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Supplementary Information:

A high-field cellular DNP-supported solid-state NMR approach to study proteins with sub-cellular specificity

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This PDF contains the following:

Supplementary Text
Figures S1 to S8
Table S1
SI References
Author contributions

Supplementary Information Text

Extended Materials and Methods

Preparation and characterization of FITC-SNAPol-1

SNAPol-FITC was obtained according to our previous method[1] for synthesis of SNAPol-1 with minor modifications. Dimethylformamide (DMF) was passed through a column of molecular sieves. N-Boc-N'-Fmoc-D-lysine, N,N-diisopropylethyl-amine (DIPEA), trifluoroacetic acid (TFA), piperidine, 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), N,N,N-Triethylamine (Et_3N), fluorescein isothiocyanate isomer I (FITC) were purchased and used without further purification. Compound 1 [2] (Fig. S1) and OX063 [3] was prepared according to the previously reported methods. High-resolution mass spectrometry was carried out employing electrospray ionization methods (ESI) for the end products and LTQ Orbitrap discovery (ESI, Thermofisher scientific) for the reaction intermediates. EPR measurements were carried out on Bruker EMX-plus X-band spectrometer. Analytical HPLC was done on an Agilent 1100 instrument equipped with a G1315B DAD detector and G1311A pump (CH_3CN/CH_3COONH_4 (20 mM), 10%-60%, 0-20 min), and data are shown below. Semipreparative HPLC was carried out on SSI 1500 equipped with a UV/Vis detector and versa-pump (Fig. S1).

Synthesis of compound 2

A solution of N-Boc-N'-Fmoc-D-lysine (45.8 mg, 0.097 mmol) and DIPEA (72 µL, 0.391 mmol) in DMF (2 mL) was dropwise added to a solution of HATU (55.8 mg, 0.146 mmol) in DMF (1 mL). Then, a solution of Compound **1** (50 mg, 0.097mmol) in DMF (1 mL) was added to the reaction mixture. After stirring at room temperature for 12 hours, the reaction mixture was treated with HCl solution (10 mL, 1 M) and extracted with ethyl acetate (3×10 mL). The resulting organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated under vacuo. The crude product was dissolved in petroleum ether and purified by silica gel column chromatography using petroleum ether/ethyl acetate (5:1) as an eluent to give a precursor of Compound **2** (Fig. S1A). Then the precursor was dissolved in DCM (1 mL) and treated with trifluoroacetic acid (TFA, 1 mL). The resulting solution was stirred at ambient temperature for 3 hours. The solvent and TFA were completely removed under vacuum. Compound **2** was obtained as a red oil (19 mg, 50% over two steps) which was used without further purification. HRMS (ESI, m/z): calculated for $C_{36}H_{51}N_4O_6^{\bullet +}$ ([M+H] $^+$) 635.3803; found, 635.3810 (Fig.S1).

Synthesis of FITC-SNAPol-1

To a solution of OX063 (26 mg, 0.019 mmol) and DIPEA (17.7 µL, 0.095 mmol) in DMF (2 mL) was dropwise added a solution of HATU (8.75 mg, 0.023 mmol) in DMF (0.5 mL). Then a solution of Compound 2 (15 mg, 0.023 mmol) in DMF (0.5 mL) was added. After stirring overnight at ambient temperature, the reaction mixture was concentrated in vacuo. The resulting residue was redissolved in phosphate buffer (10 mL, 20 mM, pH 7.4) and then purified by column chromatography on reversed phase C18 using water followed by 0-50% MeOH in water to give the Fmoc-protected trityl-nitroxide conjugate as a green solid. Then, piperidine (0.25 mL) was added to the solution of the conjugate (8 mg, 0.0047 mmol) in DMF (0.75 mL) in order to deprotect the Fmoc group. HPLC was used to monitor the reaction. After the completion of the reaction (~ 2 hours), the reaction mixture was concentrated in vacuo. The resulting residue was redissolved in DMF (1 mL) and then triethylamine (3.3 µL, 0.035 mmol) was added, followed by fluorescein isothiocyanate isomer I (FITC, 2.7 mg, 0.0069 mmol) in DMF (0.5 mL). After stirring at ambient temperature for 1 hour, the reaction mixture was concentrated in vacuo. The resulting residue was redissolved in phosphate buffer (1 mL, 20 mM, pH 7.4) and then purified by reverse-phase semi-preparative HPLC to give a yellow solid SNAPol-FITC (3.5 mg, 12% over three steps). HRMS (ESI, m/z): calculated for $C_{94}H_{114}N_5O_{26}S_{13}^{\bullet\bullet +}$ ([M+H] $^+$) 2145.4155; found, 2145.4041. FITC-SNAPol-1 exhibited a very similar room-temperature EPR spectrum compared to SNAPol30. Note: two diastereomers of FITC-SNAPOl-1 were observed in its HPLC chromatogram due to the P/M propeller

configurations of the trityl part and the chiral lysine linker and were separable using our established method.[4] In the current study, these two diastereomers were not separated and used directly (Fig.S1).

