Supporting Information

Ionic strength sensing in living cells

Boqun Liu, Bert Poolman,* and Arnold J Boersma*

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute & Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, the Netherlands.

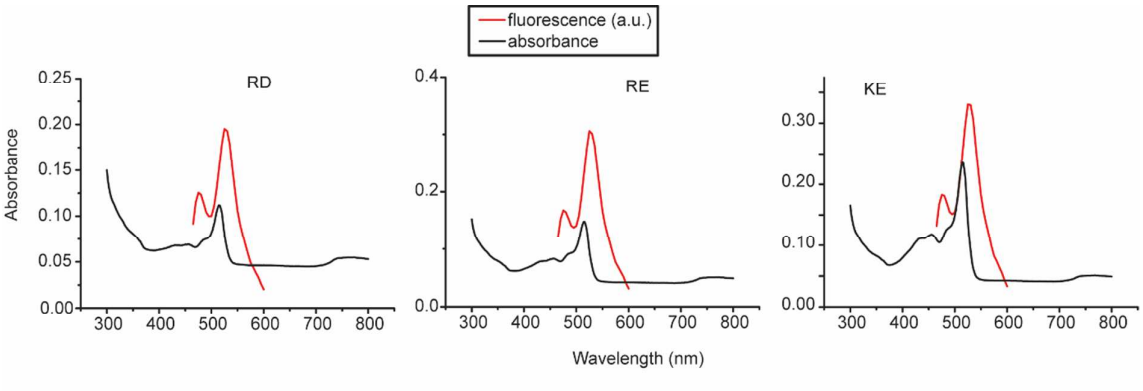

Figure S1. Absorption and fluorescence emission spectra (excitation at 420 nm) for the ionic strength sensors. The sensor concentration for the absorption measurement was ~1 μM, and for the fluorescence measurements ~100 nM. The protein concentrations were determined from the absorption at 280 nm, using the Nanodrop ND-1000 spectrophotometer, and calculated with ε_{280} = 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 ºC.

