Microfluidics delivery of DARPP-32 into HeLa cells maintains viability for in-cell NMR spectroscopy

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High-resolution structural studies of proteins and protein complexes in a native eukaryotic environment present a challenge to structural biology. In-cell NMR can characterize atomic resolution structures but requires high concentrations of labeled proteins in intact cells. Most exogenous delivery techniques are limited to specific cell types or are too destructive to preserve cellular physiology. The feasibility of microfluidics transfection or volume exchange for convective transfer, VECT, as a means to deliver labeled target proteins to HeLa cells for in-cell NMR experiments is demonstrated. VECT delivery does not require optimization or impede cell viability; cells are immediately available for long-term eukaryotic in-cell NMR experiments. In-cell NMR-based drug screening using VECT was demonstrated by collecting spectra of the sensor molecule DARPP32, in response to exogenous administration of Forskolin.
The study of protein structure under physiological conditions is the next frontier of structural biology. The intracellular environment is extremely dense and heterogeneous, providing both specific interactions that result in high-affinity protein complexes and omnipresent weak interactions that can influence protein structure and activity. The lack of bulk water changes the physicochemical properties of proteins and affects the strength of hydrophobic and electrostatic interactions that drive protein complexation. In-cell NMR provides a means to observe the atomic resolution structure of target proteins in mammalian cells.

Target proteins labeled with NMR active nuclei $^{13}$C and $^{15}$N are easily distinguished from the rest of cellular proteome and can be detected at concentrations as low as 5–10 μM. A common method for introducing labeled target proteins to cells is by overexpression in a labeled medium. However, because large protein complexes are invisible by solution NMR, there is a need to deuterate proteins to observe in-cell NMR spectra. This requirement limits protein overexpression, particularly in mammalian cells, which do not grow in perdeuterated medium. Exogenous delivery of target proteins using techniques such as microinjections, cell-penetrating peptides, creation of pores and electroporation; limit cell viability and may perturb the physiological state of the cells by impeding homeostasis and cell growth.

The ability of cells to rapidly exchange fluid with their surroundings in response to ultrafast mechanical compressions presents a robust method to deliver large extracellular molecules and particles into cells. The microfluidic technique of cell volume exchange for the convective transfer, VECT, has been used to deliver molecules intracellularly from particles suspended in extracellular fluid. The critical advantage of VECT over pore-forming techniques for protein delivery is that VECT delivers proteins without creating significant prolonged cellular stress. Microfluidic delivery does not destroy the nuclear membrane and protein is delivered only into physiologically relevant compartments.

The effectiveness of microfluidics-based delivery of target proteins into HeLa cells was tested. The viability of cells transfected by using electroporation and VECT was compared and the efficacy of in-cell NMR experiments utilizing a VECT-delivered target protein, DARPP-32, was demonstrated.

Results

VECT delivery of target protein promotes high cell viability. Membrane disruption methods are used to deliver biological target molecules intracellularly for in-cell NMR spectroscopy. In-cell NMR requires a long time, ≥3 h, to collect spectra during which cells die, lyse, and leak. Among the most commonly employed delivery techniques is electroporation, however, electroporated cells exhibit damage to membranes, mitochondria, protein, and DNA, decreases in ATP levels as well as increases in reactive oxygen species, ROS, and intracellular Ca$^{2+}$ concentrations, all of which can lead to cell death. Thus effective use of electroporation requires optimization of a number of parameters including voltage, cuvette gap size, shape, length and number of pulses, cell size and concentration, buffer and temperature, to strike a balance between transfection efficiency and cell death. This can be particularly inconvenient when the target protein is expensive to prepare or can only be purified in small quantities. The use of a bioreactor helps maintain cell viability but electroporated cells still have to recover from damage. VECT delivery, on the other hand, results in high cell viability and does not require extensive optimization; the basic procedure being applicable to most cell types and target proteins.

The experimental setup for VECT delivery of proteins into cells is shown in Fig. 1. Microliter to milliliter volumes of cells are flowed through channels ranging from tens to hundreds of micrometers in dimension. Rapid mechanical deformations cause transient cell volume exchange that facilitates the convective transfer of extracellular material into the cell. Many biological macromolecules, such as dextrans, DNA, protein, and oligomers, have been successfully transfected into a range of cell types, including HEK293 and K562 cells, primary neurons, and fibroblasts, neuron-like N1E-115 cells, dendritic cells, blood immune cells, and embryonic stem cells. Previous experiments showed that, as in electroporation, the amount of protein delivered into the cytosol is linearly proportional to the concentration of extracellular protein. Successful delivery of protein resulted in minimal, ~10%, rupture of the nuclear envelope, <5% loss of material to the cytosol, and ~2% loss of the cytosolic content during transfection.

