Electronic Supplementary Information

S.1 Methods

S.1.1. Crowding sensors

As a readout to measure changes in intracellular crowding, the compression of a random coil polyethylene glycol (PEG) was used as described previously[1]. Briefly, PEG with a molecular weight of 10 kDa was synthesized containing amino terminal modifications. Atto488 and Atto565 were conjugated terminally at the PEG chains. The PEG was obtained from Rapp Polymere (Tübingen, Germany). The PEG sensor was dissolved in DPBS at pH 7.4 (Sigma-Aldrich) prior to use. The PEG sensor was characterized in a previous publication. It was shown that it is unaffected by changes in ionic strength, pH or osmolyte concentration which were used in this study.[1]

To confirm the short-term imperfect adaptation result (Figure 1 main text), another crowding sensor was used. The same crowding sensor backbone was employed as described by Boersma et al.[2] The sensor was obtained by gene synthesis (Genscript) with Clover (GenBank JX489388.1) and mRuby2 (GenBank JX489389.1) as fluorophores in pcDNA3.1(+) for expression in mammalian cells. The final construct was Clover-(GSG)6A(EAAAK)6A(GSG)6A(EAAAK)6A(GSG)6-mRuby2 (N to C terminal). The sensor consists of a flexible domain containing (GSG)-repeats that are connected to two FRET suited fluorophores using stiff linker (EAAAK)-repeats. An increase of crowding leads to a more compact conformation that is detected via a FRET increase[2].

S.1.2 Cell culture

HeLa cells were grown in T25 culture flasks (Sarstedt) in DMEM media (Sigma-Aldrich) supplemented with 1% penicillin-streptomycin (PS) and 10% fetal bovine serum (FBS) in a humidified atmosphere, 5% CO2 and 37°C. Cells were subcultured every 2-3 days after reaching approximately 75-95 % confluency using standard trypsin digestion and split in a 1:4 to 1:5 ratio into new T25 flasks. For experiments, HeLa cells were plated 1-2 day prior to injection at a density of 1-2 x 10^5 cells on glass bottom dishes (35 mm, WPI fluorodish). One day before the experiment, the media was exchanged from growth medium (DMEM, 10% FBS, 1% PS) to growth media containing the desired concentration of salt/osmolyte to induce the long-term adaptation process (at least 16 h incubation).

S.1.3. Microinjection

HeLa cells were injected 1-2 d after plating. Prior to injection, the culture media was aseptically removed and exchanged with Leibovitz L15 (Sigma-Aldrich) supplemented with 30 % FBS and

the desired concentration of salt/osmolyte (osmolalities were controlled by vapor pressure osmometry using a Wescor Vapro 5520). Injection was performed using an Eppendorf FemtoJet connected to an Eppendorf InjectMan NI2. FemtoTips II were filled from the back with a 2 mg ml^{-1} stock solution of sensor and subsequently connected to the Eppendorf FemtoJet. The injection parameters were adjusted for each injection so that approximately 5 % of the cell volume was injected. As a starting point, an injection pressure of 100-200 hPa, a compensation pressure of 30 hPa and an injection time of 0.5 s was used.

S.1.4 Measurement procedure

Measurements were performed at room temperature with fast alternating excitation using a 470 nm and 555 nm LED at 25% power on an AxioObserver Z1 (Zeiss) inverted microscope. For the measurements, either a 40x/0.95 air (for long-term observations) or a 20x/0.8 air objective (short-term shocks for a larger field of view) was used (Zeiss). The excitation light was directed to the sample by a DFT 490+575 (HE) beam splitter (Zeiss). Emission separated by a FT 565 (HE) beam splitter (Zeiss) to separate donor and acceptor emission. Donor emission was passed through a BP 512/30 HE filter (Zeiss) and acceptor emission was passed through a BP 630/98 HE filter (Zeiss). Images from donor and acceptor emission were recorded simultaneously using two AxioCam HS (Zeiss) cameras and the AxioVision 4.8.2 (Zeiss) software.

For short-term measurements, a ~ 10 s baseline was recorded before addition of a 5 M NaCl stock solution to yield the short-term shock conditions. Osmolalities were measured using a vapor pressure osmometer (Wescor Vapro 5520). Shown in Figure S1, the osmolality linearly changes with calculated excess concentration of NaCl as expected (slope ~ 0.5). The total measurement time was 20 min and only morphological healthy cells (e.g. no blebbing) after recovery were analysed.