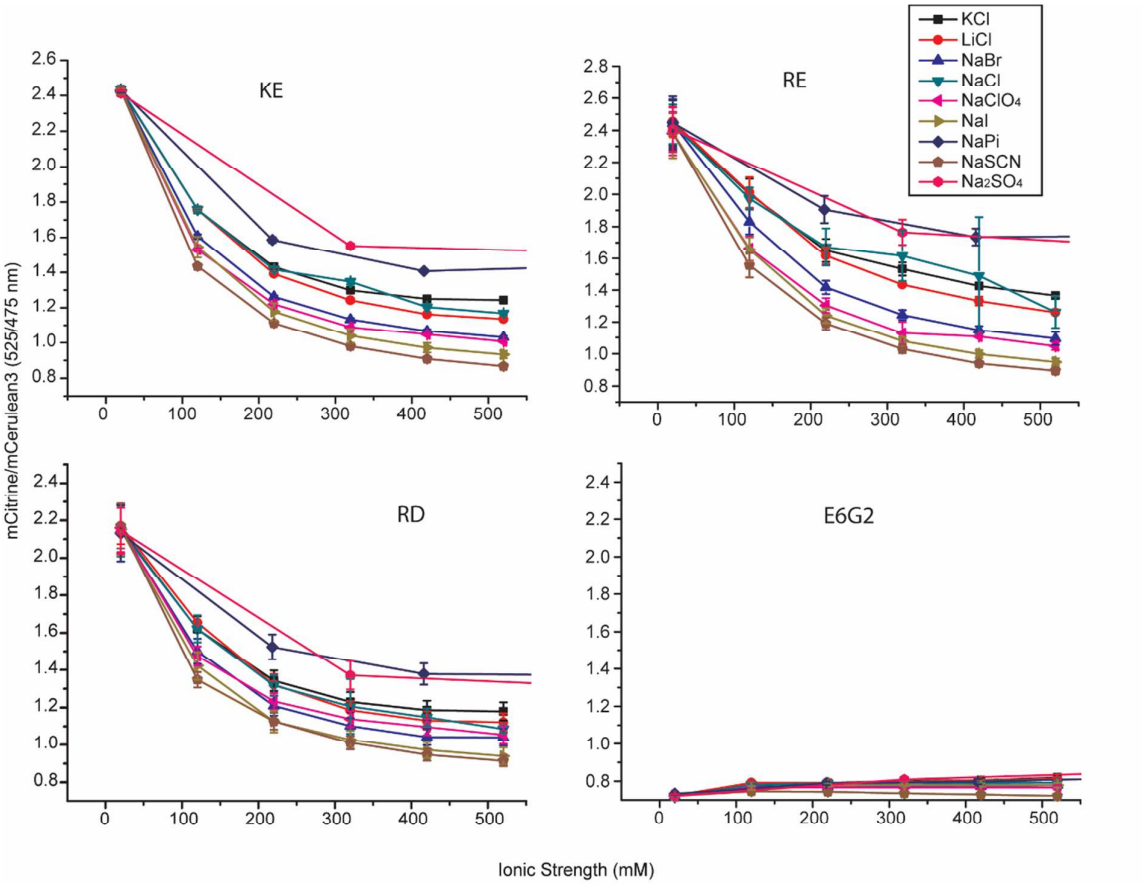

Figure S2. Dependence of the mCitrine/mCerulean3 ratio on the ionic strength for different salts. All sensors display a dependence on the identity of the ion. In all cases, the spread follows the Hofmeister series, *i.e.* SO_4^{2-}>Cl^->SCN^-. The magnitude of spread varies, and follows roughly RE>EK>RD, which corresponds to previously calculated salt bridge stabilities for these ion pairs.[2] 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 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 ºC.

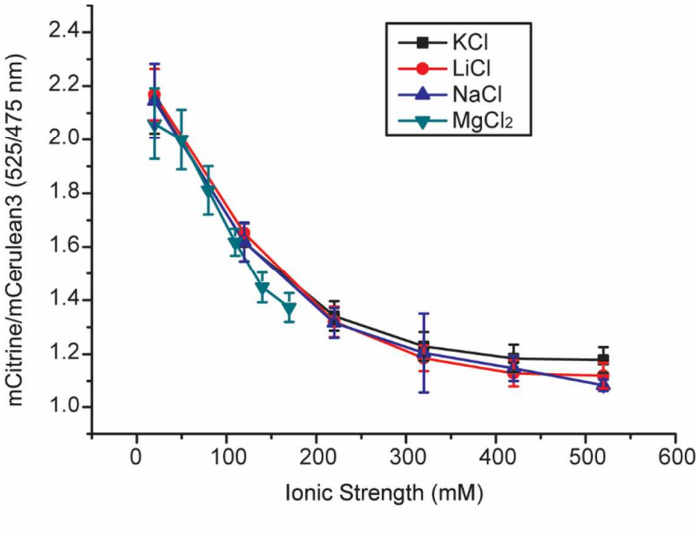

Figure S3. Dependence of the mCitrine/mCerulean3 ratio of RD on the identity of the cation. The monovalent alkali ions K^+, Li^+, and Na^+ all induce the same response. The divalent magnesium(II) gives the same response up to an ionic strength of 170 mM. This corresponds to a $MgCl_2$ concentration of 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 ºC.

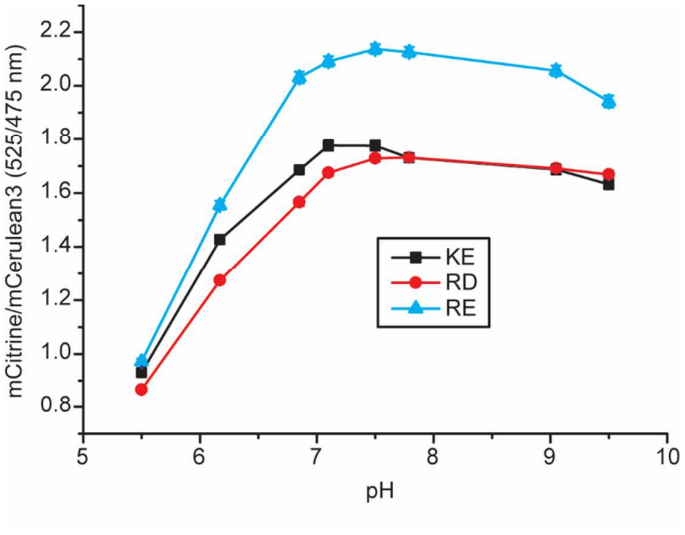

Figure S4. pH sensitivity of the probes. The pH of 10 mM NaPi was set in the presence of 100 mM KCl to account for significant ionic strength effects due to the difference in NaH_2PO_4/ Na_2HPO_4 ratio with pH. We observe that all sensors have a decreased ratio below pH 7 (the transition appears to be 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 pK_a 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 ºC.