Preparation and Characterization of TAMRA-ubiquitin (TAMRA-Ub)

The Ub(1–76) peptide sequence (25 µmol scale) with a free N terminus and protected side chains, was synthesized using a Syro II synthesizer (Multisyntech GmbH, Witten, Germany) on a preloaded Fmoc amino acid trityl resin (0.2 mmol/g, Rapp Polymere GmbH) by following Fmoc solid-phase peptide synthesis procedures as described before.[5] In brief, Fmoc was removed by treatment with 20% piperidine in NMP thrice for 3, 5 & 5 min, followed by five washing steps of NMP. Amino acids were coupled by applying a fourfold excess in the presence of 4 equivalents PyBOP and 8 equivalents DiPEA in NMP for 45 min, followed by three washing steps with NMP. After completion of all coupling cycles, the resin was washed with Et_2O and dried under vacuum.

Coupling of Tetramethylrhodamine (TMR) at the N-terminus of resin bound Ub(1-76) was carried out by reaction overnight with 1 mL DMF containing TMR (100 µmol), PyBOP (100 µmol) and DiPEA (200 µmol) at room temperature. The resin was washed with NMP, DCM and Et_2O and dried under high vacuum. The peptide was cleaved from the resin and deprotected by treatment with 2 mL trifluoroacetic acid/water/phenol/triisopropylsilane (90/5/2.5/2 vol%) for 3 h followed by precipitation into cold Et_2O/pentane (3/1 vol%). The precipitated peptide was washed twice with Et_2O, dried under vacuum, and lyophilized from water/acetonitrile (70/30 vol%). The crude product was dissolved in a minimal amount of DMSO and diluted into water, resulting in a final DMSO concentration of <5%. The peptide was purified by preparative RP-HPLC. Pure fractions were pooled and lyophilized resulting in 18.7 mg TAMRA-Ub (8%). MS ES+ (amu) calc: 8958, found: 8959 (deconv.) (see Fig. S5).

Production of [^{13}C,^{15}N] Ub

E. coli BL21 Rosetta cells were transformed with a pET (Novagen)) derived Ub-containing vector and placed on agar plate with 0.1 mg/mL ampicillin. After overnight incubation (37°C) a single colony was picked and added to a 50 mL flask of LB medium. The culture was grown in an orbital shaker at 37°C with a rotation rate of 200 rpm. A bacterial pellet was obtained when the culture reached an OD_{600} of 0.6 by centrifugation (4000 xg, 4°C, 5 mins). A 50 mL M9 culture containing unlabeled D-glucose and ammonium chloride was then inoculated at an OD_{600} of 0.1 and grown in an orbital shaker (as above) until an OD_{600} of 0.6. Following pelleting (as above), 1L of M9 minimal medium supplemented with 2g/L ^{13}C D-glucose and 0.5 g/L $^{15}NH_4Cl$ was inoculated at an OD_{600} of 0.1 with Ub expression induced at an OD_{600} of 0.6 by addition of 0.5 mM IPTG. The culture was subsequently grown (as above) for an additional 6 hrs. Following harvesting by centrifugation (4000 xg, 4°C, 15 minutes) the pellet was resuspended in 10 mL of 10 mM pH 7.4 Tris buffer supplemented with 10 mg/L of lysozyme and protease inhibitors. Cells were lysed by 2 rounds of freeze-thaw and sonication (on ice and 13 kHz - in 10 second on/off intervals until no longer viscous). The lysate was resuspended in ammonium acetate pH 5.1 to reach a final concentration of 20 mM and then titrated with 37% acetic acid to pH 4.5. Cell debris were removed by centrifugation (40000 xg, 4°C, 30 mins) followed by loading of the supernatant on to a HiTrap SP HP cation-exchange column in 20 mM ammonium acetate pH 5.1. Elution was achieved by salt gradient (0 to 1 M). Ub fractions were determined by conventional 15% Tris-tricine SDS-PAGE gels, pooled, and run down a Superdex 75 size-exclusion column in 20 mM ammonium acetate, pH 5.1, 150 mM NaCl. Ub-containing fractions were again determined by SDS-PAGE gels, pooled, and buffered exchanged into water by dialysis overnight at 4°C. [^{13}C,^{15}N] Ub was lyophilized with 15% Tris-Tricine gels again used to confirm purity (purity > 99%). Ub's fold, prior to and following lyophilization, was confirmed by SOFAST-HMBC[6] solution-state NMR experiments recorded on a Bruker Avance III spectrometer with a static field strength of 21.1 T (^{1}H - 900 MHz) equipped with a TCI cryoprobe. Experiments were

