sensors to contain a positively and a negatively charged α-helix, whose electrostatic attraction will depend on the ionic strength (Fig. 1). At the N- and C-termini of the helices are two fluorescent proteins,
mCerulean3 and mCitrine, that form a FRET pair. Increasing the ionic strength will decrease the attraction between the helices, which will result in a decrease in FRET efficiency.

To induce charge in the helices, we inserted 6 glutamates in an alanine background in one helix, and 6 lysines in the other helix (Fig. 1). The charged amino acids are in a i+5 spacing, a staircase-like configuration, ensuring that all sides of the helix are covered with charges. This prevents the helix to have a charged patch as well as preventing metal ion chelation. We further reasoned that the high intramolecular concentration of the oppositely charged -helix will outcompete electrostatic interactions with most of the charged cellular biomolecules. Because the presence of specific ion effects could be dependent on the identity of the amino acids in the helix, we designed sensors with glutamate or aspartate residues, and lysine or arginine residues, and thereby alter e.g. salt-bridge stability. With these design elements we avoid selectivity for specific ions in the sensors, allowing for ionic strength determination in living cells.

Material and Methods

Expression and purification of the ionic strength sensors. The synthetic gene (Supplementary Note) encoding the ionic strength sensors in pRSET-A was obtained from GeneArt. The plasmid was transformed into the E. coli strain BL21(DE3) pLysS (Promega). The cells were grown to an OD600 of 0.6 at 37 °C and shaking at 200rpm in LB medium (1.0% bactotryptone, 0.5% yeast extract, 1% NaCl) with 1 mg/mL ampicillin, after which the cells were induced overnight with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) at 25 °C and shaking at 200 rpm. The cell lysate was cleared by centrifugation, supplemented with 10 mM imidazole and purified by nickel-nitrilotriacetic acid Sepharose chromatography (wash/elution buffer: 20/250 mM imidazole, 50 mM NaPi, 300 mM NaCl, pH 8.0). The sensor was further purified by Superdex 200 10/300 GL size-exclusion chromatography (Amersham Biosciences) in 10 mM NaPi, pH 7.4. Fractions containing pure protein were aliquoted and stored at –80 °C.

In vitro characterization of the sensors. A 300 µL solution containing the given salt, and 10mM NaPi (adjusted the pH to 7.4 after dissolution of the salts) was added to a 96 well plate (Greiner). The purified sensor was added and the fluorescence intensity at 475 nm and 525 nm were recorded separately in a Spark® 10M microplate reader with excitation at 420 nm at room temperature. A 20 nm bandwidth for excitation and emission was applied and the average of 10 measurements of a single well was taken. The background fluorescence, buffer without sensor, was subtracted.
Transfection and imaging of HEK293 cells. Transfection and imaging of HEK293 cells was performed as described,\textsuperscript{21} with some minor modifications. HEK293 cells (ATCC CRL-1573, tested for mycoplasma contamination) were cultured in DMEM (Gibco) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine (Gibco), 100 units mL\textsuperscript{−1} penicillin (Invitrogen) and 100 μg mL\textsuperscript{−1} streptomycin (Invitrogen). For transfection experiments, HEK293 cells were plated in eight-well Labtek glass chamber slides (Thermo Scientific) at 6 × 10\textsuperscript{4} cells per well. One day after plating, the cells were transfected with plasmid DNA coding for the sensors as follows: lipoplexes composed of 1.5 μl of Lipofectamine 2000 (Invitrogen) and 0.5 μg of the pcDNA 3.1 vector carrying the corresponding sensor gene were prepared in 100 μl of serum-free DMEM, according to the manufacturer’s instructions. 25 μl of lipoplex solution was added per well and incubated for 4 h at 37 °C and 5% CO\textsubscript{2}, after which the medium was refreshed. The next day, the medium was replaced by 200 μl of DMEM with HEPES without phenol red, and sensor expression in the HEK293 cells was subsequently analyzed by confocal fluorescence microscopy. The cells were imaged directly in the eight-well Labtek glass chamber slides. The slides were mounted on a laser-scanning confocal microscope (Zeiss LSM 710), at 37 °C. The sensor was excited using a 405-nm LED laser, and the emission was split into a 450–505-nm channel and 505–797-nm channel. The fluorescence intensity of the cells was determined in ImageJ for each channel. The backgrounds for each

**Fig. 1. Design of ionic strength sensors and sensing concept.** Ions screen the attraction between the positively and negatively charged helices, reducing the FRET efficiency.
channel were subtracted and the mCitrine intensity divided by the mCerulean intensity for each cell. When the mCitrine intensity was plotted versus the mCerulean intensity, linear fits with $R^2 > 0.99$ were generally obtained.

