Chapter 6

In-Cell NMR in Mammalian Cells: Part 3

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Abstract

Irrespective of how isotope-labeled proteins are delivered into mammalian cells, laboratory routines are needed to assess the quality of the resulting in-cell NMR samples. These include methods to evaluate overall cell viability, protein transduction efficiency, intracellular protein concentration, localization, and stability. In addition, quality control experiments to assess protein leakage from manipulated cells are of particular importance for in-cell NMR experiments. The purpose of this chapter is to outline qualitative and quantitative methods to determine general biological properties of in-cell NMR samples in order to ensure the highest possible standards for in-cell NMR studies.

Key words: Trypan blue, Flow cytometry (FCM), Fluorescence live cell imaging, Immunofluorescence microscopy, Western blotting, Quantitative mass spectrometry, Quality control

1. Introduction

In the previous chapters we have outlined a collection of methods to deliver isotope-labeled IDPs into amphibian-, and cultured mammalian cells. While different protocols can be employed to prepare in-cell NMR samples, generic quality control experiments to verify the overall fitness of the obtained in-cell NMR specimens are of general importance. Questions concerning cell viability, protein delivery efficiency, as well as intracellular protein localization and stability need to be addressed in order to critically assess the biological relevance of in-cell NMR results. In the following, we will outline a compendium of stringent quality control experiments that should additionally be performed on every in-cell NMR sample. Most of the suggested experiments follow standard laboratory routines in cell biology. Therefore, we will only focus on those aspects of the protocols that are of particular importance for in-cell NMR experiments.

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First and foremost, methods to determine overall cell viability at any stage of the delivery routines-, and without having to sacrifice the sample under investigation-, are especially useful. Here, conventional phase-contrast light microscopy offers many advantages. Cytopathological changes in cell morphology, such as swelling, blebbing, or fraying of cell membranes, are readily observed and provide clear indications of imminent cell death (1). Thus, counting the fraction of morphologically intact, or compromised cells under a light microscope provides a simple means to quickly assess overall cell viability. More than 80 % of cells should score as "viable" at any point of the individual manipulation schemes, as well as before and after the in-cell NMR experiment. More specialized assays such as the Trypan blue cell staining protocol (see below) and lactate dehydrogenase (LDH)-based cytotoxicity tests should be employed at specified stages of the delivery protocols (see Chapters 4 and 5). Flow Cytometry (FCM) offers the additional advantage to test cell viability and protein uptake in parallel. Because both mammalian cell delivery protocols outlined in this volume entailed fluorescence-labeling schemes for either the CPP moiety-, or the delivered target protein-, FCM control experiments are suitable means to assess these two important parameters. In addition, the availability of fluorescence labels enables straightforward intracellular protein localization studies by live cell fluorescence microscopy imaging, which is also described in this chapter. This technique can additionally provide invaluable insights into aberrant cellular distributions of exogenously delivered proteins, which are essential for properly interpreting in-cell NMR results. While fluorescence microscopy can verify cellular protein uptake and assess cellular protein localization, it is not suited to determine intracellular protein concentrations. However, quantitative knowledge about cellular levels of delivered proteins is indispensable for evaluating protein uptake efficiencies, for optimizing individual steps in protein delivery protocols, and for ultimately determining in-cell NMR sample concentrations. Therefore, reliable methods for quantitative and semi-quantitative measurements of "delivered" protein concentrations are essential. In this chapter, we describe how specifically tailored Western-blotting routines, quantitative mass spectrometry (MS) and in-cell NMR measurements themselves can be employed to assess of the quality of mammalian in-cell NMR samples.

2. Materials

1. Equipment: Standard gel electrophoresis and Western blotting equipment, Phase contrast microscope, hemocytometer, sterile collagen-coated round 22 mm glass coverslips, 6-well cell culture plate, fluoresence confocal microscope, microscope

chamber for live cell imaging, FCM equipment, sonicator, tandem electro-spray mass spectrometer (MS), NMR spectrometer.

2. Appropriate cell culture media: For HeLa cells, complete DMEM (low Glucose, 5 mM Glutamine, 10 % Fetal Bovine Serum, (FBS), PAA Laboratories, Canada).
3. Phosphate buffered saline (PBS), cell culture grade, without Calcium/Magnesium (PAA Laboratories, Canada).
4. 0.25% Trypsin-EDTA solution in PBS (PAA Laboratories, Canada).
5. Standard SDS-PAGE solutions and buffers, Western blot incubation buffers and loading controls.
6. Denaturing lysis buffer, i.e., RIPA: Tris 50 mM, NaCl 150 mM, SDS 0.1 %, Na-Deoxycholate 0.5 %, Triton X 100 or NP40 1 %, (Protease inhibitors should be freshly added.).
7. Native phosphate lysis buffer: 20 mM Potassium phosphate, 150 mM NaCl, pH 7.5.
8. Propidium Iodide (PI) staining solution: 10 mg/mL in PBS (Sigma Aldrich, USA).
9. 7-AAD staining solution: 1 mg/mL in PBS (Sigma Aldrich, USA).
10. 0.4 % Trypan blue staining solution (Sigma Aldrich, USA).
11. Primary antibody against IDP to be delivered.
12. Appropriate secondary HRP-conjugated antibody for enhanced chemoluminescence (ECL) detection.
13. Appropriate secondary antibody, fluorescence dye conjugated.