For measurements using the genetic sensor, similar conditions were applied using transiently transfected HeLa cells. The transfection was performed 2 d prior to measurements according to the manufacturer's protocol (Lipofectamine 3000, Thermo Fisher) using 2 µg DNA. Imaging was performed using the same excitation and emission settings.

S.1.5. Cell Viability Assay

Cell viability was measured using a luminescence assay (CellTiter-Glo, Promega) according to the manufacturer's procedure. Briefly, cells were plated on white flat-bottom 96 well plates (Greiner Bio-one). For long-term viability measurements, cells were then incubated for 16 hours in presence of salt/osmolyte in a total incubation volume of 100 µL. For short-term cell viability measurements, cells were treated as described previously and elevated osmolalities were induced by addition of 5 M NaCl 20 min before performing the cell viability measurement.

Luciferase reaction was started by addition of 100 µL CellTiter-Glo reagent to each well. The plate was shaken on a double orbital shaker for 2 minutes and rested for 10 min at RT to reach

a stable luminescence signal. The luminescence was finally read out using a CLARIOstar plate reader (BMG Labtech). Each condition was at least measured in triplicates.

S.1.6. Raman microspectroscopy

Confocal Raman microspectroscopy was performed using an alpha300 RAS microscope (WITec, Ulm, Germany). For excitation, a 532 nm laser was focused on the sample using a Nikon 60x water dipping objective (NA = 1.0). All measurements were performed at constant laser power as well as constant pixel dwell time. The Raman scattered light was detected using a combination of diffraction grating and a back-illuminated CCD. Samples were prepared on glass bottom dishes (WPI fluorodish). Before measurement, the incubation media was exchanged with DPBS supplemented with the corresponding concentration of osmolytes. For the measurement, the "Sample Raster" feature of WITec Control 1.56 (2012) was used. Raman spectra were collected for a whole cell area with sufficient buffer background. The cellular region was selected by thresholding the cell based on the C-H stretching vibrations (2800-3000 cm^{-1}), which represent the intracellular macromolecules and allow a clear separation from extracellular buffer. To estimate the cellular water content, a sum filter analysis was performed from 3019.8 to 3686.5 cm^{-1}. Assuming that the extracellular water content is constant, the difference of intracellular and extracellular water spectra yields a measure for the change in intracellular water. A smaller difference would therefore indicate a higher intracellular water content.

S.1.7. Data analysis crowding sensor

Images were initially processed using ImageJ. Mean intensity values were calculated for each cellular cytosol (the weakly fluorescent nucleus region was excluded from further analysis). FRET efficiency was calculated as described, previously[1]. For all measurements, background data (from non-injected cells) was subtracted from each channel. The FRET efficiency was calculated by:[3]

$$FRET = \frac{I_{FRET} - I_{donor} \times Bleed_{donor} - I_{acceptor} \times DE_{acceptor}}{\sqrt{I_{donor} \times I_{acceptor}}}$$

I_{FRET} is the intensity of Atto565 with 470 nm excitation, I_{donor} the intensity of the donor with 470 nm excitation, $I_{acceptor}$ the Atto565 intensity with 555 nm excitation, $Bleed_{donor}$ the correction factor for Atto488 emission bleed into the Att565 channel and $DE_{acceptor}$ the correction of direct excitation of Atto565 by 470 nm excitation. Statistical evaluation was performed using Graphpad Prism 6 (GrapPad Software). Errors are represented as mean ± s.d

S.1.8. Fluorescence spectroscopy

To investigate whether the dye properties change in different solvent conditions, we performed fluorescence spectroscopy using a JASCO FP-8500 fluorescence spectrometer. Briefly, solutions containing 5 µM of Atto488 or PEG-Atto565 were investigated in DPBS (pH

7.4, physiological buffer), DPBS supplemented with 200 mM NaCl or DPBS supplemented with 250 mM TMAO. Emission and excitation scans were performed for both dyes in each condition. Emission and excitation bandwidth was set to 5 nm, respectively. The step size for each scan was 0.5 nm, the scan speed 1000 nm/minute and the acquisition accumulated 3 times. For Atto488 excitation scans, emission was set to 532 and excitation was scanned from 400 to 520 nm. For emission scans, excitation was performed at 488 nm and emission scanned from 500 to 700 nm. For Atto565 excitation scans, emission was set to 590 nm and excitation scanned from 450 to 580 nm. For emission scans, excitation was set to 564 nm and emission scanned from 580 to 650 nm. Each spectrum was normalized to its peak intensity.