Results and discussion

To determine the ionic strength sensitivity of the sensors, we performed extensive in vitro tests on isolated probes (Fig. 2A, Supplementary Fig. S1). We tested the three ionic strength sensors, that is, the KE (lysine-glutamate), the RE (arginine-glutamate), and the RD (arginine-aspartate) sensors, and compared the findings with a sensor that contains neutral helices, the E6G2 probe. We found that the FRET efficiencies of the ionic strength sensors in 10 mM NaPi buffer were much higher than that of the E6G2 probe, confirming that the helices attract each other. Addition of potassium chloride to the ionic strength sensors lead to a decrease in FRET efficiency (Fig. 2B), with the highest sensitivity between 0 and 300 mM KCl. The E6G2 probe was not sensitive, showing that we probe the ionic strength. Salt does not affect other neutral sensors with higher FRET efficiencies, and hence the FRET efficiency does not relate to the ionic strength dependence. We added a wide range of salts to test whether specific ion effects interfere with the readouts (Fig. 2C). We find that the sensitivity to the cations of KCl, NaCl, MgCl$_2$, and LiCl is the same when the ratios are plotted versus the ionic strength (Supporting Fig. S3). In contrast, the readout had a dependence on the identity of the anion (Supporting Fig. S2). The order of sensitivity followed the Hofmeister series, that is, the measure to what extent ions are hydrated, which is generally more dependent on the anion. This deviation from ideal
behavior depended on the probe, and qualitatively followed the intramolecular salt bridge strength between the two helices; the RD probe has the lowest salt-bridge strength and is least affected by the non-ideal behavior, as judged from the spread in deviation between the different ions. Likely due to the lower salt-bridge strength, the RD probe also has a lower FRET efficiency. Together, the dependence on ion identity indicates that we probe the effective ion concentration (or ionic strength) rather than total ion concentration. In all cases, the E6G2 probe was insensitive to any ion.

We investigated sensitivity to a wide variety of other parameters to make the transition to in vivo measurements. Similar to the E6G2 and GE crowding probes, mCitrine induced pH sensitivity in all the probes only below pH 7.0 (Supporting Fig. S4). The zwitterionic glycine betaine and neutral small molecules sorbitol and sucrose hardly influenced the probes (Supporting Fig. S5). The common intracellular organophosphates fructose bisphosphate, ATP, ADP, and AMP influenced the probes as can be expected from their charge, albeit that the sensitivity to the nucleotides was somewhat higher (Supporting Fig. S6). Glutathione influenced the probes according to its net negative charge (Supporting Fig. S7). The sensitivity to potassium glutamate was less than potassium chloride, as expected from the Hofmeister series, with the RD probe again deviating the least (Supporting Fig. S8). The sensors associated with the polyelectrolyte DNA in the absence of KCl, but not in the presence of 100 mM KCl (Supporting Fig. S9). Macromolecular crowding induced by Ficoll 70 compressed all the probes (Supporting Fig. S10) and hence should be taken into account when measuring in live cells. The ionic strength probes are temperature sensitive in the absence of salt, but the temperature sensitivity decreased with increasing salt concentration (Supporting Fig. S11). Increasing the sensor concentration itself did not affect the ratio (Supporting Fig. S12), ruling out interference from self-association. Hence, the sensors are sensitive to the ionic strength mainly, but other parameters such as macromolecular crowding, pH, and temperature need to be controlled for.

To demonstrate ionic strength sensing in vivo, we expressed the probes in the mammalian cell line HEK293, and imaged the cells by scanning confocal microscopy. The cells were subsequently imaged by excitation of the mCerulean3 at 405 nm, and the emission ratio of mCitrine (505–750 nm) over mCerulean3 (450–505 nm), after background subtraction, was monitored (Fig. 3A,B). The ratios obtained were very homogeneous over all the cells. When comparing the different sensors, we found that RD gave lower ratios than the KE and RE probes, similar to what we observed in vitro (Supporting Fig. S14). To quantify the readouts, we calibrated the probes in the cell by clamping the internal potassium concentration by titrating the external
medium with potassium chloride in the presence of the ionophores valinomycin plus nigericin. These ionophores equilibrate the protons and potassium over the membrane, providing a calibration curve of the corresponding sensor with a known concentration of potassium. All the ion sensors sensed the potassium concentration in a similar manner as observed in vitro. The ratios for the GE crowding sensor remained unchanged, indicating that with the calibration method we did not alter the crowding, and prevent osmotic pressure differences over the membrane by ionophore-assisted ion equilibration. The cells did change shape during the calibration procedure (Fig. 3A). We subsequently used the calibration curves to quantify the ionic strength from the readout in regular growth medium and found that the ionic strength was comparable to ~130 mM for the RD and RE sensors, and ~110 mM for the KE sensor (Fig. 3B, Supporting Fig. S14). These values are in the same range as the ion concentrations reported for