Green fluorescent protein, GFP (27 kDa), was used to quantify and compare electroporation and microfluidic protein delivery to HeLa cells. The intrinsic fluorescence of GFP facilitated imaging to assess target protein delivery and cell morphology following transfection. The concentration of extracellular GFP used for both transfection methods was 300 μM. The electroporation pulse program was the same as previously used by our group and others to electroporate HeLa cells for in-cell NMR spectroscopy, Analysis of lysed cells indicated intracellular GFP concentrations of 20 ± 10 μM and 5 ± 2.5 μM from electroporation and VECT-delivery, respectively.

VECT-transfected cells exhibited normal morphology (Fig. 2a, c) whereas electroporation-transfected cells were rounder, aggregated, and displayed more concentrated GFP signals (Fig. 2b, d). Cell attrition was assessed in the 90 min window immediately following transfection (Fig. 2e). Electroporated cells exhibited a steady decline in viability resulting in an ~25% reduction compared to VECT, which increased by ~10% over the same period. Long-term recovery showed that VECT-transfected HeLa cells were capable of exponential growth comparable to that of non-transfected cells over a 48 h period whereas the electroporated cells dropped below seeding density in 12 h, and were unable to demonstrate exponential growth by 24 h (Fig. 2f). The viability of electroporated cells after 12 h was 85–90%, comparable to the 75–85% observed by Theiliet et al., i.e., 15–25% dead cells after >13 h. Overall, the higher attrition rates of electroporated versus VECT-transfected cells were consistent with the idea that the electroporated cells were more extensively damaged.

Fig. 1 Experimental setup for cell volume exchange for the convective transfer, VECT. A syringe pump delivers the target protein into HeLa cells as it passes through the microfluidic device.
VECT-delivery of DARPP32 to HeLa cells. Dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein, DARPP-32, is a 32 kD sensor protein found in dopamine-rich areas of the brain that is extremely sensitive to cell physiology. Functional studies highlighted the role of the N-terminal region of DARPP-32 as a sensor of cell surface receptors. To investigate its structure in live cells, a C-terminally truncated DARPP-32 construct was used. In vitro characterization showed that DARPP-32 is an intrinsically disordered protein, IDP, and contains a partially folded short helix between amino acids 22 and 29. The high signal-to-noise ratio afforded by IDPs, relatively well dispersed 1H,15N correlation NMR spectrum, and high, 30 µM, physiological intracellular concentration makes DARPP-32 an attractive target for in-cell NMR analysis.

Previous characterization utilized DARPP-32 constructs from rats; in this work a human DARPP-32 construct was used (Supplementary Fig. 1). 103 out of 108 possible 1H,15N cross-peaks were assigned for the human construct in the buffer used for this study (Fig. 3a). HeLa cells were chosen to minimize specific interactions that affect the localization and activity of DARPP-32 in neuronal cells.

DARPP-32 is known to engage in an extensive interaction network that results in the formation of complexes with molecular weights that exceed the detectability limit, ~50 kDa, when using pulse programs typically employed for in vitro work. Indeed, the 1H,15N heteronuclear single quantum coherence, HSQC, NMR spectrum of HeLa cells electroporated with [U-15N] DARPP-32 resulted in no interpretable cross-peaks (Supplementary Fig. 2). Perdeuterating target proteins and collecting cross-relaxation-enhanced polarization transfer heteronuclear multiple quantum coherence transverse relaxation-optimized, 1H-15N CRINEPT-HMQC-TROSY, NMR spectra on in-cell samples can circumvent this problem by facilitating detection of high molecular weight complexes. Uniformly labeled [U-2D,15N]-DARPP-32 was delivered to HeLa cells using VECT and electroporation. 1H-15N CRINEPT-HMQC-TROSY experiments affirmed that perdeuteration was required to obtain an in-cell spectrum of DARPP-32 (Fig. 3b, c). The narrow chemical shift dispersion showed that the protein...
remained predominantly unfolded in-cell, with many of the in-cell cross-peaks lying very close to those observed in vitro (Fig. 3a). The spectra were consistent with intermediate exchange implying that DARPP-321-122 may engage in transient quinary interactions that will result in cross-peak broadening.