S.1.9. Overview sample sizes

Figure 2:

a

[NaCl]/mM	Control	TMAO
0	30	22
50	32	16
75	19	25
100	38	27
150	17	13
200	27	24

Figure 3:

a

mM	Cosolute/Osmolyte	N
0	TMAO	68
75	TMAO	46
150	TMAO	35
250	TMAO	21
100	NaCl	9
100	Sucrose	22

b

[TMAO] / mM	N
0	17
75	18
150	8
250	14

S.2 Supplementary Figures

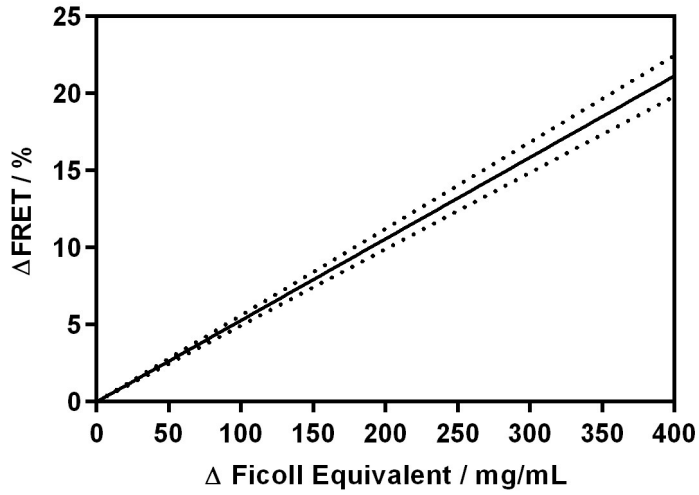

Figure S1. Crowding sensor calibration curve based on a fit of previously recorded data.[1] Only the linear fit is shown here. The dashed lines indicate the 95% confidence intervals of the fit. N=3.

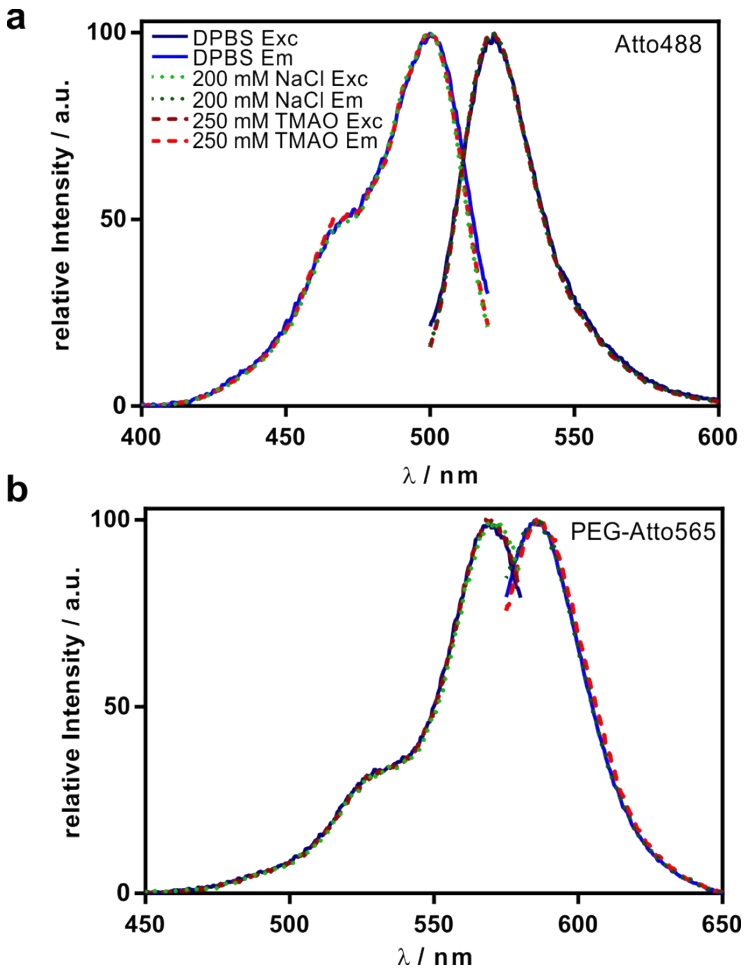

Figure S2. Emission and excitation spectra of Atto488 (**a***)* and PEG-Atto565 *(***b***)* in DPBS (pH 7.4), DPBS supplemented with 200 mM NaCl and DPBS supplemented with 250 mM TMAO. Each emission and excitation spectrum was normalized to its peak intensity.