3. Methods

3.1. Trypan Blue Staining

This section outlines a mammalian cell viability assay that can be employed in combination with cell morphology investigations using a conventional bright field light microscope. The advantage of this method is that it can easily be performed at various stages of the in-cell NMR sample preparation routines, whenever manipulated cells are in suspension. Each Trypan blue assay requires ~1×10^4 cells.

1. To suspend adherent cells: Wash cells once with pre-warmed PBS and incubate with minimum surface volume of 0.25 % Trypsin/EDTA in prewarmed PBS until all cells detach from the culture dish/flask (typically 2–4 min). Add 5× volume of prewarmed complete DMEM and collect cell suspension.

2. Remove a 20 μL aliquot, transfer to a 1.5 mL centrifugation tube and sediment cells at ~400 × *g* for 5 min.
3. Discard supernatant and resuspend cell pellet in 20 μL PBS.
4. Add equal volume (20 μL) 0.4 % Trypan blue staining solution and mix thoroughly.
5. Place a cover slip onto a hemocytometer and dispense 10–20 μL of the cell suspension into each counting chamber.
6. Count the number of viable (non-blue) and non viable (blue) cells, within 5 min of staining (over time even viable cells will begin to take up Trypan blue). The cell dilution should be adjusted to minimum cell number of ~200 on the hemocytometer grid.
7. Calculate the proportion of viable cells, adjusting for the 2× dilution factor of the Trypan blue solution in your sample.

3.2. Flow Cytometry

The Trypan blue staining procedure outlined above provides information about overall cell viability only. For in-cell NMR applications it is especially useful to correlate intracellular protein uptake to changes in cell viability. Certain protein delivery schemes may retain large numbers of healthy cells at the expense of intracellular protein uptake. In turn, high levels of protein delivery may be contrasted with deleterious effects on cell viability. In that sense, mammalian in-cell NMR sample preparation schemes need to strike a balance between levels of exogenous protein uptake-, and correspondingly intracellular protein concentration-, and overall cell viability. One way to simultaneously assess cell viability and protein uptake is flow cytometry (FCM). For FCM applications, the protein to be delivered into mammalian cells has to be labeled with a suitable fluorescent dye. Dual-channel cell sorting is achieved by the different fluorescence properties of the protein-, and cell viability dye.

1. Choose protein cargo labeling dye according to the type of FCM experiment that is to be performed (see Note 1).
2. Couple fluorescence dye to the protein to be delivered into mammalian cells (see Note 2).
3. Purify dye-coupled protein (i.e., remove nonincorporated dye) according to manufacturer's instructions and execute the cellular protein delivery protocol of choice.
4. Perform FCM measurements at desired stages of the delivery schemes and according to standard FCM protocols (2).
5. Interpret FCM results with respect to whether adherent or suspension cells were assayed (see Note 3).

6. Determine whether cells that score positive for exogenous protein uptake contain internalized protein, or whether unspecific membrane binding skews the FCM readout (see Note 4).
7. Check for changes in FCM scores upon delivery of different concentrations of exogenous protein (see Note 5)
8. Optimize individual steps of the chosen delivery protocol accordingly.

3.3. Live-Cell Fluorescence Imaging

The fluorescently labeled versions of target proteins produced for FCM measurements can also be detected inside live cells by microscopy techniques. These methods afford the possibility to qualitatively assess cellular protein uptake, as well as to determine intracellular protein localization. For the outlined protein delivery schemes, both properties are of fundamental importance. Many of the CPP-mediated protein delivery processes for example, involve endocytotic uptake routes (3). Therefore, CPP-cargo proteins are often trapped inside endosomal vesicles and inefficiently "released" into the cytoplasm (4). Similarly, toxin-mediated protein transduction procedures often result in lysosomal protein deposition, as a result of cell toxicity. Both scenarios are detrimental for in-cell NMR analyses and to know about their occurrence is important for optimizing cell delivery protocols and for assessing overall in-cell NMR sample quality.