The spectrum of electroporated cells (Fig. 3c) contained sharp cross-peaks not observed in cells containing VECT-transfected target protein (Fig. 3b). Unlike the case of VECT protein delivery, control experiments examining the supernatant of electroporated samples revealed sharp \(^{1}H\)-\(^{15}N\) cross-peaks consistent with leakage of labeled target protein from the cells (Supplementary Fig. 3). This likely reflects the loss of integrity of plasma and nuclear membranes and other organelles due to the electroporation process\(^{19,27,28}\). The combination of prolonged cell viability and the absence of cell leakage suggests that VECT is a simple and reliable method to deliver exogenous target proteins for long-duration in-cell NMR studies. It should be noted that electroporation parameters, as well as those of other delivery methods, can be optimized to minimize cell damage, and viable cells can be isolated, although this procedure requires several additional hours\(^{17}\).

DARPP-32 phosphorylation is not regulated in HeLa cells. In neuronal cells, the intracellular localization and activity of DARPP-32 is regulated by phosphorylation and dephosphorylation at several residues (Supplementary Fig. 1). Phosphorylation by cAMP-dependent protein kinase A, PKA, of residue T34 converts DARPP-32 into a potent inhibitor of protein phosphatase-1, PP1. As a PP1 inhibitor, DARPP-32 amplifies the activity of PKA at the plasma membrane and in the cytoplasm affecting a broad spectrum of potential targets and downstream functions and is a key target in combating neurological diseases\(^{40–42}\) and carcinogenesis\(^{43}\). Conversely, when phosphorylated at T75 by cyclin-dependent kinase 5, CDK5, DARPP-32 inhibits PKA signaling, abating inhibition of PP1\(^{44,45}\). Amplification of PKA activity also results in the phosphorylation of protein phosphatase 2 A, PP-2A, and subsequent dephosphorylation at T75\(^{46}\). In the cytosol, where DARPP-32 predominates, S45 and S97 (S102 in humans) are phosphorylated by casein kinase 2, CK2, and require dephosphorylation at S97 (S102) for nuclear co-localization\(^{52,54}\). CK2-mediated phosphorylation enhances phosphorylation of T34 by PKA\(^{47}\) but the functional consequences of this interaction remain unresolved\(^{48}\). Thus the state of phosphorylation determines the cellular location and consequent activity of DARPP-32.

Antibodies that recognize phosphorylated T34, T75, and S102 were used to look for evidence of biochemical modification. Western blots of cell lysates revealed weak phosphorylation at S102, indicative of constitutively expressed and active CK2\(^{48}\). The extent of phosphorylation was not quantified. No phosphorylation at T34 or T75 was detected (Supplementary Fig. 4). The lack of T34 phosphorylation by cAMP-dependent PKA, which exists as an inactive tetramer, may be due to the absence of induction and/or the intracellular localization of PKA\(^{49}\). Regulation of cAMP/PKA signaling is controlled by A-Kinase Anchor proteins, AKAPs, which confine PKA to subcellular compartments close to its targets, thus limiting its activity\(^{50}\). The lack of phosphorylation of T75 by CDK5 is likely due to the absence of regulatory neuronal activators p35, p39, and cyclin-I in HeLa cells\(^{51}\).

VECT-transfected cells were treated with 10 \(\mu\)M Forskolin, a small drug-like molecule, which activates adenylyl cyclase and downstream cAMP-sensitive enzymes such as PKA, altering metabolic fluxes in the cell. Western blots indicated no change in the extent of S102 phosphorylation after Forskolin treatment (Supplementary Fig. 4). The absence of changes suggests that the regulation of DARPP-32 activity may be cell-specific. Indeed, it is not known the extent to which DARPP-32 is expressed in HeLa cells\(^{52}\), so it is not surprising to suspect that many of the regulatory elements are not present at the required concentrations or intracellular locations. It is also possible that elements from the C-terminal half of the molecule or intact DARPP-32 is required for full regulation of phosphorylation and dephosphorylation activity.