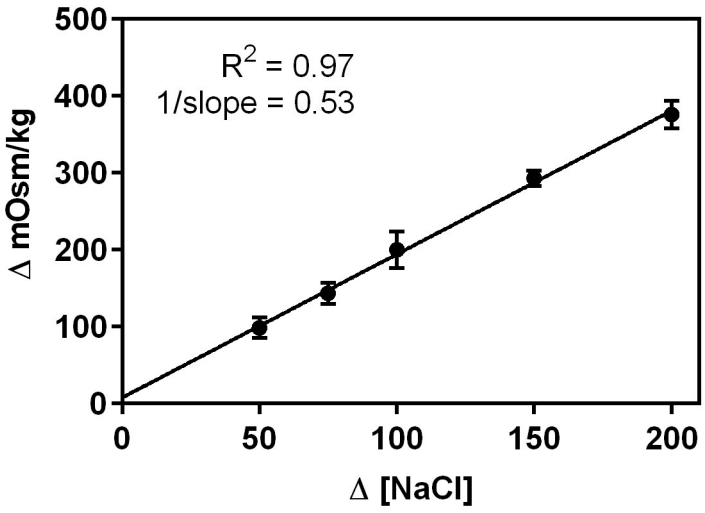

Figure S3. Measured osmolality changes upon addition of highly concentrated NaCl. The calculated excess concentration is plotted against the measured change in osmolality. Error bars represent mean ± s.d. Sample size was at least 4 independent measurements.

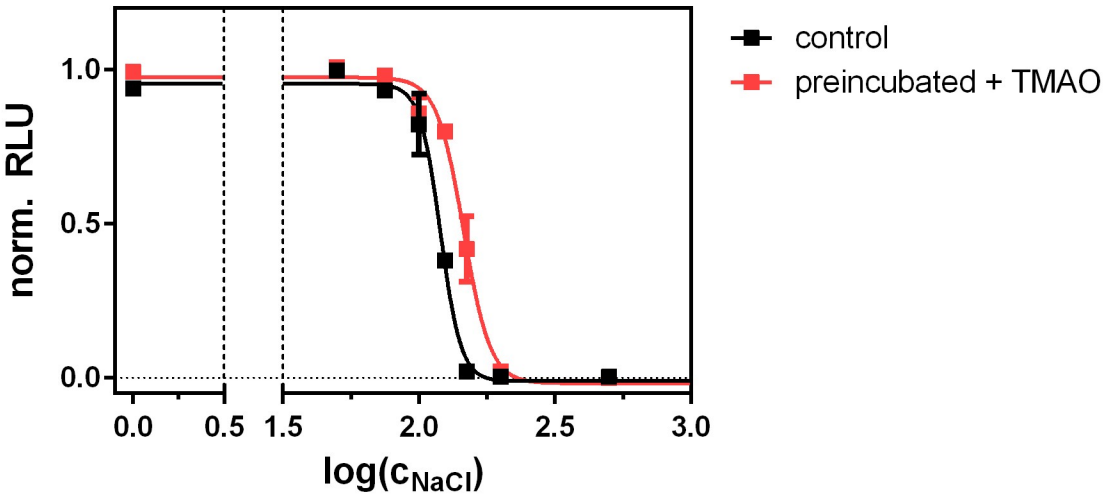

Figure S4. Cell viability after short-term osmotic stress with NaCl in presence and absence of 150 mM TMAO (> 16 h). RLU (relative luminescence units) are plotted against the excess concentration of NaCl (mM). Error bars represent mean ± s.d. Each data points represents 3-4 measurements.

