Chapter 4

In-Cell NMR in Mammalian Cells: Part 1

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Abstract

Many mammalian IDPs exert important biological functions in key cellular processes and often in highly specialized subsets of cells. For these reasons, tools to characterize the structural and functional characteristics of IDPs inside mammalian cells are of particular interest. Moving from bacterial and amphibian in-cell NMR experiments to mammalian systems offers the unique opportunity to advance our knowledge about general IDP properties in native cellular environments. This is never more relevant than for IDPs that exhibit pathological structural rearrangements under certain cellular conditions, as is the case for human α-synuclein in dopaminergic neurons of the *substantia nigra* in the course of Parkinson's disease, for example. To efficiently deliver isotope-labeled IDPs into mammalian cells is one of the first challenges when preparing a mammalian in-cell NMR sample. The method presented here provides a detailed protocol for the transduction of isotope-labeled α-synuclein, as a model IDP, into cultured human HeLa cells. Cellular IDP delivery is afforded by action of a cell-penetrating peptide (CPP) tag. In the protocol outlined below, the CPP tag is "linked" to the IDP cargo moiety via an oxidative, disulfide-coupling reaction.

Key words: Cell-penetrating peptides, Disulfide coupling, Alpha-synuclein, HIV-Tat, HeLa cells

1. Introduction

Although isotope-labeled IDPs can in principle be microinjected into mammalian cells, such procedures are hardly feasible in practice because millions of cells would need to be individually manipulated in order to produce a single in-cell NMR sample (mammalian cells are typically several orders of magnitudes smaller than *Xenopus* oocytes). For this reason, all mammalian in-cell NMR applications today exploit protein delivery schemes that target many cells in parallel, and in a batch-like manner. In essence, two different procedures for intracellular protein transduction have been described for in-cell NMR applications in mammalian cells (1, 2). The first takes advantage of the unique properties of cell-penetrating peptide

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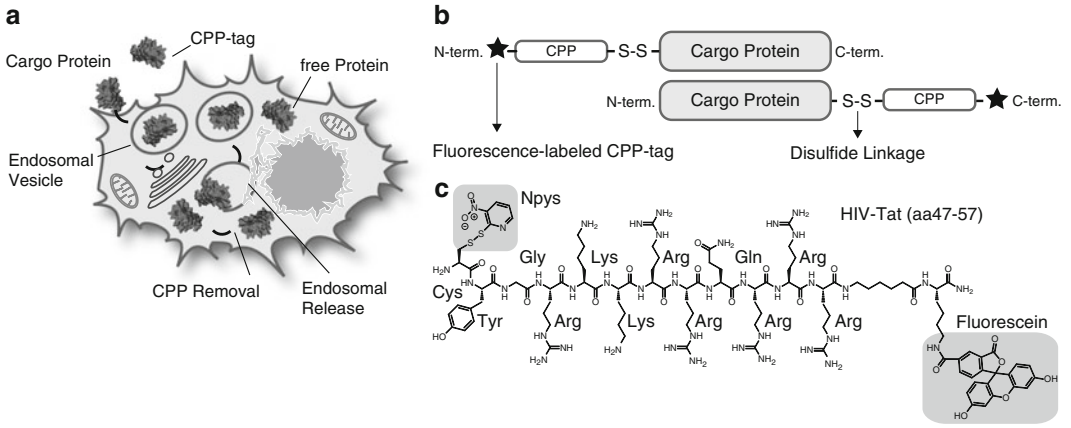

Fig. 1. (**a**) Schematic representation of CPP-mediated protein delivery into mammalian cells. CPP–cargo transduction mostly occurs via endosomal uptake routes. Once released into the reducing environment of the cytoplasm, the CPP–cargo disulfide bond is broken. This leads to the accumulation of free cargo in the cytoplasm of the host cell. (**b**) Outline of CPP–cargo constructs employed in this study. Cargo protein (α-synuclein) was produced with N- or C-terminal cysteines that are used for disulfide coupling to the CPP. The inclusion of a fluorescence label enables assessment of CPP–cargo coupling efficiency and intracellular protein uptake. (**c**) Structure of the HIV-Tat (CPP) construct described in this protocol (MW 2.4 kDa). The peptide is modified to include a N-terminal *S*-3-nitro-2-pyridinesulphenyl (Npys) activated cysteine and C-terminal Fluorescein ($\lambda_{Excitation}$ 492 nm, $\lambda_{Emission}$ 517 nm).