1. Perform live cell fluorescence microscopy imaging experiments according to standard protocols (5).
2. Correlate experimentally determined intracellular localization properties to known cellular distributions of the delivered protein (see Note 6)
3. Inspect microscopy images for unusual localization properties, in particular punctuated, or speckled intracellular distributions that might indicate organelle co-localization, or membrane attachment (Fig. 1) (see Note 7).
4. Choose alternative fluorescence dyes when in doubt about the possible effects of dye properties on intracellular protein localization behaviors (see Note 8).
5. Consider co-staining with known organelle markers such as 6-diamidino-2-phenylindole (DAPI), Hoechst 43332, or MitoTracker™, LysoTracker™ to determine the possible biological nature of non-physiological localization behaviors (see Note 9).
6. Perform time-course experiments to determine cellular protein stability and turnover rates, as well as time-dependent changes in intracellular localization (see Note 10).
7. Interpret changes in cellular localization properties with regard to expected durations of in-cell NMR experiments (see Note 11).

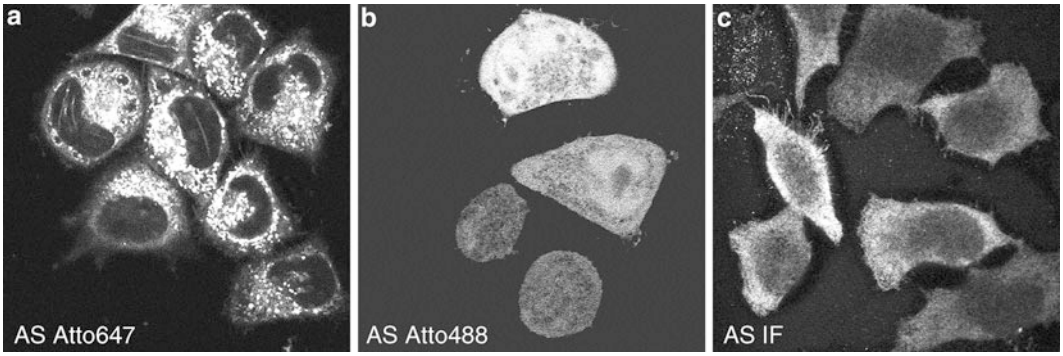

Fig. 1. (**a**) Live-cell fluorescence microscopy image of SLO transduced human α-synuclein (AS) in cultured HeLa cells. The Atto647 fluorescence dye was conjugated to recombinant AS via a lysine side-chain coupling reaction. 200 μM of exogenous AS was employed in the protein delivery protocol. A strongly punctuated, cytoplasmic staining is observed. (**b**) Live-cell fluorescence microscopy image of SLO transduced Atto488 conjugated human AS in cultured HeLa cells. The same lysine side-chain coupling procedure and protein concentration in the "delivery" solution was employed. This time, a uniform cytoplasmic distribution, as well as nuclear staining is detected. (**c**) Fixed-cell immunofluorescence (IF) microscopy analysis of SLO transduced, unmodified AS (200 μM) in HeLa cells. Primary antibodies against human AS and fluorescence labeled secondary antibodies were employed. IF-microscopy indicates a uniform cytoplasmic distribution and no nuclear localization of the protein. All microscopy images were recorded with same apertures and gain settings.

8. Determine intracellular protein localization and overall changes in cell viability upon different sample embedding procedures (see Note 12).

3.4. Fixed-Cell Fluorescence Imaging

Whenever fluorescence labeled proteins are not available, or when conjugating proteins to fluorescence dyes results in non-physiological intracellular protein distributions (see above), cellular protein uptake and localization can also be verified by fixed-cell, immunofluorescence (IF) microscopy using antibodies against the delivered protein. This procedure requires cells to be "fixed," which denotes a process that involves immobilizing the antigen (i.e., the protein of interest) inside the cell and to permeabilize the plasma membrane so that the antibody can enter the cytoplasm and bind to the antigen. Therefore, fixed-cell microscopy employs specimens that are no longer viable. In addition, it has been demonstrated that different fixation protocols can lead to drastic differences in observed intracellular localization properties of CPP-delivered proteins, for example (6, 7).

1. Carefully choose a suitable fixation protocol for the envisaged IF microscopy experiment (see Note 13).
2. Perform fixed-cell IF experiment according to standard protocols (5).
3. Correlate experimentally determined intracellular localization properties to known cellular distribution characteristics of

the delivered protein and to the live-cell imaging results (see Note 14).

4. Determine the effect of different fixation protocols on intracellular protein localization (see Note 15).
5. Check for changes in intracellular protein localization at different intracellular concentrations of delivered protein and during time-course experiments (see Note 16).

3.5. Semi-quantitative Western Blotting

Cellular protein detection by Western blotting provides a simple routine to qualitatively and semi-quantitatively assess intracellular protein concentrations, as well as address potential protein leakage problems (8, 9). Antibodies against targeted proteins need to be available in order to perform such experiments. The main purpose of this section, is to provide a general protocol to accurately determine the amount of successfully delivered protein in mammalian cells. In the following section, we demonstrate how Western-blot signals, obtained with lysates of protein-transduced mammalian cells, can be employed to semi-quantitatively determine intracellular concentrations of successfully delivered proteins.

