Fundamental molecular mechanism for the cellular uptake of guanidinium-rich molecules

Supplementary Information

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Materials and methods

Peptides: 5-carboxytetramethylrhodamine (TAMRA)-TAT (GRKKRRQRRR) peptides were synthesized as D-isomers and coupled directly to TAMRA at the N terminus, while R5, R8, R12, K5, K10 and K12 were synthesized as L-isomers and coupled directly to fluorescein-isothiocyanate (FITC) at the N terminus (Peptide Specialty Laboratories GmbH, Heidelberg, Germany). R10 peptides were synthesized as D-isomers and coupled directly to fluorescein-isothiocyanate (FITC) at the N terminus while the cR10 peptides were synthesized intercalating L- and D-isomers as described in [1]. TATssPL* was synthesized by Biosyntan GmbH (Berlin, Germany).

Table 1. Summary of peptides. The fluorophores 5-carboxytetramethylrhodamine (TAMRA) and fluorescein-isothiocyanate (FITC) were used for labeling the peptides. L amino acids are spelled using a capital letters while D amino acids are not capitalized and (-CC-) refers to a disulfide bridge.

| Name     | Sequence                |
|----------|-------------------------|
| TAT      | TAMRA-rrrrrrkkrg        |
| R5       | FITC-RRRRR              |
| R6       | FITC-RRRRRR             |
| R8       | FITC-RRRRRRRR           |
| R10      | FITC-rrrrrrrrrrrrrrrrrr |
| R12      | FITC-RRRRRRRRRRRRRRRRRR |
| cR10     | FITC-KrRrRrRrRrRrRE-(cyclic) |
| K5       | FITC-KKKKK              |
| K10      | FITC-KKKKKKKKKKK        |
| K12      | FITC-KKKKKKKKKKKKKKKKKK |
| TATssPL* | RRRQRRKKG-CC-SAVLQKKITKYFPKKG-EDA-TAMRA |
Reagents: Olive oil was purchased from Oliveira da Serra, product name “1ª Colheita” (Figueira dos Cavaleiros, Portugal). Refined Castor oil from Caesar & Loretz GmbH (Hilden, Germany) was purchased from Kronen Pharmacy (Darmstadt, Germany). Octanol, oleic acid and other reagents were purchased from Sigma Aldrich and used without further purification.

Buffers: All buffers used for peptide studies were prepared with 140 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 5 mM glycine, and the pH was adjusted with NaOH or HCl.

Peptides absorption into a hydrophobic phase as a function of pH: Peptides were diluted to a final concentration of 10 µM at each pH. Equal volumes of the buffer-peptide mix at each pH and of octanol plus 1 % of oleic acid were placed into individual microcentrifuge tubes (as shown if Figure 1 (a)). Each pH mix was vortexed for 5 min and centrifuged for 2 min with a centripetal force of 2200 g to quickly separate the octanol from the aqueous phase. Although the fraction of peptides absorbed into the octanol phase could be inferred from the fraction left into the aqueous phase we extracted it from the octanol phase and measure it. To measure directly the relative concentration of peptide absorbed in the octanol phase we used the fact that no peptide absorption into the octanol phase was detected at pH 4. Therefore, the octanol phase was extracted with a pipette and mixed with a buffer at pH 4 separately for each pH into microcentrifuge tubes, which were also vortexed and centrifuged. In this way the fraction of peptide previously absorbed in the octanol phase for each pH was reabsorbed in a pH 4 buffer and now the octanol phase with little or no traces of the TAT peptides was again removed with a pipette and discarded. The peptide in each buffer solution was measured using a fluorescent spectrometer, using as a reference a buffer solution of 10 µM peptide at pH 4, measuring the relative fluorescent intensity emission between the reference solution and the solution of interest, exciting with a laser wavelength of 543 nm and measuring the emission wavelength of 575 nm. Similarly the peptides that remained in the aqueous phase were compared to a reference solution of 10 µM peptide at each pH without having been in contact with the octanol phase.

The protocol for the control experiment to determine the behavior of the TAMRA dye with pH (Figure S1) was done analogously. In this case to extract and directly measure the relative fluorescence intensity of the dye absorbed in the octanol phase, it was used a buffer at pH 9 to reabsorbe the dye previously absorbed into the octanol phase (at low pH in this case).