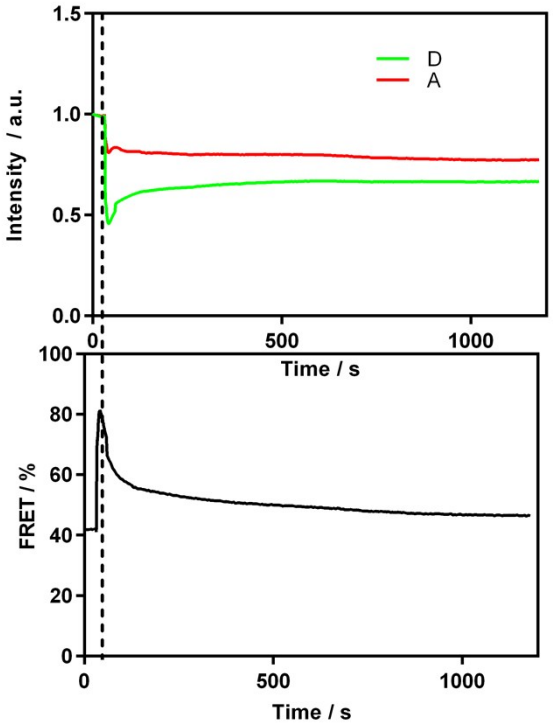

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Figure S5. Exemplary, uncorrected donor and acceptor intensity traces and the corresponding FRET curve.

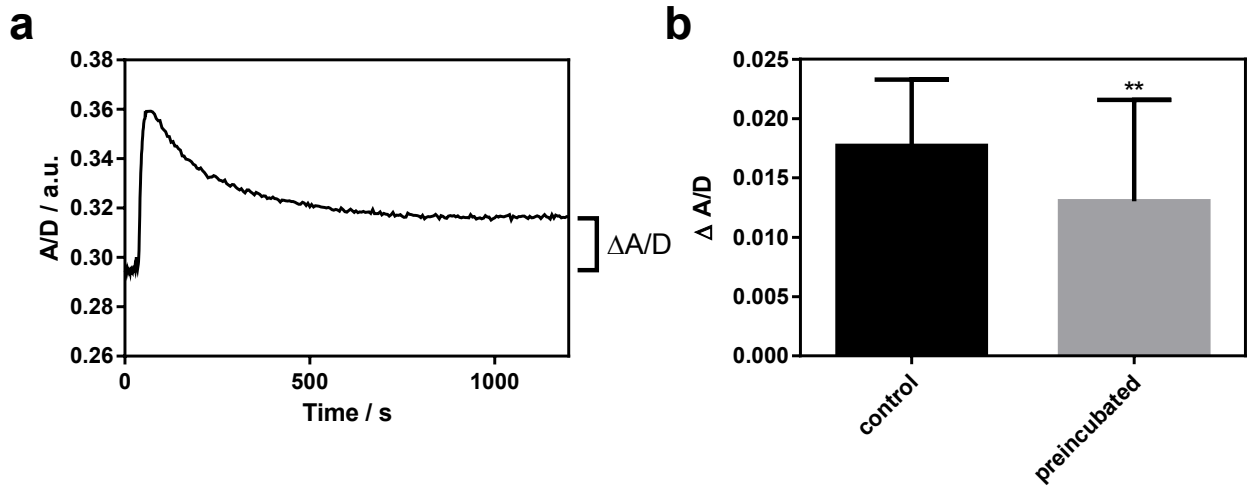

Figure S6. Short term adaptation response observed by the genetically encoded crowding sensor. **a** Acceptor to donor intensity ratio are plotted against the time. Exemplary curve of a cell expressing the genetic crowding sensor. The cell was shocked using 100 mM NaCl after ~ 20 s.[2] An increase of the ratio indicates a higher crowding. After 20 min (1200 s), the imperfect adaptation is quantified as the difference between initial and final baseline. As shown for the PEG based crowding sensor (Figure 1 main text), crowding only imperfectly adapts after 100 mM NaCl stress. **b** Comparison of the imperfect adaptation of untreated (N = 43) and preincubated cells (150 mM TMAO for more than 16 h, N = 51). A significant decrease ($p < 0.01$, Mann-Whitney test, 95% confidence interval) was observed for pre-treated cells in accordance with the PEG based crowding sensor results in Figure 2 (main text).