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**Fig. 3. Ionic strength determination in vivo and observation of dynamic changes observed in scanning fluorescence confocal microscopy of HEK293 cells expressing the RD sensor.**

**A:** Fluorescence and mCitrine/mCerulean3 ratio images of sensor calibration with KCl, nigericin, and valinomycin. **B:** Calibration curve of the mCitrine/mCerulean3 ratio versus potassium concentration; the red line is the ratio of cells in growth medium (red shading is the standard deviation within a population, n = 20). **C:** Fluorescence and mCitrine/mCerulean3 ratio images of cells before and 10 minutes after addition of 450 mM sorbitol. See Supporting Fig. S13 for more detail. **D:** mCitrine/mCerulean3 ratio upon addition of sorbitol as a function of time; the data of 20 individual cells and the average are shown. Error bars are standard deviation within a single population of cells. The standard deviations of the averages of independent biological repeats are smaller (±0.03, n = 4).

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the monovalent ions in various cells, and the concentration of “free” ions is somewhat lower. The total ion concentration would be 140 mM and 12 mM for potassium and sodium, respectively. Divalent cations were not monitored because they are mostly bound to the biopolymers. Organophosphates form complexes with magnesium and may bind nonspecifically to proteins, and the molecules thus contribute less to the ionic strength than is expected from their total concentrations. Single cell measurements as function of time indicate that the ionic strength is determined with a precision better than 10 mM (that is, changes in mCitrine/mCerulean3 of less than 0.02). Thus, we conclude that the probes function in HEK293 cells and we can determine the ionic strength in vivo.

To further demonstrate the potential of the sensors in vivo, we monitored changes in ionic strength over time after an osmotic upshift by adding 450 mM sorbitol to HEK293 cells (Fig. 3C,D). We expected the short-term response to be an initial increase in crowding and ionic strength, followed by a regulatory volume increase by uptake of potassium and chloride from the medium, retaining the abnormally high ionic strength but decreasing the crowding. We assume that the response mechanism of the sensor is much faster than the biological events. We saw with the GE crowding probe that the crowding indeed increased with 450 mM sorbitol (Supporting Fig. S15), as we observed previously, after which a slow decrease in crowding took place. In the timeframe of ~20 minutes, the crowding was not yet fully recovered. The RD sensor showed that the ionic strength increased with osmotic upshift and remained at this level. It showed no apparent sensitivity to in vivo crowding, contrary to the KE and RE sensors that showed an initial increase in FRET directly after the osmotic upshift. Interestingly, an increase in ionic strength of ~190 mM to 320 mM can be inferred with the RD calibration curve, which is similar to the expected increase in osmolarity simply based by equating the sum of K and Cl to the number of sorbitol molecules added. We could monitor single cells in time and found that the distribution of FRET values increased after the osmotic upshift and the adaptation process that followed. This shows that the cells are affected differently by the osmotic upshift, which could for example be related to the cell cycle, or intrinsic variation in fitness in the population of cells that we analyzed. Hence, the probes can sense the ionic strength on the single-cell level during dynamic changes in the ionic strength.

Conclusions

In conclusion, we present here the first sensors to determine the ionic strength that function in living cells. Taking into account changes in
macromolecular crowding and pH, the sensors allow facile determination of the intracellular ionic strength on the single-cell level in changing environments or intracellular conditions. Given the wide variety of processes that are influenced by the ionic strength, these sensors will aid future investigations on the importance of the ionic strength during a wide variety of conditions such as cell volume regulation, disease, and environmental stresses.

Reference

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Supporting information:
Ionic strength sensing in living cells

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**Fig. S1. Absorption and fluorescence emission spectra (excitation at 420 nm) for the ionic strength sensors.** The sensor concentration for the absorption measurement was ~1 µM, and for the fluorescence measurements ~100 nM. The protein concentrations were determined from the absorption at 280 nm, using the Nanodrop ND-1000 spectrophotometer, and calculated with ε280 = 48,000 M⁻¹ cm⁻¹, which was estimated with the ExPASy ProtParam tool. The intensities of the fluorescence and absorption spectra were adjusted for comparison. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 °C.