Peptide translocation across a hydrophobic phase from high to low pH: As described in Figure 3 (a), two separate compartments contained in a bigger compartment were built by
cutting the top of two 0.5 ml microcentrifuge tubes and inserting them into a polypropylene BD falcon round-bottom tube. The top of the falcon tube was cut leaving 2 cm from the bottom. One of the interior tubes was filled with 200 µl of the buffer at pH 7.5 plus the peptide and the other interior tube was filled with a buffer at pH 4. Before adding the octanol/oleic acid mix, the space between the two compartments and the falcon tube was filled with glycerol to reduce the amount of octanol solution required to connect the high and low pH chambers. This step was important to reduce the hydrophobic volume accessible to the peptide and speed up the peptide translocation. On top was added a solution 400 µl of octanol plus 1 % of oleic acid previously vortexed and each one was closed with a cap to avoid evaporation. Five of these experimental setups were built for each experiment repetition. One was left untouched for photographic imaging and each one of the others were dismantled every 2 hours, by removing and discarding the octanol phase and measuring the relative fluorescence of each compartment using a fluorescence spectrometer, exciting with a laser wavelength of 543 nm and measuring the emission wavelength of 575 nm.

**Cells and Culture Conditions:** Human cell lines HeLa and HepG2 were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10 % fetal calf serum, 50 µg/ml gentamicin and 2mM glutamine. Mouse myoblasts C2C12 [2] cells were cultured in DMEM supplemented with 20 % fetal calf serum, 50 µg /ml gentamicin and 2mM glutamine. Mouse fibroblasts Flp 3T3 was grown in similar conditions but supplemented only with 10 % FCS. Baby hamster kidney (BHK) cell line was grown in Glasgow minimal essential medium supplemented with 10 % fetal calf serum, 50 µg/ml gentamicin, 2 mM glutamine and 150 µg/ml hygromycin B [3]. All mammalian the cell lines were grown at 37° C in a humidified atmosphere with 5 % CO₂. Insect cells, from Spodoptera frugiperda pupal ovarian tissue (SF9) were cultured in Ex-Cell 420 media supplemented with 10 % FCS, without CO₂ at a 27° C, in suspension cultures shaked permanently at 105 rpm. Bright yellow (BY-2) tobacco cells [4] were grown in suspension in the dark at 25 °C on a rotary shaker (115 rpm) in modified Murashige-Skoog basal medium, supplemented with 1.5 mM KH2PO4, 3 mM thiamine, 0.55 mM inositol, 87 mM Suc, and 1mM 2,4-dichlorophenoxy acetic acid. Media components were purchased from Sigma-Aldrich, Steinheim, Germany.

**Confocal Microscopy:** Confocal images were collected using an Ultra VIEW VoX spinning disc system (Perkin Elmer) on a Nikon Ti microscope equipped with an oil immersion Plan Apochromat VC ×60/1.45 NA (pixel size in XY = 111 nm, Z-step = 0.3–1 µm), a non-immersion Plan Apochromat VC ×20/0.75 NA and a Plan Fluor 20XMI/0.75 NA multi-immersion objective.
lenses, with laser lines at 488 nm, 561 nm, a differential interference contrast (DIC) setup, and a temperature, humidity and CO₂ incubation control (ACU control, Olympus).

**TAT cellular uptake in HeLa cells:** Cells were seeded at 60 % confluence in a tissue culture treated 6 channel µ-Slide VI (from Ibidi GmbH, Germany) 24 hours before peptide treatment. The uptake imaging at different pHs was done by washing two times with the HEPES buffers at the pH of interest and replacing the buffer solution with the HEPES buffer with the peptide added at a final concentration of 2 µM. The sample was taken to the microscope and imaged at equally spaced intervals of 2 min. This was done simultaneously for pH 6, 7.5 and 9 to compare the relative peptide uptake side by side. HeLa cells were imaged by swapping at each time point between two objectives, a x60 and a x20 immersion oil. This was done to be able to perform two types of time-lapse analysis over the same sample to compute the cellular uptake of the TAT peptide. With the x20 objective is possible to simultaneously visualize several cells but is not easy to separate the fluorescence intensity from internalized peptide from the fluorescent intensity of membrane bound peptide. With the x60 objective a fewer number of cells are visualized but the relative intensity of free peptide can be computed by measuring the fluorescence intensity of peptides accumulated at the nucleolus, since peptide bound to the cell plasma membrane or trapped in endosomes cannot reach the nucleolus [1]. Using the x20 images, the uptake was computed by measuring the average background fluorescence intensity in an area of the images without cells and subtracting this value from the average intensity of the whole image. Using the x60 images, the uptake was computed by measuring the average background fluorescence intensity in an area without cells and subtracting this value from the average intensity in the nucleus. The nucleus area was obtained using the DIC channel. This experiment was repeated 3 times and the average and the standard error plotted. In each experiment, after 30 min the cells were washed with DMEM cell culture media and calcein was added to detect cell viability at a final concentration of 5 µM. In live cells, the non-fluorescent calcein AM is converted to green-fluorescent calcein, after acetoxymethyl ester hydrolysis by intracellular esterases. This was incubated for 30 min and then imaged. Cell viability was also assessed using the DIC images used to detect the cell morphology along the experiments. To further evaluate the viability of cells after uptake of the TAT at pH 9, cell division was monitored for 16 hours.