(CPP) tags (1). The second exploits bacterial pore-forming toxins that will be discussed in the next chapter (2).

CPPs come in many different flavors (3). They usually comprise 10–15 amino acid residues, are rich in basic amino acids, sometimes occur in the form of an amphipathic alpha-helix (4), and have the ability to translocate through biological membranes (5, 6). For these reasons they are often employed as "carrier peptides" to deliver biomolecules into live cells (Fig. 1a). A CPP–cargo construct can be produced several ways. In the following sections, we will describe the coupling of a synthetic CPP to a recombinantly produced cargo IDP (specifically human α-synuclein). Coupling is achieved via engineered cysteines in the CPP-, and cargo protein moieties. As this method involves the separate production of the CPP and the cargo protein, it offers several advantages for in-cell NMR experiments. First, only the IDP portion can be prepared in an isotope-labeled form and thus constitutes the only detectable species in the in-cell NMR experiment (the CPP remains "invisible"). Second, because the CPP is coupled to the IDP in a separate reaction, a number of different CPPs can be prepared in advance and then coupled to the IDP cargo of interest to quickly assess the best CPP–cargo combination for optimal protein delivery (see below). Third, because the method described here involves CPP production by chemical solid-phase peptide synthesis (SPPS), additional chemical entities like a fluorescence label, a biotin tag, or a sulfhydryl activating group, can easily be incorporated and exploited to verify cellular uptake (see Chapter 6). Fourth, once

the CPP–cargo has been successfully delivered into mammalian cells, the reducing environment of the cytoplasm breaks the disulfide bond between the CPP and the cargo and thereby ensures efficient cargo release. Alternative CPP "coupling" methods such as the recombinant production of a single CPP–cargo fusion protein will not be discussed.

CPPs mediate cellular protein transduction by different biological mechanisms and by different uptake routes (7–11). Thus, for any given CPP–cargo–cell line combination, uptake efficiencies may vary greatly (12). In most instances, the choice of cargo protein and cell line to be targeted is given by the nature of the research project. Therefore, the choice and design of the CPP–cargo construct should be evaluated carefully. As the method presented here may be applied to different CPPs and IDPs we have chosen general terms throughout the protocol. In our case, "cargo" refers to isotope-labeled human alpha-synuclein that has been engineered to contain a N- or C-terminal cysteine residue to which the CPP can be coupled (Fig. 1b). "CPP" refers to SPPS synthesized HIV-Tat (aa 47–57) containing a fluorescein dye and a *S*-3-nitro-2-pyridinesulphenyl (Npys) activating group to enhance the coupling efficiency (Fig. 1c).

2. Materials

1. Equipment: The following protocol assumes that standard laboratory equipment for recombinant protein production and purification, including a fast protein liquid chromatography (FPLC) system, is available. In addition, CPP synthesis requires a SPPS setup including a reversed-phase high-pressure liquid chromatography (HPLC) system, or access to a commercial peptide production facility. Cell culture equipment for maintaining and manipulating mammalian cells, including a sterile workbench, a CO_2 incubator and a basic tissue culture microscope are needed. Access to high-field (>500 MHz) solution-state NMR spectrometers must be available.
2. Cargo: Cargo proteins should contain single cysteines at either the N- or the C-terminus. Cysteines that are part of the cargo protein sequence may also be used for coupling. The cargo protein should be available in an appropriately isotope-labeled form for the envisaged in-cell NMR experiment. Detailed protocols for recombinant protein expression and purification (13), as well as for stable-isotope labeling for NMR purposes (14) are described elsewhere.
3. CPP: The CPP to be coupled to the cargo protein must contain a cysteine residue that is preferably *S*-3-nitro-2-pyridinesulphenyl

(Npys) activated. An additional fluorescence dye can be incorporated for convenient in-cell detection by microscopy methods (see Chapter 6). Other chemical entities like a Biotin tag for example, can also be added.