**Fig. S2. Dependence of the mCitrine/mCerulean3 ratio on the ionic strength for different salts.** All sensors display a dependence on the identity of the ion. In all cases, the spread follows the Hofmeister series, i.e. SO₄²⁻->Cl⁻->SCN⁻. The magnitude of spread varies, and follows roughly RE>EK>RD, which corresponds to previously calculated salt bridge stabilities for these ion pairs. The data for the E6G2 crowding sensor is given as control, showing that RE, EK and RD sensors are uniquely dependent on ionic strength and that the sensing...
Fig. S3. Dependence of the mCitrine/mCerulean3 ratio of RD on the identity of the cation. The monovalent alkali ions K+, Li+, and Na+ all induce the same response. The divalent magnesium(II) gives the same response up to an ionic strength of 170 mM. This corresponds to a MgCl2 concentration of 50 mM (+10 mM NaPi pH 7.4). Higher concentrations are not feasible in NaPi buffer, which induces precipitation upon adjusting the pH. Divalent cations zinc and calcium were insoluble in NaPi buffer in the mM range at pH 7.4. Error bars are the standard deviations of three independent replicates. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 °C.

Fig. S4. pH sensitivity of the probes. The pH of 10 mM NaPi was set in the presence of 100 mM KCl to account for significant ionic strength effects due to the difference in NaH2PO4/Na2HPO4 ratio with pH. We observe that all sensors have a decreased ratio below pH 7 (the transition appears to be between pH 6.85 and 7.1), similar to the observations previously made on the GE sensor.1 Likely, the acid sensitivity is caused by the high pKa of the fluorophore in mCitrine, reducing its fluorescence. The signal is stable between pH 7 and ~9. Conditions: 10 mM NaPi, 100 mM KCl, temperature of 26±1 °C.

mechanism originates from the charged helices. Error bars are the standard deviations of three independent replicates. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 °C.
Fig. S5. Effect of neutral carbohydrates sorbitol and sucrose, and the zwitterionic osmolyte glycine betaine on the mCitrine/mCerulean3 ratio of the sensors. The small decrease at high concentrations could be due to an increase in preferential hydration of the peptides and decrease in salt-bridge strength. The differences are very small compared to the ionic strength effects, considering the high concentrations of osmolyte and the absence of a background electrolyte (e.g. 100 mM KCl) that would otherwise buffer most of the specific solute effects. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 °C.

Fig. S6. Dependence of the response of the RD sensor on the identity of physiological phosphate anions. The net charge of the ions at pH 7.4 was taken to be -3.3 for ATP (adenosine triphosphate), -2.8 for ADP (adenosine diphosphate), -1.8 for AMP (adenosine monophosphate), -1.6 for Pi (phosphate), and -3.6 for FBP (fructose 1,6-bisphosphate).
Fig. S7. Dependence of the mCitrine/mCerulean3 ratio of the sensor on the glutathione concentration. Glutathione is one of the most common small molecules of living cells and typically present at millimolar concentrations. A small decrease is observed, which is at least partly caused by the higher ionic strength, i.e. the addition of NaOH to neutralize the negative charge on glutathione and to obtain pH 7.4. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 ºC.

Fig. S8. Response of the sensors to potassium glutamate versus potassium chloride. Glutamate is the principle small anion of many types of cells. Glutamate decreases the mCitrine/mCerulean ratio less than chloride does, and this is expected according to the Hofmeister series. The ratios of the RE and KE sensors decrease little with glutamate, and at >300 mM glutamate the ratios even seem to level off and increase. The ratios obtained with the RD sensor for glutamate and chloride ions are more similar, but the decreased sensitivity for glutamate remains. In general, the difference in spread between the sensors is comparable to that observed for the other anions. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 ºC.

Corresponding sodium salts or protonated forms were dissolved in 10 mM NaPi adjusted to pH 7.4 with NaOH. The curves for ATP, ADP and AMP overlay when corrected for the ionic strength. The response to these salts is higher than for NaCl, which could be caused by some additional interactions of the nucleotides with the helices (e.g. due to chelation effects). The bisphosphate FBP and phosphate induce less of a change in the sensor, which is in accordance with the Hofmeister series. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 ºC.
Fig. S9. Sensitivity of the sensors to the presence of DNA. There is a strong decrease in mCitrine/mCerulean ratio in the presence of DNA and in the absence of KCl, which is not observed for the neutral crowding sensors. To test whether this behavior is caused by association of the positively charged helix of the sensor to DNA, we added 100 mM KCl and indeed we find a strong screening effect. Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 °C.