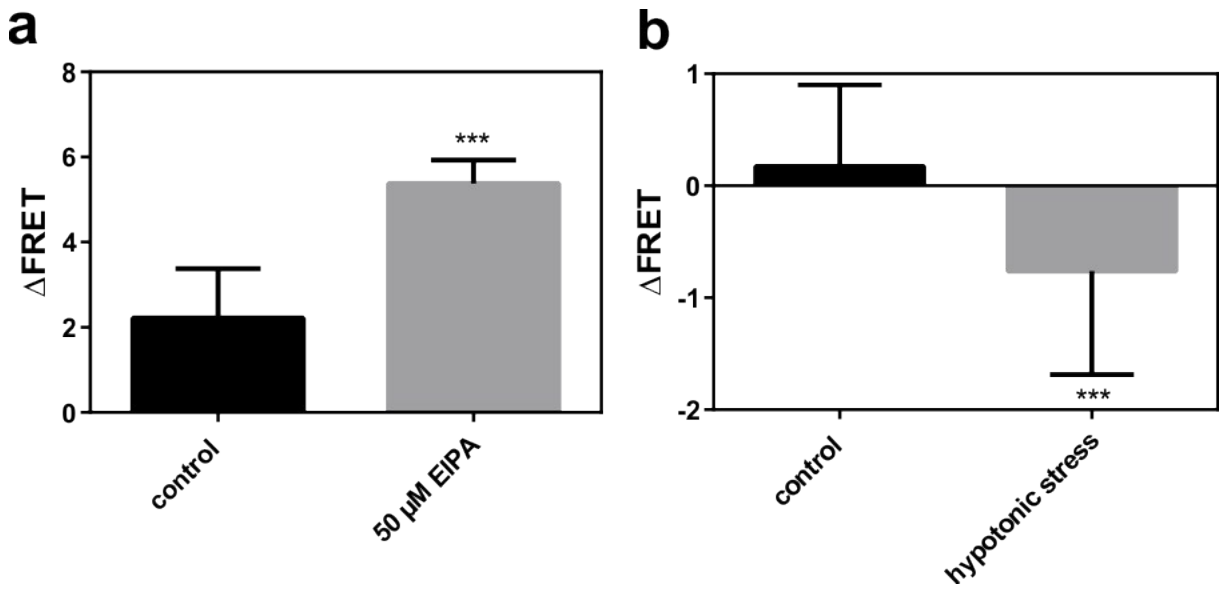

Figure S7. a Quantification of cellular crowding after addition of 100 mM NaCl in absence and presence of 50 µM EIPA. N=18-27. Error bars represent mean ± s.d. Statistical significance was tested by a nonparametric Mann-Whitney test (95% confidence interval). *** $p < 0.001$ **b** Quantification of cell crowding after hypotonic stress (-120 mOsm/kg difference to basal conditions) induced by addition of MilliQ water. The ΔFRET after 20 min was quantified. N=30-33. Error bars represent mean ± s.d. Statistical significance was tested by a nonparametric Mann-Whitney test (95% confidence interval). *** $p < 0.001$

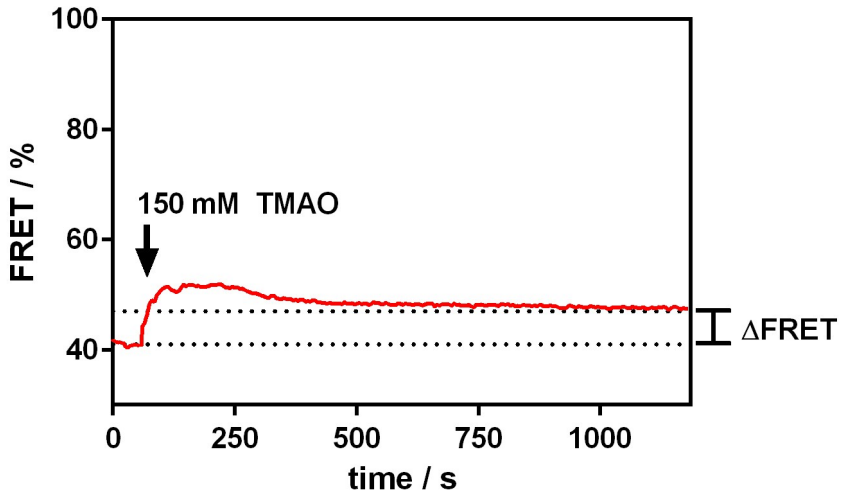

Figure S8. Exemplary short-term response of a cell after addition of 150 mM TMAO (black arrow). The dashed line indicates the initial and final baseline used for determination of ΔFRET. Similar to NaCl, TMAO addition leads to an increase in crowding and imperfect adaptation within 20 min.