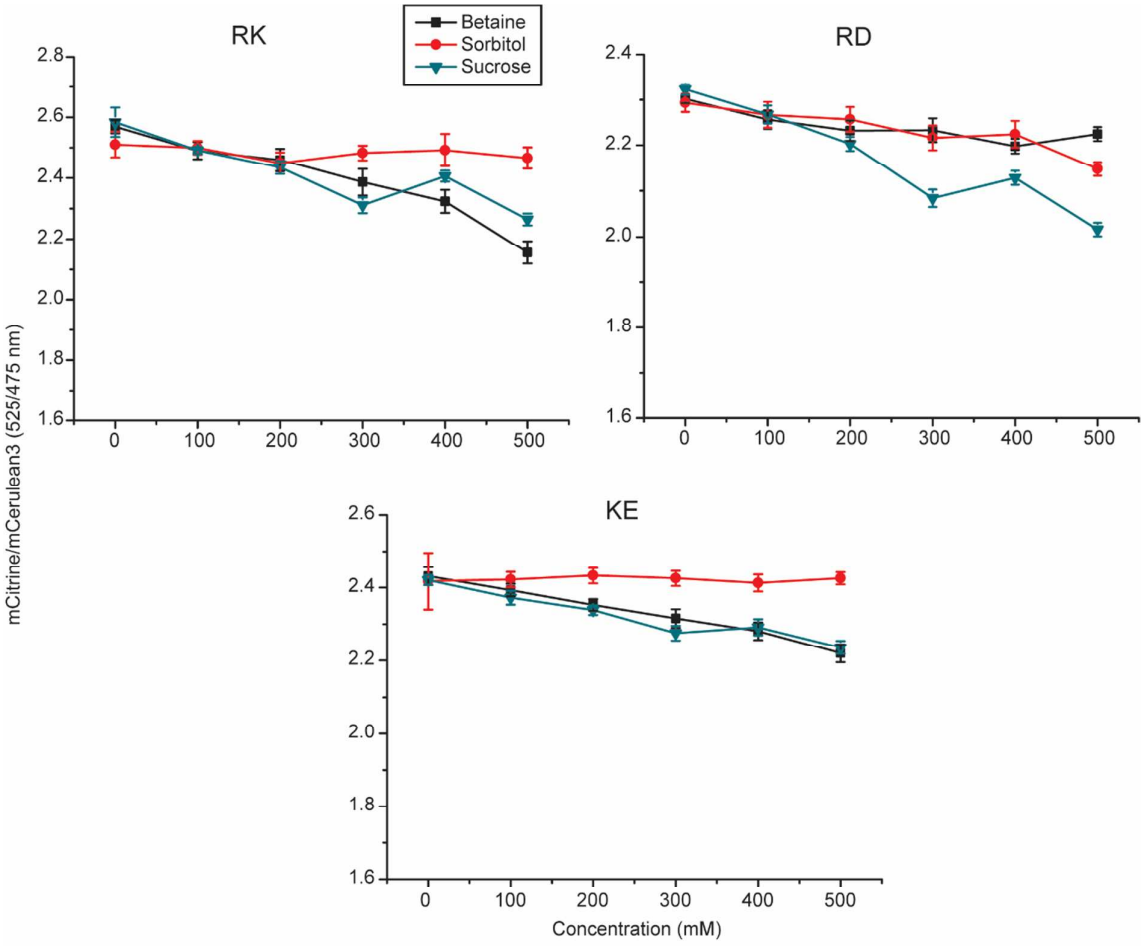

Figure S5. Effect of neutral carbohydrates sorbitol and sucrose, and the zwitterionic osmolyte glycine betaine on the mCitrine/mCerulean3 ratio of the sensors. The small decrease at high concentrations could be due to an increase in preferential hydration of the peptides and decrease in salt-bridge strength. 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 ºC.