1. Choose an appropriate lysis buffer for cell extract preparation (see Note 17).
2. Prepare ~2 $\times$ 10^6 protein-transduced cells. If adherent cells are to be analyzed, this number corresponds to two 9.6 cm^2 cell culture dishes, 80 % cell density, cell volume 1–4 pL.
3. Detach cells by Trypsin treatment, sediment by centrifugation (~400 $\times$ *g*) and wash cell pellet twice with PBS.
4. Resuspend cells in 500 µL PBS (1 $\times$ 10^6 cells/mL), and transfer 20 µL to a hemocytometer to count cell numbers (see Note 18).
5. Collect 1 $\times$ 10^6 cells accordingly, sediment, and discard supernatant.
6. Resuspend cell pellet in 100 µL lysis buffer (see Note 19).
7. Lyse cells by brief sonication or repeated freeze–thaw cycles (see Note 20).
8. Centrifuge extract at 16,000 $\times$ *g* for 10–20 min at 4 °C and collect supernatant (i.e., the final cell lysate).
9. Determine the total protein concentration by a Bradford assay, or other appropriate techniques (see Note 21).
10. Load ~4 µL (per slot) of cell lysate (with appropriate sample buffer) onto a SDS-PAGE (~20 µg total protein/lane). Add a reference concentration series of known amounts of input protein (10–300 ng) on the same gel.
11. Run gel electrophoresis, transfer proteins onto an appropriate membrane (i.e., Nitrocellulose, or PVDF). Perform Western-

blotting routines by standard protocols (10) using primary antibodies against the protein of interest and relevant HRP-conjugated secondary antibodies.

12. Quantify Western signal intensities by densitometry, or by commercial software packages when using digital chemoluminescence detection setups.
13. The intracellular protein concentration is determined based on the calibration curve given by the input reference samples. For a detailed outline of the individual calculation steps, refer to the indicated note (see Note 22).
14. Optimize delivery protocols accordingly.

3.6. Quantitative Mass Spectrometry

Given that ^{13}C and ^{15}N labeled proteins possess similar physical and chemical properties as their unlabeled counterparts, stable isotope labeling in combination with mass spectrometry (MS) has been widely used for relative quantifications of proteins (11). Absolute quantification methodologies based on isotope dilution strategies allow the determination of protein concentrations in biological samples (12, 13). Intracellular concentrations of proteins can also be accurately determined by MS using labeled peptides as internal standards (14, 15). In fact, MS turns out to be especially useful for assessing intracellular protein concentrations of in-cell NMR samples, because levels of isotope-labeled proteins can be correlated to known quantities of non-isotope-labeled internal standard proteins. To this end, cell lysates prepared from in-cell NMR samples are "spiked" with defined amounts of unlabeled protein and jointly analyzed by MS after proteolytic digestion with Trypsin (or an alternative enzyme such as chymotrypsin, Lys-C, Asp-N, or others). Within a certain dynamic range, the mass spectrometer can recognize both the labeled and unlabeled forms of tryptic peptides and relative quantification can be achieved by comparing their respective signal intensities. If so, the mean ratio of all quantified peptides accurately reflects the heavy/light ratio of a protein species, which can be used to calculate in-cell NMR sample concentrations.

1. For a given protein to be delivered into mammalian cells, defined mixtures of unlabeled (UL) and stable-isotope labeled (SIL) forms of the protein are prepared first. Concentration ratios may range from 0.001 to 100 (UL/SIL). Absolute concentrations are less critical since MS is highly sensitive and detection limits are not usually a problem. 25–100 ng is a good starting point (see Note 23).
2. Separate mixtures by SDS-PAGE, Coomassie stain and excise the corresponding protein band (see Note 24).
3. Digest proteins in-gel with appropriate enzyme(s) (see Note 25).