Purified \([U-\text{\(^{15}\)N}]\)-DARPP-321-122 was treated in vitro with PKA, which phosphorylates T34, and CK2, which phosphorylates S102. \(^{1}H\)-\(^{15}N\) HSQC spectra were assigned to account for chemical shift changes associated with the biochemical modifications and to help identify modified \(^{1}H\)-\(^{15}N\) cross-peaks in in-cell spectra (Supplementary Fig. 5). \(^{1}H\)-\(^{15}N\) cross-peaks corresponding to phosphorylated T34 and S102 were observed in the \(^{1}H\)-\(^{15}N\) HSQC spectra (Supplementary Fig. 5, Fig. 4a) but not in the in-cell \(^{1}H\)-\(^{15}N\) CRINEPT-HMQC-TROSY spectrum of \([U-\text{\(^{2}\)D, \(^{15}\)N}]\)-DARPP-321-122 treated with Forskolin (Fig. 4b). This is not surprising since phosphorylation of T34 was not detected by Western blot analysis (Supplementary Fig. 4) and phosphorylation of S102 was sub-stoichiometric in HeLa cells.

The in-cell spectrum of \([U-\text{\(^{2}\)D, \(^{15}\)N}]\)-DARPP-321-122 treated with Forskolin is more extensively broadened than in untreated cells (Fig. 4b). Overlays of selected \(^{1}H\)-\(^{15}N\) cross-peaks obtained in vitro and in-cell in the absence and presence of Forskolin reveal differential changes in chemical shifts and intensities...
Fig. 4 Forskolin treatment results in broadening of the DARPP-321-122 in-cell NMR spectrum. a In vitro 1H-15N HSQC spectrum of [U-15N]-DARPP-321-122 treated with CK2. The cross-peak associated with phosphorylated S102 is in red. Bold residues are the same as in the insets on the right. b In-cell 1H-15N CRINEPT-HMQC-TROSY spectrum of [U-2D,15N]-DARPP-321-122 treated with Forskolin. Boxed regions show overlays from the in vitro 1H-15N HSQC spectrum of [U-15N]-DARPP-321-122 (black) and the in-cell 1H-15N CRINEPT-HMQC-TROSY spectrum of [U-2D,15N]-DARPP-321-122 acquired in the absence (blue) and presence (red) of Forskolin.

Discussion
Microfluidics-based delivery of target protein into HeLa cells was tested and found to be a simple reliable method that preserves cell physiology for long-term in-cell NMR experiments. Direct comparison of protein delivery into cells showed that electroporation may deliver up to four times more protein under the same conditions. However, unlike electroporation and other pore-forming delivery techniques, which result in cell mortality within 24–48 h and can rupture internal membranous structures, VECT-transfected cells maintained viability for at least the 90 min window required to prepare them for NMR spectroscopy. Beyond that, reduced intracellular oxygen and ATP were likely to cause the health of the cells to decline during the time required to collect the data, which may contribute to the spectral changes.

Methods
Chemicals and reagents. All chemicals used were of molecular biology grade or better.

Plasmid construction. Synthetic DNA encoding for the 13 kDa N-terminal human DARPP-32 fragment, DARPP-321-122, was subcloned into pET-28a(+) (Novagen) using Ndel and Xhol restriction sites (Genscript). The resulting plasmid, pET-28trDARPP-32 confers kanamycin resistance and expresses N-terminally 6His-tagged DARPP-321-122 from the T7 lac promoter.

Protein overexpression. Reduced proton density, REDPRO, uniformly labeled [U-2D,15N]-DARPP-321-122 was prepared as previously described. Briefly, E. coli strain BL21(DE3) Codon+ was transformed with pET-28trDARPP-32 and 50 mL of Miller Lyogeny Broth (LB) containing 75 µg/mL of kanamycin was inoculated using a single colony and incubated overnight at 37 °C. The overnight culture was transferred into 1 L of fresh LB medium containing 75 µg/mL of kanamycin and allowed to grow at 37 °C to an OD600 of 0.7–0.9. Cells were centrifuged at 200 × g for 20 min at 37 °C and washed twice with minimal, M9, medium, and resuspended in 1 L of deuterated M9 medium containing 1 g/L of [15N]NaCl and 0.2% glucose as the sole nitrogen and carbon sources. The culture was incubated for 15–20 minutes at 37 °C to facilitate cell acclimation. Protein expression was induced by adding isopropl β-1-thiogalactopyranoside to a final concentration of 1 mM and induction was allowed to proceed for 2–4 h. For experiments to assign backbone nuclei, 13C-glucose was used in place of the 0.2% glucose as the sole carbon source to prepare [U-15N, 13C] DARPP-321-122, and the final culture was overexpressed in non-deuterated M9 medium.