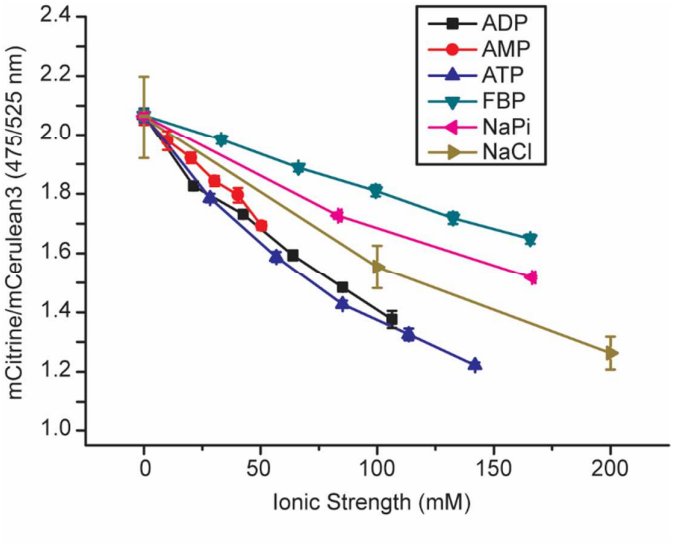

Figure S6. Dependence of the response of the RD sensor on the identity of physiological phosphate anions. The net charge of the ions at pH 7.4 was taken to be -3.3 for ATP (adenosine triphosphate), -2.8 for ADP (adenosine diphosphate), -1.8 for AMP (adenosine monophosphate), -1.6 for Pi (phosphate), and -3.6 for FBP (fructose 1,6-bisphosphate). The 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.

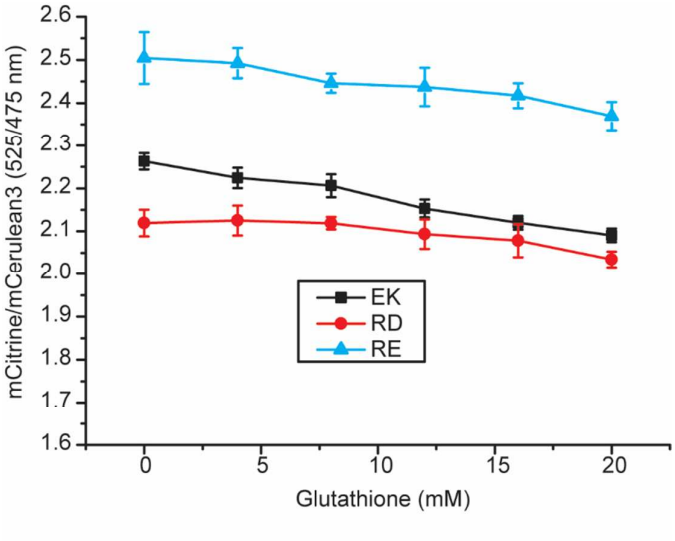

Figure S7. Dependence of the mCitrine/mCerulean3 ratio of the sensor on the glutathione concentration. Glutathione is one of the most common small molecules of living cells and typically present at millimolar concentrations. A small decrease is observed, which is at least partly caused by the higher 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.

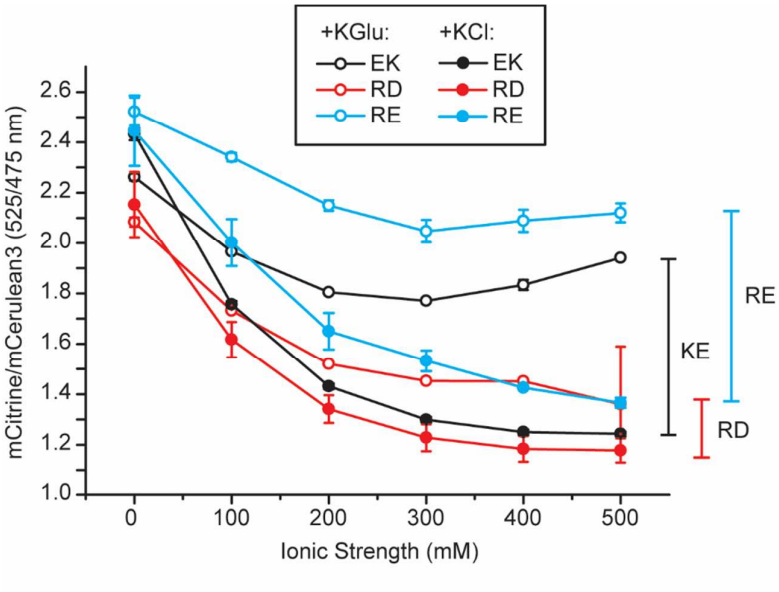

Figure S8. Response of the sensors to potassium glutamate *versus* potassium chloride. Glutamate is the principle small anion of many types of cells. Glutamate decreases the mCitrine/mCerulean ratio less than chloride does, and this is expected according to the Hofmeister series. The ratios of the RE 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.