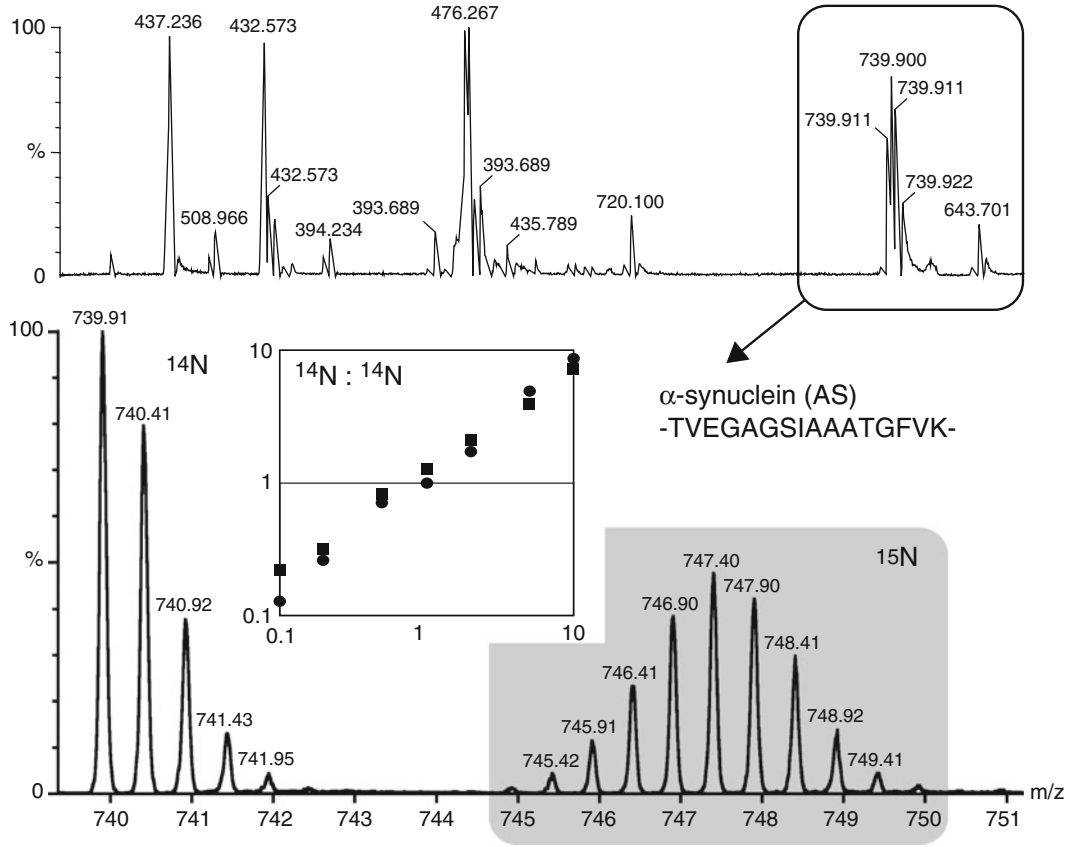

Fig. 2. Quantification of stable-isotope labeled protein concentrations by quantitative mass spectrometry (MS). NanoLC tandem MS run of an in-gel Trypsin digested mixture (equimolar) of ^{15}N-labeled and non-labeled (^{14}N) α-synuclein (AS) (*upper panel*). Several tryptic AS peptides and their corresponding masses are detected. The *lower panel* depicts the MS/MS fragmentation run and isotope-resolved distribution pattern of the 739.9 Da peptide species (corresponding to aa 81–96 of human AS). Clear separation of stable isotope-labeled (^{15}N) and non-isotope-labeled (^{14}N) signals enables accurate concentration measurements over a dynamic range spanning two orders of magnitude (*inset*).

4. Analyze peptide mixtures by nanoLC tandem MS (preferably nanoLC coupled with an ESI-QTOF or ESI-LTQ-Orbitrap instrument) and select suitable proteolytic (tryptic) peptides to confirm peptide sequences by MS/MS fragmentation(s) (Fig. 2) (see Note 26).
5. Analyze MS spectra of selected peptides (see Note 27).
6. Integrate MS signals from unlabeled and the corresponding labeled species (see Note 28).
7. Inspect the quality of the fit of experimentally obtained-, versus calculated concentration ratios. Choose a suitable dynamic range for all subsequent steps. In most instances, ratios between 0.1 and 10 (UL/SIL) yield good correlations and produce the most accurate results (Fig. 2).

8. For in-cell NMR sample analysis, prepare a cell lysate from a defined number of stable-isotope labeled, protein transduced cells.
9. Measure the total protein concentration of the cell lysate and add defined amounts (~25–100 ng) of unlabeled target protein. An estimate for the intracellular protein concentration of the labeled component may already be available at this point (from Western-blot analyses, for example). This will help to determine the most suitable concentration of unlabeled protein to be added for optimal correlation results (however, this is not strictly required) (see Note 29).
10. Separate protein lysate by SDS-PAGE, Coomassie stain and excise gel band at the expected molecular weight of the delivered protein (see Note 30).
11. Digest proteins in-gel with appropriate enzyme(s), extract isotope-labeled and non-isotope-labeled peptide fragments, and analyze peptide mixture by nanoLC tandem MS as indicated above (steps 3–5).
12. Integrate MS signals and calculate heavy/light ratios as indicated above. Determine lysate concentration of isotope-labeled protein using the mean value of all quantified peptides. Refer to Subheading 3.5 for calculating the effective intracellular protein concentration of the delivered substrate based on the number of cells initially used to prepare the lysate.