**TAT cellular uptake in different species and kingdoms:** The cellular uptake in the rest of the cell lines was monitored with a single objective through each experiment (either a x20 or a x60). The TAT peptide concentration in each cell line uptake experiment was adjusted to obtain
peptide uptake in more than 60% of the cells at pH 9 within 30 min. Peptide uptake was monitored simultaneously for pH 6, 7.5 and 9 to compare the relative peptide uptake side by side. Experiments were repeated 3 times for each cell line and at least 300 cells. The proportion of cells displaying uptake was visually counted and the average and standard error values computed.

**Cellular uptake in fatty acid enriched cells:** A HEPES buffer at pH 7.5 was mixed with 0.2% volume of oleic acid. Cells were washed twice and incubated for 15 min with this buffer. Next, cells were washed once with a HEPES buffer at pH 7.5 (without oleic acid) and the TAT peptide mixed with this last buffer was added at different concentrations. Cells were washed after 5 min with DMEM cell culture media two times and imaged. This was followed by the calcein viability test as described previously.

**Umbrella sampling computation of TAT free energy insertion into model bilayers:** Molecular dynamics simulations were performed to study the translocation energetics of the protein transduction domain of the HIV-1 TAT across model membranes, the TAT peptide water-octanol/fatty acid partition, and the energetics of arginine and lysine interactions with deprotonated fatty acids [5-7].

The free energy of insertion of a TAT peptide into model lipid membranes was measured by placing the TAT peptide in a periodically repeating box containing a pre-equilibrated lipid bilayer composed of 64 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) lipids, 48 oleic acids (protonated and/or deprotonated), neutralizing potassium counter ions and 8700 water molecules. In different umbrella sampling calculations the TAT peptide was placed at 31 windows separated by 0.075 nm intervals from the center of mass of the bilayer in a direction perpendicular to the bilayer plane. The center of mass of each peptide was restraint by a harmonic potential at each position with a force constant of 3000 kJ/(mol nm²). The weighted histogram analysis method (WHAM) was used to compute the free energy of insertion of the peptide along this path [8]. Each independent simulation was equilibrated for at least 300 ns and the data collection was done over an extra 40 ns. The total computed time for each free energy calculation expanded 10.2 µs. Free energy block averages of 4 ns was computed over the last 100 ns to obtain the standard deviation.

**Molecular dynamics computation of TAT partition into water/octanol phases:** The partition of the TAT peptide into the water/octanol phases was studied by mixing 4 TAT peptides, 64 oleic acids (all protonated or all deprotonated), 16000 octanol molecules, 24000 water
molecules and counter ions to neutralize the systems (chloride or potassium respectively). Experimentally, the octanol and water phases separate into two bulk phases by the effect of the gravitational force, the less dense octanol on top of the denser water phase. The same effect is produced by a centripetal force. Therefore, we modeled a centripetal force by adding an external force in the z direction over each atom given by $F=k \ m \ (z-L_z/2)$, with $m$ being the mass of each atom, $k=0.01 \ kJ/(mol \ nm^2)$, and $L_z$ the dimension of the system box in the direction z. Each system was simulated for 300 ns.