Protein purification. DARPP-321-122 was purified using a Ni-NTA column under denaturing conditions. Cells were resuspended in lysis buffer, 100 mM NaPO4, pH 8.0, 10 mM Tris, 8 M urea, and sonicated using a Model 250 Digital Sonifier (Branson) for seven cycles at 40% power using 0.5 s pulses and a 1.0 s rest between pulses for 60 total seconds of pulse time. The lysate was clarified by centrifugation at 30,000 × g for 45 min and loaded onto a column pre-equilibrated with lysis buffer. The column was washed with 50 mL of wash buffer, 100 mM NaPO4, pH 6.3, 10 mM Tris, 8 M urea, and the protein eluted with 20 mL of elution buffer, 100 mM NaPO4, pH 4.5, 10 mM Tris, 8 M urea. The eluent was dialyzed into buffer A, 50 mM NaPO4, pH 7.0, 50 mM NaCl, loaded onto a GE HiTrak™ Q HP Column, and eluted with a 300 mL linear gradient from buffer A to buffer B, 50 mM NaPO4, pH 7.0, 1 M NaCl, using a Biorad DualFlow chromatography system (Biorad). The purified protein was exchanged into storage buffer, 10 mM sodium phosphate, pH 7.0, 100 mM NaCl, 0.01% sodium azide, and 20% glycerol, and concentrated to 100 mM by using an Amicon Ultra-15 Centricon (Millipore) for storage at ~80 °C. Emerald GFP, EmGFP, was overexpressed from plasmid pRSET-EmGFP and purified as previously described and exchanged into the storage buffer.

Protein transfection by electroporation. HeLa cells (Sigma) were prepared by seeding 4 × 10⁶ cells into five 15 cm Corning culture plates. Cells were incubated for 2–3 days in culture medium, Dulbecco’s Modified Eagle Medium, DMEM (Gibco), containing 4.5 g/L d-glucose, 110 mg/L sodium pyruvate, and supplemented with 10% fetal bovine serum, FBS (Gibco), to ~80% confluence (~12 × 10⁶ cells/plate). Cells were harvested as previously described by exposure to 0.25% trypsin EDTA (Gibco) for 3 min at 37 °C, pelleted by centrifugation at 200 × g for 6 min at 25 °C, washed twice with 5 mL of phosphate-buffered saline, PBS, and counted. Cells were resuspended in 100 µL of electroporation buffer, 100 mM NaPO4, pH 7.0, 5 mM KCl, 15 mM MgCl2, 15 mM HEPES, 5 mM ATP, 5 mM reduced glutathione and 50% Amaza Nucleofector Solution R (Lonza, Inc). Purified [U-2D,15N]-DARPP-321-122, or GFP, in storage buffer, was added to a final concentration of 300 µM. Aliquots of 100 µL containing ~2 × 10⁶ cells were transferred into 1 mm cuvettes (Lonza) and electroporation was performed using...
an Amaxa Nucleofector 2b apparatus (Lonza) set to the B-28 pulse program as previously described. Each cuvette was pulsed twice, with gentle agitation between the pulses. 1 mL of prewarmed culture medium was added to each cuvette immediately following the second pulse and the cell suspension was transferred into a 50 mL centrifuge tube and incubated at room temperature for 20 min to maximize transfection and facilitate cell recovery. Cells were centrifuged at 200 × g for 3 min at RT, and washed twice using 2 mL of culture medium to remove residual protein. Samples were prepared for in-cell NMR by resuspending the cell suspension in 450 μL of culture medium and 50 μL of D2O, and transferring the suspension to a 5 mm NMR tube. For plate reading to determine GFP concentrations, aliquots of 2 × 106 cells were resuspended in 1 mL of RIPA buffer, 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and frozen at −80 °C.