4. CPP coupling buffer (CB): 20 mM Phosphate or 50 mM HEPES, 150 mM NaCl, pH 7.0.
5. Appropriate gel-filtration (size-exclusion) and ion-exchange columns (GE Healthcare, USA).
6. SDS-PAGE equipment.
7. Coomassie Blue staining solution.
8. Immobilized-TCEP reducing column (Thermo scientific, USA, Meridian Rd.).
9. Suitable cell culture medium for HeLa cells: Complete DMEM (low Glucose, 5 mM Glutamine, 10 % Fetal Bovine Serum, (FBS) (PAA Laboratories, Canada)).
10. Phosphate buffered saline (PBS), cell culture grade, without Calcium/Magnesium (PAA Laboratories, Canada).
11. 6-Well cell culture plates/T175 cell culture flasks.
12. 0.25× Trypsin-EDTA (PAA Laboratories, Canada).
13. Low melting agarose (USB Affymetrix, USA, California).
14. In-cell NMR buffer: DMEM (serum-free), 5 mM HEPES, pH 7.2, 90 mM D-glucose, 5 % D_2O, or DMEM (serum-free), 10 % D_2O.
15. RIPA buffer (denaturing): Tris 50 mM, NaCl 150 mM, 0.1 % SDS, 0.5 % Na-Deoxycholate, 1 % Triton X 100 or NP40, protease inhibitors.
16. Hemocytometer.

3. Methods

3.1. CPP–Cargo Coupling

1. Before starting the procedure, carefully assess the required number of mammalian cells to prepare the in-cell NMR sample (see Note 1), the target volume of the final in-cell NMR sample (see Note 2) and the target concentration of the isotope-labeled protein inside the cells (see Note 3).
2. Produce the CPP by SPPS. Extend the CPP sequence by a N- or C-terminal *S*-3-nitro-2-pyridinesulphenyl (Npys) activated cysteine. Reversed-phase HPLC purify the CPP. 10–40 mg of lyophilized CPP is needed as a starting material for a single in-cell NMR sample (see Note 4).

3. Produce NMR isotope-labeled, recombinant cargo protein. The cargo protein must contain at least one cysteine residue for CPP coupling. This can also be engineered by mutagenesis (preferable at the N- or C-terminus of the protein). The cysteine residue must be in a reduced state (i.e., as sulfhydryl) prior to coupling. The required concentration of cargo protein before coupling is ~2 mM in a total volume of 2 mL CB (see Note 5).
4. Initially test CPP–cargo coupling efficiencies in small-scale experiments. Non-isotope-labeled cargo proteins may be used at this point. For small-scale coupling trials, reduce all volumes indicated below by a factor of 10.
5. Dissolve CPP to a final concentration of 4 mM in 2 mL of CB, adjust pH to 7.0 (see Note 6).
6. Mix 2 mL CPP and 2 mL cargo stock solution (2 mM). The CPP will be in ~4-fold molar excess over the cargo protein (see Note 7).
7. Incubate the coupling reaction at room temperature (RT) for 10–60 min. Note that steps 5–7 will require optimization to ensure preferential formation of the desired CPP–cargo product (see Note 8).
8. Determine coupling efficiency by running a nonreducing SDS-PAGE (Fig. 2) (see Note 9).
9. Optimize coupling conditions accordingly and proceed to large scale coupling reaction (step 5).
10. After coupling, concentrate CPP–cargo mixture to a final volume of 2 mL using a centrifugal filter unit, or any other appropriate device.
11. Apply and separate by size-exclusion chromatography (see Note 10).
12. Collect fractions containing the desired CPP–cargo and further purify by ion-exchange chromatography (see Note 11).
13. Concentrate CPP–cargo to 1 mM (~2 mL) (see Note 12).