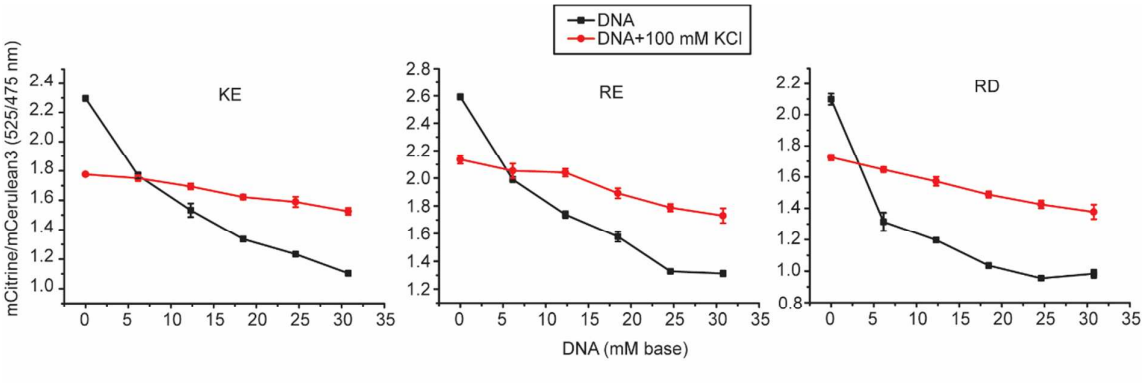

Figure S9. Sensitivity of the sensors to the presence of DNA. There is a strong decrease in mCitrine/mCerulean ratio in the presence of DNA and in the absence of KCl, which is not observed for the neutral crowding sensors[1]. To test whether this behavior is caused by association of the positively charged 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 ºC.

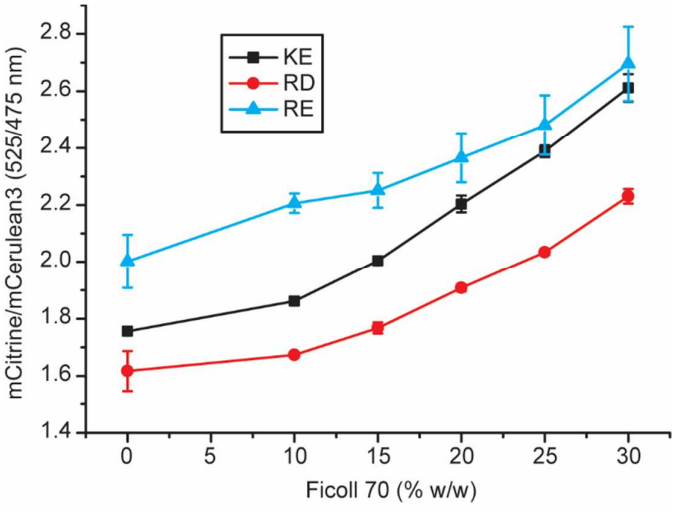

Figure S10. Sensitivity of the ionic strength sensors to macromolecular crowding induced by Ficoll 70. The KE, RD and RE sensors are compressed by high concentrations of Ficoll 70, similar to the neutral FRET sensors.[1] The sensitivity to macromolecular crowding needs to be taken into account when measuring *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 ºC.

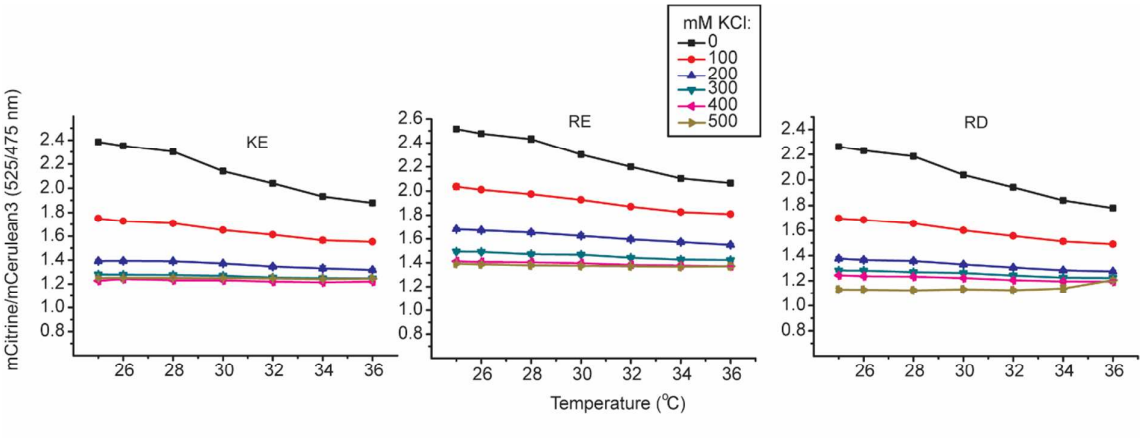

Figure S11. Temperature dependence of the ionic strength sensors at varying KCl concentrations. The temperature dependence could originate from salt-bridge stability, helix stability, altered buffer pH, Coulombic screening interactions, altered Debye lengths, and other effects. Such effects may oppose 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.