3.7. In-Cell NMR Spectroscopy

In the previous Subheadings 3.5 and 3.6 we outlined methods to experimentally determine solid estimates for intracellular protein concentrations (C_{Cell}) of successfully delivered substrates in mammalian in-cell NMR samples. By using the equation provided in Chapters 4 and 5, Subheading 3.1 Note 3, we can now calculate the effective NMR concentrations (C_{NMR}) that the different in-cell NMR samples should have. In order to assess whether expected-, and experimentally observed in-cell NMR signal intensities match, simple 1D in-cell NMR experiments provide a good starting point (see below). Why are those correlations important? Above all, because expected and experimentally determined in-cell NMR signal intensities will only match when most of the delivered protein is "tumbling freely" in the cytoplasm of the targeted cells. Protein interactions with cellular components like membranes, or other large macromolecular biomolecules (such as cytoskeletal proteins or DNA, for example) will result in severe NMR line broadening (16) so that NMR signal intensities will be greatly diminished. In-cell NMR measurements themselves can thereby identify possible biological interactions of intracellular proteins and provide important clues towards the qualitative nature of the environment that the delivered protein experiences.

1. Based on the methods outlined above, determine the intracellular protein concentration (C_{Cell}) of the in-cell NMR sample and correspondingly the expected, effective NMR concentration (C_{NMR}).
2. Prepare a "mock" in vitro in-cell NMR sample to which the isotope-labeled protein sample is directly added at the effective NMR concentration (C_{NMR} see Note 31).
3. Run 1D hetero-nuclear NMR experiments with settings that will also be applicable and used for the true in-cell NMR specimen (see Note 32).
4. Determine experimentally obtained NMR signal intensities as reference for the in-cell NMR sample.
5. Run mammalian in-cell NMR sample with identical settings as previously employed for the reference sample.
6. Compare NMR signal intensities and interpret accordingly (see Note 33).
7. Proceed to multi-dimensional NMR experiments when results are satisfying.
8. Consider re-running intermittent 1D NMR quality tests to evaluate changes in NMR signal intensities over time and interpret accordingly (see Note 34).

4. Notes

1. The choice of fluorescence dye is determined by the desired cell viability assay that the FCM experiment is supposed to report. Because dual-channel FCM measurements will be employed, the spectroscopic properties of the two different fluorescence dyes (i.e., absorbance and emission wavelengths) must be sufficiently set apart in order to enable their simultaneous readouts. Standard cell viability dyes for FCM analyses include 7-AAD, or propidium iodide (PI) (17).
2. Different fluorescence dyes require different reactive groups for coupling (i.e., lysine or cysteine side-chains, for example). Therefore, check for the availability of these residues in the protein of interest. Note that certain residues might be important for protein function and/or localization. Nuclear localization sequences (NLS), for example, are rich in lysines and arginines. To modify their chemical properties will impair nuclear import of the target protein and should therefore be avoided. Note that certain buffers are not compatible with these coupling reactions, for example Tris (TBS), glycine and ammonium salt buffers contain free amide groups, that greatly affect coupling efficiencies.

3. For protein delivery into adherent mammalian cells, keep in mind that non-viable cells typically detach from cell culture dishes/flasks and are conveniently removed by the individual wash steps of the respective protocols. For suspension cells, this is not the case. Therefore, adherent cell viability counts are usually larger than for suspension cells, although this does not necessarily reflect the true situation of the manipulated system.
4. An important point to consider is that fluorescence protein signals detected by FCM measurements may originate from molecules inside the manipulated cells, as well as from molecules stuck to the extracellular cell membrane, thereby scoring as protein uptake positive cells without containing properly internalized protein. This problem may become particularly evident for cellular protein transduction schemes that involve membrane interactions (i.e., CPP-mediated protein delivery). A way to test whether FCM results are skewed by unspecific protein/membrane binding events is to employ a Trypsin treatment step. Trypsin proteolytically degrades cell-surface-bound proteins, but does not affect successfully delivered, intracellular proteins (18). A direct comparison of Trypsin-treated versus non-reacted cells provides an accurate measure for intracellular protein uptake, and the degree of unspecific membrane binding. (This step is more important for suspension cell lines, as adherent cells will be treated with Trypsin to detach them from the culture dishes prior to FCM measurements.)
5. Increasing the intracellular concentration of a delivered protein eventually leads to toxicity problems. As the range of intracellular protein concentrations for in-cell NMR samples is typically high (up to hundreds of μM) different proteins may lead to cytotoxicity problems at different intracellular protein concentrations. It is therefore necessary to carefully assess maximally tolerable protein levels for every protein that is delivered and for every cell line that is targeted.
6. Most proteins to be studied by in-cell NMR spectroscopy have been characterized by other biological methods, including cell microscopy. To assess the physiological relevance of cellular distribution patterns of exogenously delivered proteins, it is important to know beforehand where the protein of interest localizes to in its native setting (also in respect to the cell type to be targeted). Note that for CPP-mediated protein delivery protocols for example, the fluorescence dye is part of the CPP moiety and not of the cargo protein. Intracellular release of the CPP from the cargo, by nature of the reducing environment of the mammalian cytoplasm and reduction of the disulfide bond in between the CPP and the protein, means that the observed fluorescence signal most likely originates from free cytosolic CPP. The distribution of the fluorescence signal and its cellular

lifetime will therefore reflect properties of the CPP, rather than those of the delivered protein. In order to overcome this problem, the cargo protein may be directly detected by immunofluorescence microscopy, see Subheading 3.4.