3.2. Cellular CPP–Cargo Delivery

1. To determine the efficiency of cellular CPP–cargo delivery, small-scale trials are carried out first. Seed 2.5–3 $\times$ 10^5 cells per well in a 6-well plate (surface area 9.6 cm^2/well) (see Note 13).
2. Incubate cells for 24 h in complete DMEM at 37 °C in a CO_2 incubator. Cells should be ~80 % confluent at the beginning of the experiment (~5–6 $\times$ 10^5 cells) (see Note 14).
3. *Optional* Remove culture medium and wash twice with PBS (3 mL/well). Add 0.5 μL/well of 50–250 μM Pyrenebutyrate (PA) in PBS. Incubate for 3–10 min at 37 °C (see Note 15).
4. Add 0.5 mL/well of serial CPP–cargo dilutions (0.05–0.5 mM) in prewarmed PBS to test for optimal uptake concentrations.

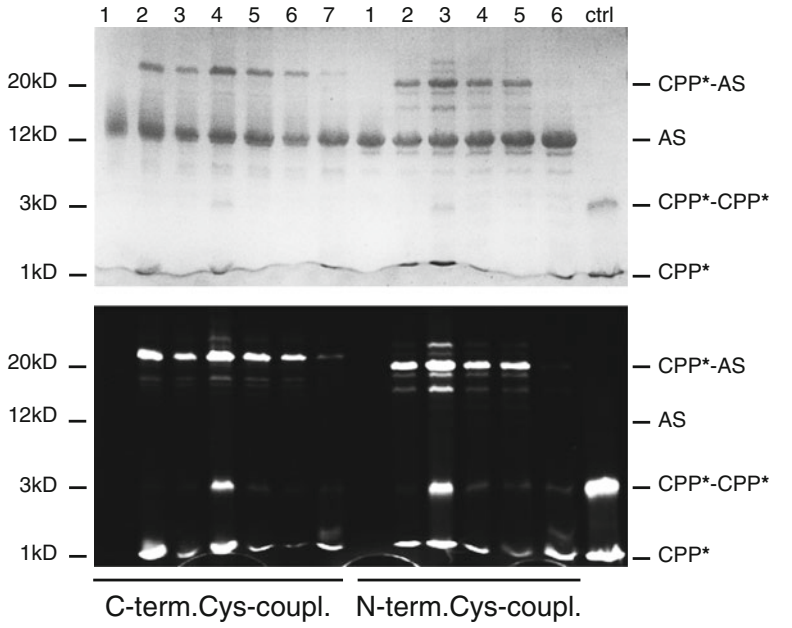

Fig. 2. Assessment of CPP–cargo coupling efficiencies by nonreducing SDS-PAGE. *Top panel*: Coomassie stained PAGE of C-, or N-terminal cysteine α-synuclein (AS)/CPP coupling reactions. Lanes 1 are AS input samples. Differences in AS/CPP coupling efficiencies and levels of CPP–CPP dimers are readily discerned for coupling reactions at 37 °C with incubation times of 10 min (*lane 2*), 30 min (*lane 3*) ,and 1 h (*lane 4*). Reactions at 4 °C, ranging from 10 min (*lanes 6 and 7*) to 1 h (*lane 5*) display similar differences in coupling efficiencies. The control lane at the far right (ctrl) indicates the extent of CPP–CPP dimer formation in the absence of AS (37 °C for 1 h). The bottom panel shows the same PAGE under UV illumination. The CPP moiety contains the fluorescein label (CPP*).

5. Incubate cells with CPP–cargo mixtures for 10–60 min at 37 °C in a CO_2 incubator (see Note 16).
6. *Optional* Cellular CPP–cargo incubations can be repeated multiple times for enhanced protein uptake. If this option is chosen, allow cells to recover for 1 h in complete DMEM at 37 °C in between the individual incubation steps (see Note 17).
7. Remove and collect CPP–cargo solution and wash cells twice with prewarmed PBS. Add fresh complete DMEM and allow cells to recover for 1 h at 37 °C in a CO_2 incubator (see Note 18).
8. Determine cellular CPP–cargo uptake by semiquantitative Western blotting of lysates prepared from manipulated cells, or by suitable alternative methods (see Note 19).