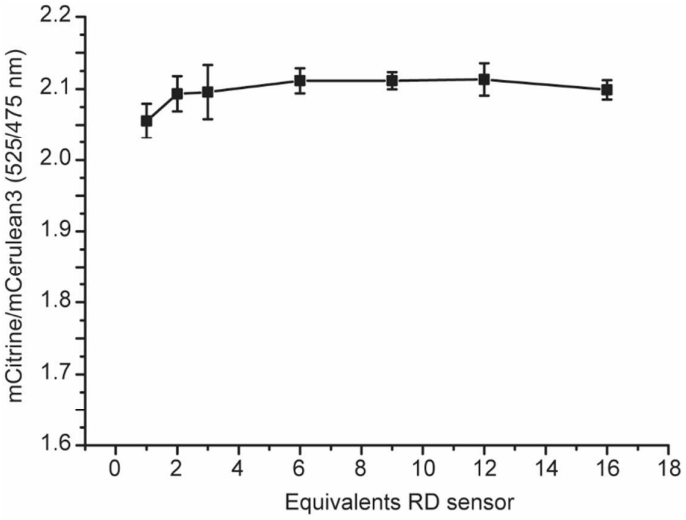

Figure S12. Dependence of the mCitrine/mCerulean3 ratio on the concentration of the RD sensor. The FRET ratio does not change by adding up to 16 equivalents of RD sensor, when starting with 30 nM. If self-association would occur, intermolecular FRET would increase the FRET ratio. Hence the absence of 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 ºC, pH 7.4.

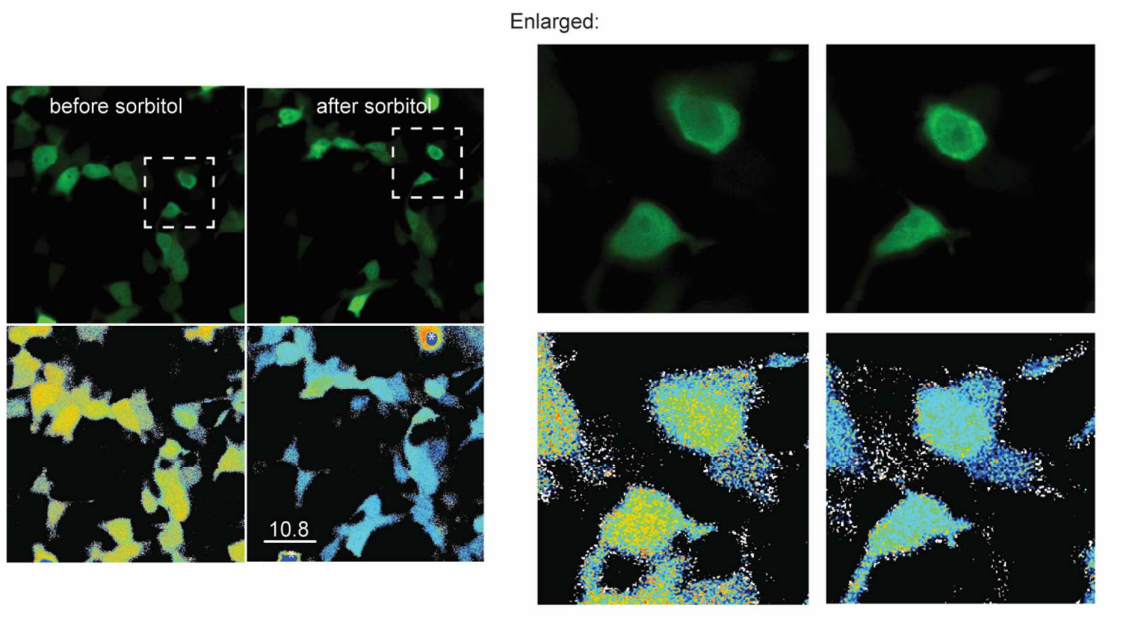

Figure S13. Enlarged images of cells of Figure 3C. The left panel depicts figure Figure 3C, with dashed squares denoting the enlarged area displayed in the right panel. The ratiometric images (mCitrine/mCerulean3, bottom) are smoothed in ImageJ. We assign ratiometric variations in a single cell to instrumental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