7. As stated before, whenever a delivered protein displays intracellular localization characteristics that are not in line with its known physiological properties, it should be a matter of concern. Unusually high intracellular protein concentrations-, as required for in-cell NMR measurements-, may lead to non-physiological protein distributions. A notion that is also well known in transient protein overexpression studies in cell biology. In that sense, irregular protein localization may result as a consequence of intracellular protein levels. In a few instances, aberrant intracellular protein targeting may also be artificially introduced by the chemical nature of the fluorescence dye that is used to visualize the delivered protein. In one such example, we found that α-synuclein (AS), lysine-coupled to Atto488, or to Atto647 displayed vastly different localization properties in human HeLa cells. Depending on which fluorescent dye was used, AS strongly colocalized with lysosomal vesicles, i.e., Atto647 (Fig. 1a), or displayed a uniform distribution in the cytosol and cell nucleus, i.e., Atto488 (Fig. 1b). By contrast, immunofluorescence microscopy detection of the non-modified protein using AS-specific-antibodies and fixed HeLa cells revealed a homogenous cytoplasmic staining with no nuclear localization (Fig. 1c).
8. Whenever experimental evidence suggests that the chemical properties of a fluorescence dye might influence the intracellular localization behavior of a delivered protein, alternative dyes may be chosen for microscopy analyses. It is important to emphasize, however, that intracellular localization properties may be different for non-fluorescence dye coupled proteins. As the actual in-cell NMR experiment will not be performed with dye-coupled-, but rather with non-modified protein, localization studies need to be considered as indicative of possible intracellular localizations only.
9. Several dyes are commercially available to quickly identify cellular structures/organelles by co-staining procedures. This is especially helpful for identifying potential causes for non-physiological protein localizations. In many instances, such analyses will reveal protein co-localization with lysosomes, vesicular organelles that function as cellular “trash-bins” processing “superfluous” biomolecular materials. This may indicate rapid cellular clearance of the delivered protein, which is detrimental for in-cell NMR analyses.

10. Time-dependent evolution of changes in intracellular localization behaviors are of particular importance for in-cell NMR studies as samples are typically analyzed over extended periods of time (up to several hours). Therefore, it is advisable to perform live cell imaging analyses at time intervals that correspond to expected experimental NMR settings. In this regard, it is also suggested to analyze samples under "mock" in-cell NMR conditions, i.e., after incubating the manipulated cells in NMR sample tubes to mimic the effects of limited aeration and nutrient supply.
11. Most cytotoxic effects accumulate over time and consequently lead to increased problems in intracellular protein stability. It is important to assess beforehand the level of in-cell NMR sample deterioration that is expected for an envisaged in-cell NMR experiment.
12. As both mammalian in-cell NMR sample preparation protocols contain optional steps for embedding manipulated cells in high-density support matrices (i.e., low-melting agarose, or Redigrade™) check how these procedures affect intracellular protein localization and cell viability. Manipulated cells can be mock treated with embedding material on microscopy cover slips and similarly analyzed by fluorescence imaging. For this, apply the staining procedure with organelle-specific dye (e.g., DAPI, Hoechst 43332, LysoTracker™), then harvest cells and prepare solid support matrix.
13. Fixation methods commonly utilize a combination of organic solvents and cross-linking reagents. Many different combinations are available which can be applied to best suit the target antigen.
14. Similar to live cell fluorescence imaging experiments outlined above, intracellular protein distribution must be assessed against the known localization properties of the delivered protein. Comparing live-cell and fixed-cell imaging results may help to identify problems in fixation procedures, as well as possible sources for imaging artifacts.
15. As different fixation procedure can lead to different localization properties, it is advisable to test several routines in parallel.
16. Similar to live-cell imaging experiments, localization analyses by IF microscopy should also be performed with samples containing different intracellular concentrations of delivered protein and at different time intervals (see above).
17. There are essentially two options: Manipulated cells can be lysed with either a denaturing (i.e., RIPA) or a non-denaturing buffer. Denaturing buffers will solubilize all proteins, while non-denaturing buffers will only yield the soluble fraction of cytoplasmic proteins (i.e., no membrane-, or

membrane-attached, or vesicular proteins). For determination of the total intracellular protein concentration of a delivered substrate, a denaturing buffer should be employed. If an estimate for the fraction of soluble delivered protein is to be obtained, a non-denaturing buffer may be chosen instead, or in addition. Note that proteins observable by in-cell NMR spectroscopy should largely be contained in the soluble protein fraction, but may not necessarily do so. It is therefore advised to comparatively analyze manipulated cells by preparing extracts with both types of buffer.