3.3. In-Cell NMR Sample Preparation

1. Once optimal delivery conditions have been found, the protein transduction procedure is scaled up for in-cell NMR sample preparation. To this end, seed 4–5 × 10^6 cells in one T175 cell culture flask. (It should be noted that a fluorescent tag is not necessarily desired on the CPP–cargo construct used for the final in-cell NMR sample preparation).

2. Incubate cells for 24 h in complete DMEM at 37 °C in a CO_2 incubator. Cells should be ~80 % confluent at the beginning of the experiment (~0.8–1 × 10^7 cells).
3. Replace medium with 4 mL/flask prewarmed PBS, containing CPP–cargo at the concentration that was determined to yield the best protein uptake results.
4. Incubate cells with CPP–cargo under optimized conditions (see Note 20).
5. Remove and collect CPP–cargo solution and wash cells twice with prewarmed PBS. Add fresh complete DMEM and allow cells to recover for 1 h at 37 °C in a CO_2 incubator.
6. In order to transfer the manipulated cells to the NMR tube, they first have to be detached from the cell culture flask. Wash once with prewarmed PBS to remove FBS.
7. Incubate with 4 mL (minimal surface volume) 0.25 % Trypsin/EDTA in prewarmed PBS for 3 min (see Note 21).
8. Detach cells by tapping the side of the cell culture flask.
9. Add 20 mL (5× volume) prewarmed complete DMEM to inactivate Trypsin.
10. Transfer the cell suspension to a 50 mL centrifugation tube. Remove a 20 µL aliquot and sediment by low-speed centrifugation (~400 × *g*).
11. Resuspend the pellet in 20 µL PBS and add 20 µl 0.4 % Trypan Blue in PBS.
12. Mix cells carefully and remove 10 µL for a Trypan Blue cell viability test using a hemocytometer (see Note 22).
13. Sediment the remaining ~24 mL of the original cell suspension (step 10) by low-speed centrifugation (~400 × *g*).
14. Wash cell slurry twice in the final in-cell NMR buffer (see Note 23)
15. Resuspend cells in 500 µL (or 300 µL, depending on the size of the NMR tube) in-cell NMR buffer (see Note 24).
16. Transfer cells to the NMR tube and proceed to in-cell NMR experiments.
17. *Optional* Test for protein leakage and cell viability before and after in-cell NMR measurements as outlined in Chapter 6.

4. Notes

1. On average, 1–3 × 10^7 cells are needed for a single in-cell NMR sample. This number depends on the kind of cells that are used and on their individual cell volumes, i.e., ~2 pL for human HeLa cells (15).