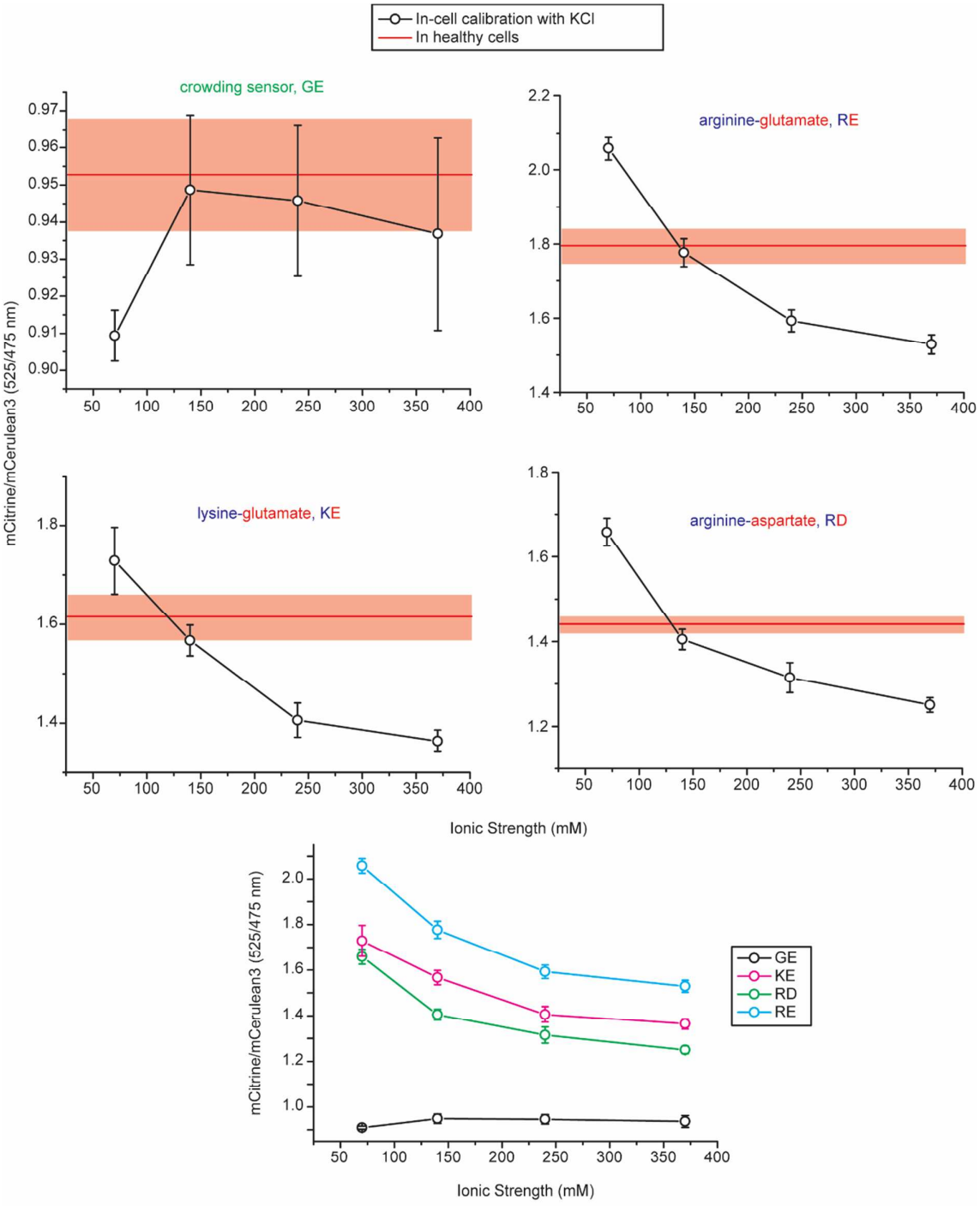

Figure S14. Calibration of the ion strength sensors in HEK293 cells. The calibration was done in the presence of set amounts of KCl (depicted on the x-axis), and using valinomycin (10 μM, K^+ ionophore), nigericin (5 μM, K^+/H^+ ionophore), and 10 mM NaPi, pH 7.4, at 37 ºC. The presence of ionophores 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 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 Figure 2B, where KCl was added to the purified sensors. Error bars are standard deviation within a single population of cells.