18. It is important to precisely determine the number of cells used to prepare the extract. This number will be used to finally calculate the molar concentration of delivered protein substrate per cell. Cell counting is performed in a Neubauer hemocytometer (a cell counting chamber) under a light microscope according to the manufacturer's instructions.
19. Protease/phosphatase inhibitors should be added freshly. Note that detergents such as TX-100, NP40, Tween20 and SDS may not be suitable for lysate protein concentration measurements by Bradford-type assays (see below).
20. When non-denaturing buffer conditions are used, complete cell lysis should be confirmed by light microscopy.
21. The total protein concentration of the cell lysate will be ~5 μg/μL at this point. The linear range for Bradford assays is typically 0.1–1 μg/μL. Dilute an aliquot of the cell lysate accordingly and measure total protein concentration of the resulting extract.
22. If, for example, 40 ng of delivered protein in 4 μL of cell lysate has been determined in this way, the effective lysate concentration is 0.01 μg/μL. Since 1×10^6 cells (~2 pL individual cell volume, 2 μL total cell volume) were initially employed to prepare the extract (in 100 μL) a ~50-fold dilution factor going from cell volume to extract volume has to be taken into account. Doing so, we obtain a total protein concentration of 0.5 μg in 1×10^6 cells (or 0.5 μg/μL). On a single cell basis, this equates to 1 pg of protein/cell (2 pL cell volume). Assuming a protein molecular weight (MW) of 14,500 Da for example, we reach an intracellular protein concentration (C_{Cell}) of 35 μM.
23. These mixtures should contain different ratios of concentrations in order to initially determine the dynamic range over which MS produces accurate quantification results.
24. For a mixture of pure proteins, there should only be one protein band on the gel.
25. Use Trypsin wherever applicable and follow standard laboratory routines for MS-based protein identification and quantification used in proteomic studies (19–21).

26. Multiple proteolytic peptide species should be sequenced and selected for most accurate quantification results.
27. MS spectra display characteristic isotopic patterns of MS signals. These reflect natural abundance isotope distributions (mainly C^{12}/C^{13}), as well as different ionization species. Because recombinantly produced proteins are never 100 % isotope-labeled, this results in an additional mass distribution of the "heavy" peptide species (Fig. 2).
28. For accurate results integrate and sum up all signals of the isotopic pattern of the individual species and calculate heavy/light ratios for each of the selected peptide using at least three proteolytic peptides.
29. This corresponds to the expected range of concentrations of successfully delivered proteins in in-cell NMR sample lysates (with ~20 μg of total protein). Note that the targeted cell line may already contain endogenous amounts of non-isotope-labeled protein. In such instances, first determine the concentration of the endogenous protein by spiking untreated cell extracts with defined amounts of isotope-labeled protein (i.e., the reverse reaction). Analyze endogenous protein levels accordingly and subtract from in-cell NMR sample calculations.
30. Whenever target proteins are posttranslationally modified by cellular enzymes, their SDS-PAGE migration behaviors-, as well as their individual molecular masses-, will change in unpredictable manners and complicate MS analyses. Check for PTM events by appropriate alternative means.
31. It is important to mimic in-cell NMR sample conditions as closely as possible. If, for example, cells are embedded in low-melting agarose or resuspended in Redigrade™, then the corresponding "mock" sample should also contain these components. This will also enable to determine the NMR behavior of leaked protein molecules in the true in-cell NMR sample.
32. Employ settings (especially temperature and pH conditions) that will be used for and experienced by the final in-cell NMR sample. It is essential to avoid differences in chemical exchange behavior in these analyses, as these will affect NMR signal intensities and compromise quantitative comparison.
33. Note that some differences in signal intensities are to be expected. These result from sample and magnetic field inhomogeneities, as well as from differences in intracellular viscosity and macromolecular crowding that are unavoidable in in-cell NMR samples. These differences should, however, be within an expected range of effects and not orders of magnitude off the anticipated results.

34. As sample aging is clearly observed in many in-cell NMR studies, it is advisable to repeat the outlined 1D experiments at different time points of in-cell NMR measurements. An overall decrease in experimentally obtained signal intensities with time will be apparent and conversely reflect changes in overall cell viability parameters. Based on these observations, suitable time frames for in-cell NMR measurements in mammalian cells should be chosen.

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