2. The final volume of the in-cell NMR sample is determined by the kind of NMR tube that is to be used. On narrow-bore NMR probes, standard 5 mm (~500 μL), or Shigemi™ NMR tubes (~300 μL) may be employed. Their difference in volume will require different numbers of cells.
3. The effective NMR concentration (C_{NMR}) of the final in-cell NMR sample will be determined by the intracellular concentration of the "delivered" protein (C_{Cell}), the number of cells in the NMR sample (N_{Cell}), their individual cell volume (V_{Cell}), the final NMR sample volume (V_{NMR}) and the corresponding volume dilution factor, VDF ($V_{NMR}/V_{Cell} \times N_{Cell}$). According to $C_{NMR} \times V_{NMR} = C_{Cell} \times (V_{Cell} \times N_{Cell})$ the effective NMR concentration can be calculated as $C_{NMR} = C_{Cell}/\text{VDF}$. For example, in order to obtain a spectrum with an effective NMR concentration (C_{NMR}) of 10 μM of isotope labeled protein in 300 μL of NMR sample volume (V_{NMR}), for 1×10^7 cells (N_{Cell}) with an average cell volume (V_{Cell}) of 2 pL (i.e., 2×10^{-6} μL) a total intracellular protein concentration (C_{Cell}) of 150 μM of isotope-labeled IDP must be reached (VDF in this case is 15). It is therefore suggested to first determine the minimum protein concentration that is required for suitable in vitro NMR results (i.e., the lower limit of C_{NMR}). This will define the benchmark intracellular protein concentration (C_{Cell}) that has to be reached in a defined number of cells (N_{Cell}) of volume (V_{Cell}), for satisfactory in-cell NMR results.
4. The indicated amount of CPP starting material is based on average yields of CPP-coupled cargos that can be obtained with the outlined protocol. Individual coupling efficiencies, losses during CPP–cargo purification steps and overall yields may therefore vary for different CPP–cargo combinations. Additional chemical entities like a fluorescence label for in-cell detection by microscopy methods, or a biotin-tag for affinity pull-down experiments, may be incorporated at this point.
5. The required amount of isotope-labeled cargo protein is based on average coupling yields (>60 %). Efficient reduction of cysteine residues is achieved by running the cargo through a Tris(2-carboxyethyl)phosphine (TCEP) column prior to the coupling reaction. No other reducing agents must be present at this point.
6. Some CPPs may not be sufficiently soluble in aqueous solutions to achieve the suggested concentration of the stock solution. In such instances, test different pH ranges for solubilizing and coupling.
7. A molar excess of CPP generally ensures that the desired species, i.e., CPP–cargo is preferably formed. Ratios from 1:1 to 4:1 (CPP–cargo) are good starting points. One drawback of

using large excess of CPP is the generation of CPP–CPP disulfide dimers that constitute unwanted side-products and have to be removed in turn (see below).

8. The following parameters have been shown to greatly influence coupling efficiencies: incubation time, i.e., minutes to hours, incubation temperature, i.e., 4–37°C, and CPP to cargo molar ratio. These parameters should be optimized in parallel for best coupling results.
9. To determine overall coupling efficiencies, small aliquots of the coupling reaction are removed at different time-points and run on a SDS-PAGE that is then stained with Coomassie Blue. Successful coupling is assessed by the appearance of the desired CPP–cargo protein band. Whenever the CPP contains a fluorescence dye, coupling efficiency can also be determined by UV illumination. In most instances, two protein populations will be present: High molecular weight (MW) components, i.e., CPP–cargo, cargo–cargo, and uncoupled cargo and low MW species, i.e., free CPP and CPP–CPP dimers.
10. To initially separate high-, from low-MW components (see above) a gel filtration chromatography (GF) step is employed. Some FLPC systems allow for detection of the emission wavelength of the CPP fluorophore, which may assist in the identification of the CPP–cargo product. After the GF run, the high MW fractions containing the desired CPP–cargo, as well as free cargo molecules are pooled. Fractions containing the low MW species, i.e., CPP and CPP–CPP dimers can be discarded.
11. In order to purify the CPP–cargo from the pool of nonreacted cargo molecules an additional chromatography step is employed. The positive charge of the CPP usually alters the p*I* of the CPP–cargo product in a manner that is sufficient to separate it from free cargo molecules by ion exchange chromatography. The optimal loading pH and slope of the salt gradient to elute the desired species must be experimentally determined for best separation results. If the final eluate contains a salt concentration above 150 mM, dialysis of the CPP–cargo solution is suggested.
12. When measuring final CPP–cargo concentrations, beware that the presence of a fluorescence label on the CPP will necessitate a correction factor for accurately determining the precise amount of product by UV/VIS spectrophotometry.
13. Seeding density will vary depending on the cell line used. The number given here is based on the average cell size of human HeLa cells. It is generally recommended to employ cells with a low passage number.
14. HeLa cells have an average doubling time of 24 h. Therefore, this incubation time will vary depending on the cell line used.