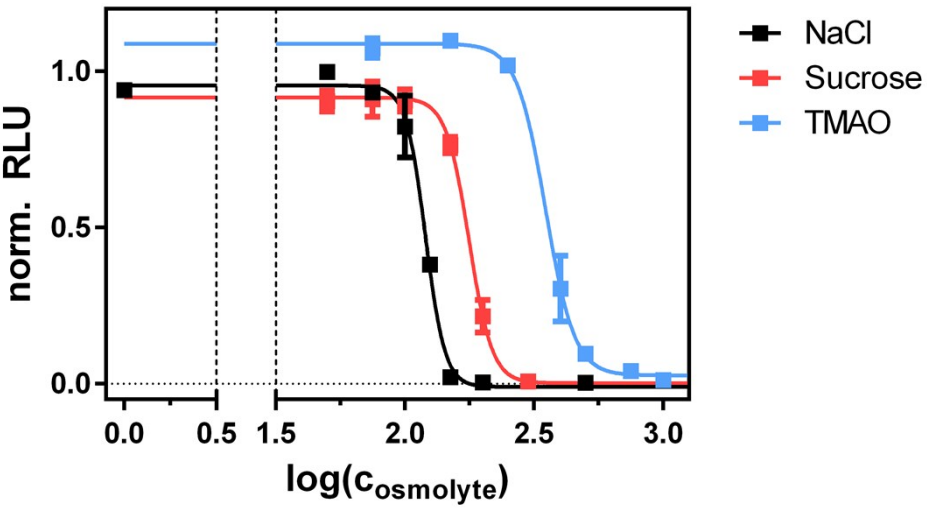

Figure S9. Cell viability assay in presence of osmolytes. Cells were cultured in standard DMEM growth medium supplemented with different concentrations of osmolytes or salt. The log of the excess concentration of osmolyte is plotted against the relative normalised luminescence (normalized against untreated cells) obtained from the CellTiter-Glo luminescence assay. Concentrations were given in mM. Error bars represent mean ± s.d. Each data points represents 3-4 measurements.

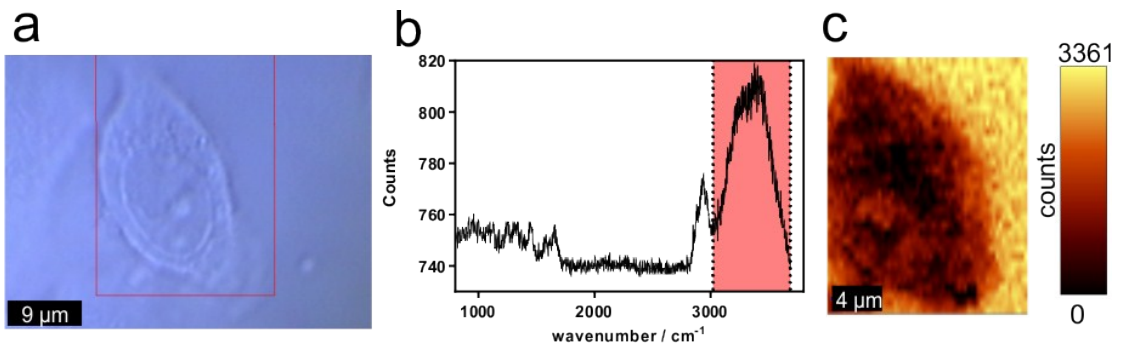

Figure S10. Raman microspectroscopy to quantify cellular water content. **a** Representative brightfield image of a HeLa cell in DPBS. The red area shows the region used for Raman microspectroscopy. A spectrum was recorded for every pixel. **b** A sum-filter analysis was performed for every pixel for the OH-stretching vibration, marked in red. **c** Results of the sum filter analysis are shown for every pixel. The difference between intracellular and extracellular counts is used as a measure for the intracellular water content (ΔI_{OH}). For further analysis, thresholding was applied to separate the intracellular from the extracellular region.

S.3 Supplement References

1. D. Gnutt, M. Gao, O. Brylski, M. Heyden and S. Ebbinghaus, *Angew. Chem. Int. Ed.*, 2015, **54**, 2548-2551.
2. A. J. Boersma, I. S. Zuhorn and B. Poolman, *Nat. Methods*, 2015, **12**, 227-229, 221 p following 229.
3. Z. Xia and Y. Liu, *Biophys. J.*, 2001, **81**, 2395-2402.