**Computation of free energy differences between lysine and arginine amino acids:** The free energy of interaction profile as a function of the distance between an oleic acid and an arginine (or lysine) amino acid was calculated in systems composed of a single arginine (or lysine) amino acid a single oleic acid, and 2200 water molecules. The distance was restrained using a harmonic potential with a force constant of 3000 $kJ/(mol \ nm^2)$ at 30 positions separated by 0.025 nm, staring from a distance of 0.2 nm, between the first carbon atom of the oleic acid and the central carbon of the guanidinium group (or nitrogen of the amino group). The weighted histogram analysis method (WHAM) was used to compute the free energy of insertion of the peptide along this path.[9] Each independent simulation was equilibrated for at least 30 ns and the data collection was done over an extra 30 ns. The total computed time for each free energy calculation expanded at least 1.8 μs.

**Molecular dynamics software and parameters:** The simulations were performed using the GROMACS package, version 4.5.6 [10, 11]. The overall temperature of the water, lipids, and peptides was kept constant, coupling independently each group of molecules at 323 K with a v-rescale thermostat. The pressure was coupled to a Berendsen barostat at 1 atm separately in the direction perpendicular to the bilayer plane and in the directions parallel to the bilayer. The temperature and pressure time constants of the coupling were 0.2 and 2 ps, respectively. The integration of the equations of motion was performed by using a leap frog algorithm with a time step of 2 fs. Periodic boundary conditions were implemented in all systems. A cutoff of 1 nm was implemented for the Lennard–Jones and the direct space part of the Ewald sum for Coulombic interactions. The Fourier space part of the Ewald splitting was computed by using the particle-mesh Ewald method [12], with a grid length of 0.11 nm on the side and a cubic spline interpolation. We used the CHARMM TIP3P [13] water model, for the lipids and peptides it was used the force field CHARMM 36.
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Supplementary Figures