Fig. S10. Sensitivity of the ionic strength sensors to macromolecular crowding induced by Ficoll 70. The KE, RD and RE sensors are compressed by high concentrations of Ficoll 70, similar to the neutral FRET sensors. The sensitivity to macromolecular crowding needs to be taken into account when measuring in vivo; the change in FRET due to crowding is relatively small compared to the ionic strength effect. If the change in crowding as determined with the GE or E6G2 probe is not insignificant, then calibration of the sensors in vivo is needed (see main text). Conditions: 10 mM NaPi, pH 7.4; temperature of 26±1 °C.

Fig. S11. Temperature dependence of the ionic strength sensors at varying KCl concentrations. The temperature dependence could originate from salt-bridge stability, helix stability, altered
Supporting information: Ionic strength sensing in living cells

Fig. S12. Dependence of the mCitrine/mCerulean3 ratio on the concentration of the RD sensor. The FRET ratio does not change by adding up to 16 equivalents of RD sensor, when starting with 30 nM. If self-association would occur, intermolecular FRET would increase the FRET ratio. Hence the absence of an effect indicates that in the concentration range of 30 to 500 nM RD self-association does not play a role. Conditions: 10 mM NaPi, 26±1 °C, pH 7.4.

![Graph showing the dependence of mCitrine/mCerulean3 ratio on the concentration of RD sensor](image)

Fig. S13. Enlarged images of cells of Fig. 3C. The left panel depicts Fig. 3C, with dashed squares denoting the enlarged area displayed in the right panel. The ratiometric images (mCitrine/mCerulean3, bottom) are smoothed in ImageJ. We assign ratiometric variations in a single cell to instrumental noise.

![Images showing enlarged cells before and after sorbitol treatment](image)

buffer pH, Coulombic screening interactions, altered Debye lengths, and other effects. Such effects may oppose and cancel each other. In practical terms, the temperature dependence is only present at low ionic strength, and the sensors should be calibrated at the same temperature as the in vivo experiment. Conditions: 10 mM NaPi, pH 7.4.
Fig. S14. Calibration of the ion strength sensors in HEK293 cells. The calibration was done in the presence of set amounts of KCl (depicted on the x-axis), and using valinomycin (10 µM, K+ ionophore), nigericin (5 µM, K+/H+ ionophore), and 10 mM NaPi, pH 7.4, at 37 °C. The presence of ionophores balances the potassium and pH in the cell with the external environment, and the concomitant equilibration of chloride from the medium upon 20 min incubation. It can be seen that RE, KE, and RD respond to the increase in ionic strength, while the neutral GE probe does not. The GE probe functions as a control sensor to confirm that crowding remains the same during the calibration procedure. We do see that the shape of the cell does change under the calibration conditions. In lieu of a biophysical model to describe the sensor mechanism, we use the connections between the individual data points to calibrate the in-cell readout to an ionic strength. The red line is the measurement of the sensors in healthy cells in DMEM/Hepes medium (Dulbecco’s Modified Eagle Medium with L-glutamine, high...
Fig. S15. The response of the sensors in HEK293 cells upon changing the osmotic conditions.
The red data points are the average over 20 cells and the error bar is the standard deviation. The individual cells are the grey lines. Cells are imaged in 200 µL DMEM/Hepes medium at 37 °C, after which 20 µL of a 5M sorbitol solution was added. The solution was carefully mixed by pipette, and imaged every 2 minutes. Given the addition and mixing procedure, an error bar of ~0.5 minute is appropriate on the first data point after addition of sorbitol. The GE probe shows that crowding increases, followed by a slow recovery, in a similar fashion as we reported previously.1 The ionic strength sensors RE and KE show a fast initial increase in mCitrine/mCerulean ratio, followed by a decrease. This initial increase could be due to nonideal ion effects to which the RE and KE probes are more sensitive than RD (see studies with isolated probes above), in combination with crowding changes. The RD probe behaves most ideal and does not show the initial increase. The high accuracy of the measurements can be seen from the low noise in the single cell ratios in time. Furthermore, although most cells yield similar ratios, 3 out of 20 cells deviate strongly in their response to osmotic upshift. This could have various reasons, including differences in cell cycle, or inherent cell-to-cell variation. Error bars are standard deviations within a single population of cells.

(glucose, HEPES, and without phenol red). The transparent red bar is the standard deviation over a single population of cells. The crossing of the graphs is taken to be the readout of the ionic strength in vivo. In the bottom panel all the calibration lines are compiled in a single graph for comparison. Note the strong similarity to Fig. 2B, where KCl was added to the purified sensors. Error bars are standard deviation within a single population of cells.
References