**Protein transfection by microfluidics.** HE-La cells (Sigma) were prepared by seeding 4 × 106 cells into three 15 cm dishes (Corning). Seeded cells were incubated for 2–3 days in culture medium to −80% confluence (~1.2 × 107 cells/plate). Cells were harvested as described above, passed through a 40 μm filter to reduce clumping, and counted. Approximately 3 × 106 cells were resuspended in 3 mL of cell flow buffer, 0.4% BSA, 0.04% EDTA, 20% Percoll, 5 μL of Tween 20, and purified [U-2D,15N] DARPP-32, 122, or GFP, in storage buffer, was added to a final concentration of 300 μM. A three-channel polydimethylsiloxane, PDMS, Volume Exchange for Convective Transfer, VECT, device with rigid, 9.6-μm microchannels was prepared as previously described. The VECT device was placed in a vertical position (0°) and the suspension was allowed to flow through the device and purged to remove trapped air and any existing manufacturing debris using passivation buffer, 1% BSA in phosphate-buffered saline (Gibco). The cell suspension was transferred to a 3 mL syringe, connected to a New Era Pump Systems Model 300 syringe pump set to a flow rate of 100 μL/min, and the flow commenced while observing for bubbles and blockages. The cell suspension eluent was collected and allowed to equilibrate for 20 min in a 37 °C room to maximize cell transfection and facilitate cell recovery. Cells were centrifuged at 200 × g for 6 min at 25 °C and washed twice with 5 mL of PBS. Samples were prepared for in-cell NMR by resuspending transfected cells in 450 μL of culture medium with or without 10 μM Forskolin, and 50 μL of D2O, and transferring the suspension to a 5 mm NMR tube. For plate reading to determine GFP concentrations, aliquots of 2 × 106 cells were resuspended in 1 mL of RIPA buffer, and frozen at −80 °C. About 5 × 106 cells were reserved for plate reading as described above. Following VECT delivery of DARPP-32, 122, ~5 × 106 cells were resuspended in 1 mL of RIPA buffer and frozen at −80 °C in preparation for future Western blotting.

**NMR experiments.** All NMR spectra were recorded at 298 K on either a 700 MHz Avance II NMR spectrometer (Bruker) equipped with a TXI cryoprobe or a 600 MHz Avance III NMR spectrometer equipped with a QCI-P cryoprobe. All in vitro samples were prepared by combining 450 μL of purified labeled DARPP-32, 122, in NMR buffer, 50 mM Na3PO4, pH 6.8, with 50 μL of D2O, to a final concentration of 100 μM. In-cell samples were prepared by combining 450 μL of culture medium and 50 μL of D2O. Spectra were processed with Topspin (version 3.2, Bruker) and analyzed using CARA software. Heteronuclear single quantum coherence, [1H-15N] HSQC, experiments were performed with Watergate water suppression and the spectra were acquired with 64 transients and 1024 and 128 points in the H and 15N dimensions, respectively. The spectra were zero filled in the H and 15N dimensions were 14 and 35 ppm, respectively.

Cross-relaxation-enhanced polarization transfer heteronuclear multiple quantum coherence transverse relaxation-optimized, [1H-15N] CRIPPS, experiments were performed with Watergate water suppression and CRIPPS transfer delays of 1.5 ms and a recycle time of 300 ms. 512 transients were used to acquire 1024 and 128 points with spectral widths of 14 and 35 ppm in the proton and nitrogen dimensions, respectively. A standard array of triple resonance experiments, NCABC, CBCACOH, HNCA, HNCO, HNCOA, HNCCO, (H)CC(CO)NH, and (H)CC(CO)NH, were used to assign backbone nuclei of both unphosphorylated and phosphorylated [U-15N] DARPP-32, 122. Assignments were accomplished using CARA software.

Biological replicates HSQC experiments were performed after transfecting DARPP-32, 122 into HE-La cells using VECT. CARA software was used to obtain the intensity values for each experiment. The intensities were normalized using a glutamine amidine side chain at 7.49 ppm and 11.2 ppm in the proton and nitrogen dimensions, respectively, that did not undergo changes in chemical shift. The errors in the ratios were derived by propagating the errors in the individual cross-peak intensities. All experiments were performed independently at least twice and the results were combined for analysis by using the ANOVA statistical package in Prism 6.0 (Graphpad, Inc.).

**Phosphorylation of DARPP-32, 122.** Phosphorylation of DARPP-32, 122 was performed as previously described. Purified [U-2D,15N] DARPP-32, 122 was exchanged into NE Buffer (New England Biolabs) 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35, and 200 μM ATP. 8 μg/mL of protein kinase A, PKA (New England Biolabs), or casein kinase 2, CK2 (New England Biolabs), was added to initiate the reaction. Each reaction was allowed to proceed at 30 °C for 90 min and the phosphorylated DARPP-32, 122 was immediately combined into 450 μL of NMR buffer and 50 μL of D2O for NMR spectroscopy, or 1:1 with Laemmli buffer for SDS-PAGE and Western blotting. SDS-PAGE band intensities were measured by using ImageJ software.