**Figure S1.** Arginine-rich peptides drive the fluorescent dyes used in this study towards the aqueous or octanol phase. TAMRA (5-Carboxytetramethylrhodamine) and FITC (Fluorescein isothiocyanate) fluorescent dyes get protonated as the pH is lowered becoming more hydrophobic and prompt to partition into the hydrophobic phase. This is the opposite behavior as when they are coupled to arginine- and lysine-rich peptides (Figure 1, 2, 3 and 7) indicating that the peptides drive the partition of the dyes into the aqueous phase at low pH and into the hydrophobic phase at high pH. While the partition fluorescein dyes has already been reported (Oba, Y et al. Geochem J, 2012. 46: p. 517-20), to our knowledge, there is no study on the partition of TAMRA. Therefore, as a control experiment, here we studied the partition of TAMRA following the same protocol as in Figure 1 (a). The photograph and the plot show that for a pH less than 6 TAMRA (10 µM) partitions partially into the octanol phase, while for any larger pH partitions exclusively in the aqueous phase. This partitioning is essentially determined by the protonation state of the carboxyl acid groups (pKa <5), as these groups become protonated at low pH the fluorescent dyes partition into the hydrophobic phase.
Figure S2 Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show the TAT (2 µM) uptake in living cells at pH 6, 7.5 and 9. The lower plot shows the average (over three independent repetitions) of the overall fluorescence intensity minus the background intensity, and standard error of the mean, as a function of time. After 30 min the fluorescence increases several folds at pH 9 relative to pH 6 and pH 7.5. The images were acquired with x60 objective magnification, to quantify only free intracellular distributed peptide, and it was measured over time the fluorescence intensity at the nucleolus relative to the background. Scale bar 15 µm.
**Figure S3.** Cells display enzymatic activity after being exposed for 30 min to buffers at pH 6, 7.5 and 9, containing the TAT peptide. After imaging HeLa cells in Figure 4 and Extended Data Figure 2, cells were incubated for an extra 30 min in standard culture media containing calcein AM and imaged again. Calcein AM is a common cell-permeant dye used to determine cell viability in most eukaryotic cells. In live cells the non-fluorescent calcein AM is converted to a green-fluorescent calcein after acetoxyethyl ester hydrolysis by intracellular esterases. Images were acquired using a x20 objective (scale bar 75 µm) and the insets were acquired using a x60 objective (scale bar 15 µm).
Figure S4. Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides with different structures, lengths and chirality. Fluorescence images show the uptake of the peptides listed in Table 1 (2 µM) in living cells (HeLa) at pH 6, 7.5 and 9 after 30 min. For each pH is shown the fluorescent confocal image of the peptides (green) and an image composed of an overlay of the DIC, the peptides and the nucleus (red). Cells permanently expressing Proliferating Cell Nuclear Antigen (PCNA) labeled with Cherry or GFP were used to facilitate the detection and visualization of confocal planes across the nucleus. This helped to easily classify and count cells containing transduced peptides from the cells that only contain membrane bound peptides. Although, this is clear when the peptides are in D form (R10 or cR10) since they are stable and distinctly label the nucleolus, the peptides in L form are being actively degraded and the signal quickly redistributes more homogeneously within the cell making more challenging to distinguish membrane bound peptides form internalized peptides. Cells were counted as positive when the peptide signal colocalized with the PCNA signal. The percentage of cells counted with intracellular peptide at pH 9 was consistently larger relative to pH 6 and 7.5 for all arginine rich peptides, while the poly-lysine peptide K10 displays no uptake at all pHs. The uptake efficiency also increases with the number of arginine amino acids and by cyclization. The images where acquired with a x60 objective magnification. Each experiment was repeated 3 times and the percentage represents the average over more than 400 cells in each case. Scale bar 15 µm.
Figure S5. Cellular uptake of the TAT peptide in HepG2 (human liver hepatocellular) cells at pH 6, 7.5 and 9. Live cell confocal microscopy images obtained after 30 min incubation with the TAT peptide (2 μM). In the first column are shown fluorescence images of the TAT peptide labeled with TAMRA indicating the percentage of cells counted with internalized peptide over 3 independent experiments for each pH (standard deviations are shown between parenthesis). In the second column are shown DIC images and in the third column is shown the overlay of the first two columns. Scale bar 75 μm.
Figure S6 Cellular uptake of the TAT peptide in C2C12 (mouse myoblast) cells at pH 6, 7.5 and 9. Live cell confocal microscopy images obtained after 30 min incubation with the TAT peptide (2 µM). In the first column are shown fluorescence images of the TAT peptide labeled with TAMRA indicating the percentage of cells counted with internalized peptide over 3 independent experiments for each pH (standard deviations are shown between parenthesis). In the second column are shown DIC images and in the third column is shown the overlay of the first two columns. Scale bar 75 µm.
Figure S7  Cellular uptake of the TAT peptide in BHK (baby hamster kidney fibroblasts) cells at pH 6, 7.5 and 9. Live cell confocal microscopy images obtained after 15 min incubation with the TAT peptide (2 µM). In the first column are shown fluorescence images of the TAT peptide labeled with TAMRA indicating the percentage of cells counted with internalized peptide over 3 independent experiments for each pH (standard deviations are shown between parenthesis). In the second column are shown DIC images and in the third column is shown the overlay of the first two columns. Scale bar 75 µm.
Figure S8 Cellular uptake of the TAT peptide in Sf9 (Spodoptera frugiperda pupal ovarian) cells at pH 6, 7.5 and 9. Live cell confocal microscopy images obtained after 30 min incubation with the TAT peptide (0.5 µM). In the first column are shown fluorescence images of the TAT peptide labeled with TAMRA indicating the percentage of cells counted with internalized peptide over 3 independent experiments for each pH (standard deviations are shown between parenthesis). In the second column are shown DIC images and in the third column is shown the overlay of the first two columns. Scale bar 15 µm.
Figure S9  Cellular uptake of the TAT peptide in tobacco plant BY-2 cells at pH 6, 7.5 and 9. Live cell confocal microscopy images obtained after 45 min incubation with the TAT peptide (4 µM). In the first column are shown fluorescence images of the TAT peptide labeled with TAMRA indicating the percentage of cells counted with internalized peptide over 3 independent experiments for each pH (standard deviations are shown between parenthesis). In the second column are shown DIC images and in the third column is shown the overlay of the first two columns. Scale bar 75 µm.
Movie Legends

Movie S1. Structures and free energy computation of the TAT peptide insertion into a phospholipid bilayer containing deprotonated fatty acids using molecular dynamics simulations. In the upper panel are shown molecular conformations of the systems with the peptide constrained along the direction perpendicular to the bilayer. The systems are composed of a TAT peptide, 8700 water molecules, 68 DOPC, 48 oleic acid molecules (all protonated). The systems are neutralized with the addition of potassium or chloride ions. Water is represented by a blue surface, water molecules at less than 3 Å from any atom of the peptide or lipid bilayer are explicitly drawn in blue. DOPC and oleic acid molecules are shown with a white surface. Phosphate atoms are shown in yellow, protonated fatty acid carboxyl groups are shown in grey and the TAT is shown in red. In the bottom panel is shown the free energy profile as a function of the distance of the center of mass of the TAT peptide from the center of mass of the lipid bilayer. The total computed time for this free energy calculation profile expanded 10 µs.