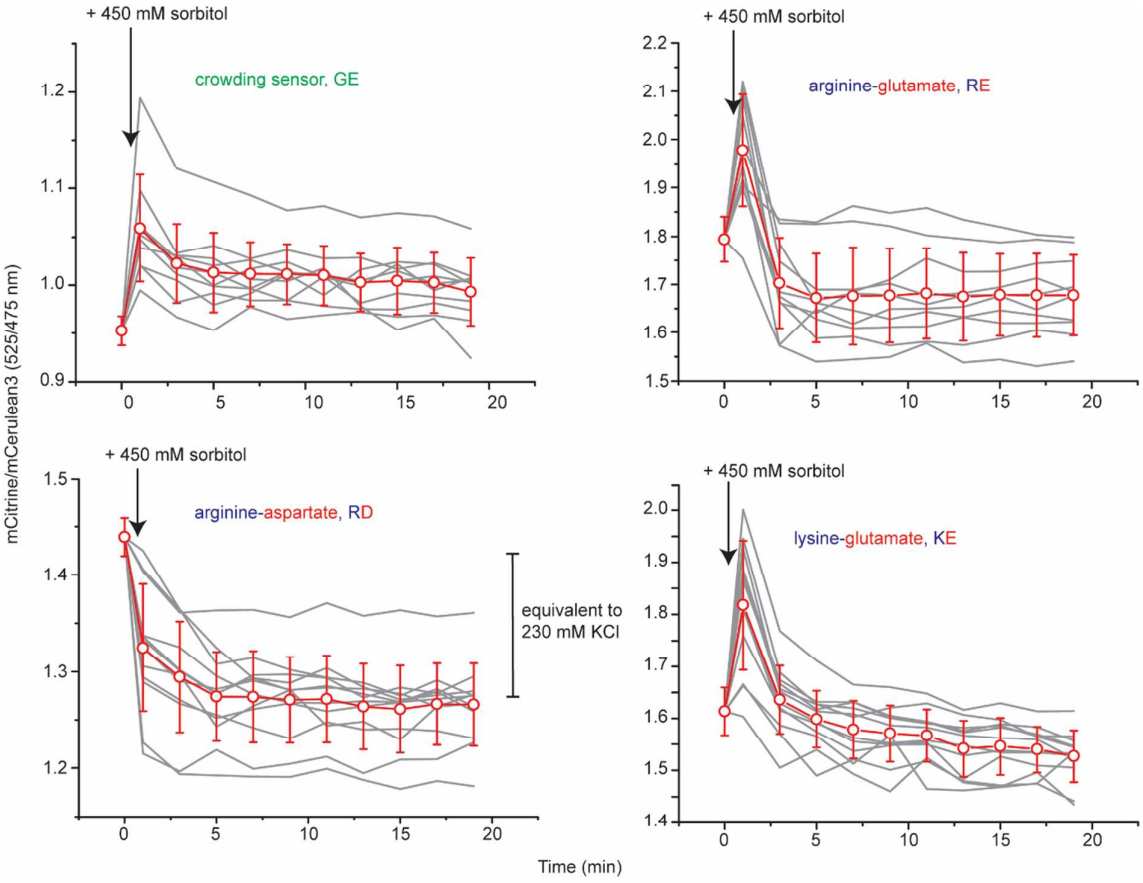

Figure S15. The response of the sensors in HEK293 cells upon changing the osmotic conditions. The red data points are the average over 20 cells and the error bar is the standard deviation. The individual cells are the grey lines. Cells are imaged in 200 µL DMEM/Hepes medium at 37 ºC, after which 20 µL 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.

References

1. Boersma, A. J., Zuhorn, I. S., and Poolman, B. (2015) A sensor for quantification of macromolecular crowding in living cells. *Nat. Methods. 12*, 227-229.

2. White, A. D., Keefe, A. J., Ella-Menye, J. R., Nowinski, A. K., Shao, Q., Pfaendtner, J., and Jiang, S. (2013) Free energy of solvated salt bridges: A simulation and experimental study. *J. Phys. Chem. B. 117*, 7254-7259.

DNA sequences of the ionic strength sensors

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GCTGGCATTACACTGGGCATGGACGAGCTGTACAAATAAAAGCTT

RE in pRSET A (*E. coli*, codon-optimized):
CATATGCATCATCATCACCATCATGTGAGCAAAGGTGAAGAACTGTTTACCGGTGTTGTTCCGATTCTGGTTGAACTGGATGGTGACGTTAATG
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