**Forskolin treatment of HE-La cells.** Aliquots containing ~5 × 106 HE-La cells that had undergone VECT delivery of [U-2D,15N] DARPP-32, 122, were suspended in 1 mL of culture medium. The cells were treated with 10 μM Forskolin (TCI) and incubated at 37 °C for 30 min. The cells were centrifuged at 200 × g for 6 min at 25 °C, resuspended in 1 mL of RIPA buffer, and stored at −80 °C for SDS-PAGE and Western blotting.

**Cell viability and morphology assays.** Cells were collected at the end of the 20 min rest period following protein delivery to measure the initial survival rates and viability of VECT- and electroporation-transfected cells. Individual samples were combined into a 50 mL conical tube (Thermo), washed twice with 10 mL of PBS (Gibco) to remove residual protein, and resuspended in 2 mL of culture medium in duplicate. To assess initial survival rates in the critical 90 min window where cells are prepared for in-cell NMR, a 10 μL aliquot of cell suspension was removed every 10 min, diluted 1:10 (v/v) with 0.4% Trypan blue (Thermo Fisher) and counted with a hemocytometer (Reichert). To assess long-term viability (48 h), eight 35 mm tissue culture dishes (Corning) were individually seeded with 0.3 × 106 cells across three conditions (electroporation-, VECT- and non-transfected cells). Plates were prepared for cell counting at 12 h intervals. Cell images were taken using an Evos™ FL auto imaging system (Thermo Fisher Scientific) 12 h after protein delivery to assess morphology and fluorescence.

**Western blotting.** HE-La cell samples from electroporation- and VECT-transfections, ±Forskolin, were individually thawed and lysed using a Model 250 Digital Sonifier (Branson). The lysate was centrifuged at 200 × g for 30 min to pelletize cellular debris and the supernatant was decanted. A 1:1 dilution of the clarified lysate was prepared for electrophoresis using 2x Laemmli buffer. The same procedure was followed to create a control sample using ~5 × 106 HE-La cells that had not undergone transfection. Whole-cell lysates and samples from in vitro phosphorylation reactions were subjected to SDS-PAGE (Mini PROTEAN Tetra Cell, Bio-Rad). Protein transfer to a 0.2 μm nitrocellulose membrane (Biorad) was performed at 20 V for 16 h. Four membranes were blotted using recombinant anti-DARPP-32 rabbit antibody (EP720Y/AB40801, 1:1000 dilution, Abcam), phospho-DARPP-32 (Ser97 in rat or S102 in human) rabbit monoclonal antibody (D11A5, 1:1000 dilution, Cell Signaling Technology), phospho-DARPP-32 (T34) rabbit monoclonal antibody (D27A4, 1:1000 dilution, Cell Signaling Technology), and phospho-DARPP-32 (T75) rabbit polyclonal antibody (AB51114, 1:3000 dilution, Abcam). Anti-DARPP-32 was used to initially determine the effectiveness of the blotting protocol. Chemiluminescence was generated using ECL western blotting substrate (Promega) and detection and imaging was performed by using a ChemiDoc™ MP imaging system (Bio-Rad).

**Fluorometric quantitiation of intracellular GFP concentrations.** Duplicate sets of six samples of ~2 × 106 cells each from the electrophoresis and microfluidics transfections, along with a control sample of ~2 × 106 non-transfected HE-La cells, were thawed and lysed using a Model 250 Digital Sonifier (Branson). Each of the samples was centrifuged at 200 × g for 30 min at room temperature to pelletize cellular debris and the supernatant was collected. About 150 μL of each of the transfected, control, and background samples were transferred into a 96-well plate (Model 3603, Costar). Fluorescence was detected using a Synergy HT plate reader (BioTek). A calibration curve was generated using purified GFP. To calculate the final concentration of GFP delivered per cell, a HE-La cell volume of 2500 μm3 was assumed.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All data are available upon request. The source data behind the graphs and unedited gel images are included in Supplementary Data 1 and Supplementary Fig. 7, respectively.

**Materials availability.** All materials are available upon request.

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