15. Incubation of cells with pyrenbutyrate (PA) has been shown to enhance CPP–cargo uptake (16). It is recommended to perform initial PA toxicity tests to determine suitable PA concentrations and incubation times for a given cell line. The initial PBS wash is required to remove FBS, as it will diminish PA activity. If PA is applied, a cell recovery step (30–60 min) in DMEM (serum-free) is suggested (1). The CPP–cargo incubation solution that is then added must also contain PA.
16. Cellular CPP–cargo uptake will vary depending the CPP–cargo concentrations and chosen incubation times. It is therefore advisable to optimize these parameters accordingly. In our experience, incubation with 300 μM CPP–cargo results in 10–20 % uptake efficiency. Thus, a single incubation step yields an intracellular CPP–cargo concentration of 30–60 μM and a resulting effective NMR concentration (C_{NMR}) of 4–8 μM, in a 300 μL in-cell NMR sample volume (V_{NMR}) with 1×10^7 HeLa cells (N_{Cell}).
17. Inomata et al. have reported four rounds of CPP–cargo incubations for their mammalian in-cell NMR samples (1). They achieved an effective NMR concentration (C_{NMR}) of 30 μM in 200μL of NMR sample volume (V_{NMR}). With a total number of 1×10^7 cells (N_{Cell}) this equates to an intracellular protein concentration (C_{Cell}) of approximately 150 μM, according to the equation outlined above. Assuming equal amounts of intracellular protein deposition for every round of incubation, this relates to a protein uptake efficiency of 15 % in each incubation step.
18. CPP–cargo solutions can be recycled for additional incubation steps. As only 10–20 % of CPP–cargo is taken up per round of incubation, substantial amounts of noninternalized CPP–cargo remain present in the supernatant. This portion of CPP–cargo can easily be repurified by conventional chromatography methods and reused at later points in time.
19. To prepare cell lysates for Western-blotting, add RIPA buffer directly to the cells and detach them from the culture dish. Separate proteins by SDS-PAGE and perform Western-blotting according to standard protocols (17) with antibodies against the cargo protein. Determine cellular protein uptake by comparing cargo signal intensities to a series of known cargo concentrations run on the same gel. Quantification is afforded by commercial imaging readout software packages. Additional methods for the quantification of CPP–cargo uptake are outlined in Chapter 6.
20. At this point, optimal conditions for efficient cellular CPP–cargo uptake should be known. If PA treatment, and multiple rounds of CPP–cargo incubations, were advantageous for cellular protein uptake in small-scale experiments, they should also be used here.

21. Trypsin incubation times may vary for different cell lines. Cell morphology/viability should be assessed at this point by bright field microscopy. Another beneficial aspect of the Trypsin treatment is the proteolytic degradation of membrane-attached, but not internalized CPP–cargo molecules that might compromise the in-cell NMR readout.
22. This cell count assay is used to determine overall cell viability after CPP–cargo delivery (18). On average, more than 80 % of the cells should score as viable (i.e., nonblue) in the Trypan Blue assay.
23. Inomata et al. use 5 mM HEPES (pH 7.2) with 90 mM D-glucose (supplemented with 5 % D_2O) as the final in-cell NMR buffer (1). In our hands, serum-free DMEM (10 % D_2O) works equally well.
24. Alternatively, cells can be embedded in a solid support matrix to avoid sedimentation during the in-cell NMR experiment. This often yields better overall in-cell NMR results. We sometimes employ low-melting agarose as a biologically and spectroscopically inert embedding material. The low-speed centrifugation cell pellet obtained in step 13 is resuspended in half of the final in-cell NMR sample buffer (i.e., 250 µL or 150 µL, depending on the size of the NMR tube). An equal volume of a 0.4 % low-melting agarose in PBS (20 % D_2O) solution (37 °C) is added. After careful mixing of the two suspensions, the sample is transferred to the NMR tube and solidified by briefly placing it at 4 °C. The resulting 0.2 % agarose mixture is sufficient to prevent sedimentaion of 1×10^7 HeLa cells for up to 24 h.

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