Movie S2. Structures and free energy computation of the TAT peptide insertion into a phospholipid bilayer containing protonated and deprotonated fatty acids using molecular dynamics simulations. In the upper panel are shown molecular conformations of the systems with the peptide constrained along the direction perpendicular to the bilayer. The systems are composed of a TAT peptide, 8700 water molecules, 68 DOPC, 48 oleic acid molecules (24 protonated and 24 deprotonated). The systems are neutralized with the addition of potassium or chloride ions. Water is represented by a blue surface while water molecules at less than 3 Å from any atom of the peptide or lipid bilayer are explicitly drawn in blue. DOPC and oleic acid molecules are shown with a white surface. Phosphate atoms are shown in yellow, protonated (deprotonated) fatty acid carboxyl groups are shown in grey (green) and the TAT is shown in red. In the bottom panel is shown the free energy profile as a function of the distance of the center of mass of the TAT peptide from the center of mass of the lipid bilayer. The total computed time for this free energy calculation profile expanded 10 µs.
**Movie S3.** Structures and free energy computation of the TAT peptide insertion into a phospholipid bilayer containing deprotonated fatty acids using molecular dynamics simulations. In the upper panel are shown molecular conformations of the systems with the peptide constrained along the direction perpendicular to the bilayer. The systems are composed of a TAT peptide, 8700 water molecules, 68 DOPC, 48 oleic acid molecules (all deprotonated). The systems are neutralized with the addition of potassium or chloride ions. Water is represented by a blue surface, water molecules at less than 3 Å from any atom of the peptide or lipid bilayer are explicitly drawn in blue. DOPC and oleic acid molecules are shown with a white surface. Phosphate atoms are shown in yellow, protonated fatty acid carboxyl groups are shown in grey and the TAT is shown in red. In the bottom panel is shown the free energy profile as a function of the distance of the center of mass of the TAT peptide from the center of mass of the lipid bilayer. The total computed time for this free energy calculation profile expanded 10 µs.

**Movie S4.** Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show no cellular uptake of the TAT (2 µM) in living cells at pH 6. The upper image shows time-lapse confocal fluorescent images of the tat peptide coupled to TAMRA and the lower DIC image captures the morphology of the cells. The images where acquired with a x20 objective magnification. Scale bar 75 µm.

**Movie S5.** Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show no cellular uptake of the TAT (2 µM) in living cells at pH 6. The upper image shows time-lapse confocal fluorescent images of the tat peptide coupled to TAMRA and the lower DIC image captures the morphology of the cells. The images where acquired with a x60 objective magnification. Scale bar 15 µm.

**Movie S6.** Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show cellular uptake of the TAT (2 µM) in some living cells at pH 7.5. The upper image shows time-lapse confocal fluorescent images of the tat peptide coupled to TAMRA and the lower DIC image captures the morphology of the cells. The images where acquired with a x20 objective magnification. Scale bar 75 µm.
**Movie S7.** Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show cellular uptake of the TAT (2 µM) in some living cells at pH 7.5. The upper image shows time-lapse confocal fluorescent images of the tat peptide coupled to TAMRA and the lower DIC image captures the morphology of the cells. The images where acquired with a x60 objective magnification. Scale bar 15 µm.

**Movie S8.** Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show cellular uptake of the TAT (2 µM) in all living cells at pH 9. The upper image shows time-lapse confocal fluorescent images of the tat peptide coupled to TAMRA and the lower DIC image captures the morphology of the cells. The images where acquired with a x20 objective magnification. Scale bar 75 µm.

**Movie S9.** Increasing the extracellular pH consistently increases the transduction efficiency of arginine rich peptides. Time-lapse fluorescence images show uptake of the TAT (2 µM) in all living cells at pH 9. The upper image shows time-lapse confocal fluorescent images of the tat peptide coupled to TAMRA and the lower DIC image captures the morphology of the cells. The images where acquired with a x60 objective magnification. Scale bar 15 µm.

**Movie S10.** Cells remain morphologically healthy and keep undergoing cell division after TAT peptide uptake at pH 9. Time-lapse fluorescence images show the TAT peptide (2 µM) after uptake at pH 9. The DIC image captures the morphology of the cells and cells undergoing division are encircled with dotted lines. The images where acquired with a x20 objective magnification every 30 min for 16 hours. Scale bar 75 µm.