recorded on 500 μM Ub dissolved in phosphate buffered-saline pH 7.4 (Sigma-Aldrich) and 10% D_2O with 8 scans, 0.6 sec recycle delay, 2048 point in the direct dimension, and 128 points in the indirect dimension.

Preparation of microcrystalline [^{13}C,^{15}N] Ub

[^{13}C,^{15}N] Ub microcrystals were prepared by batch crystallization in 2-methyl-2,4-pentanediol, as described in Ref. [7]. Approximately 20 mg of crystalline material was required to fill a 3.2 mm MAS rotor.

Mammalian cell culture

2D adherent HeLa cells (ATCC CCL-2) were cultured at 5% CO2 atmosphere in Dulbecco's modified Eagle medium (DMEM, 4,5 g/L D-Glucose, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (Gibco, fetal bovine serum, qualified, performance).

Nuclear isolation and enrichment

At 80% confluency, HeLa cells were harvested by trypsinization, pelleted (400 x g, 5 mins, 4 °C), and counted by trypan blue staining; 16 million live cells were then washed thrice with Dulbecco's phosphate buffered saline (PBS) (without calcium or magnesium). After the last centrifugation step, excess PBS was aspirated and 0.1% NP-40 PBS was added to the pellet.[8,9] Using a 1 mL pipette, the pellet was resuspended with 10 strokes. After mild centrifugation (400 x g, 5 mins, 4 °C) the supernatant was carefully removed (cytoplasmic fraction 1). The pellet was again treated with 1 mL of 0.1% NP-40 containing PBS and resuspended with 10 pipette strokes followed by mild centrifugation (400 x g, 5 mins, 4 °C) (cytoplasmic fraction 2). The nuclei-enriched pellet was washed thrice with PBS to remove NP-40, the final pellet was obtained by centrifugation (400 x g, 5 mins, 4 °C). Nuclei were subsequently resuspended in 200 μL of PBS supplemented with Hoechst (1 μg/ml), 0.01 mM $CaCl_2$, and protease-inhibitor. 10 μL was placed on a hemacytometer and counted revealing near quantitative isolation efficiency (>90%). This was done with both Hoechst and Trypan blue exclusion staining.

Electroporation

Electroporation of 1.2 mM [^{13}C,^{15}N] Ub or 1.2 mM N-terminally tagged TAMRA-Ub into HeLa cells utilized the methodology previous described by Selenko *et al.*,[10] and Narasimhan *et al.*[11] HeLa cells were grown to 80% confluency, harvested by trypsinization, resuspended in pre-warmed DMEM- 5% CO_2 equilibrated medium, and counted by trypan blue exclusion staining. For whole-cell samples 8 million cells and for nuclei-enriched samples 16 million cells were electroporated in 4 million cell batches. After 5 hrs of recovery in prewarmed and 5% CO_2 equilibrated medium, the cells were again harvested by trypsinization and washed thrice with PBS (400 x g, 5 mins, 4 °C).

Notably, nuclei account for 8 to 24% of the total cellular volume,[12] therefore utilizing the same effective coil volume as with in cell samples required a larger number of nuclei - which after accounting for nuclei swelling in 'DNP juice' equated to $1.6x10^7$ isoalted cell nuclei.

Whole-cell DNP rotor packing

Electroporated whole-cells were resuspended in 30 μL of modified 'DNP juice' (60% ^2H, ^{12}C enriched-glycerol, 30 mM SNAPol-1/AMUPol, 1 x Hanks buffered saline salts (HBSS), and 40% D_2O) and spun into a sapphire DNP rotor by mild-centrifugation (400 x g, 5 mins, 4 °C). Following removal of excess 'DNP Juice', Teflon spacer insertion, cap placement, and MAS mark placing the sample was flash frozen in liquid nitrogen.

Isolated-nuclei DNP rotor packing

For nuclei-enriched samples, the electroporated whole cell pellet was subject to fractionation as described above. After removal of NP-40 from the nuclear-fraction, by washing thrice with 0.01 mM $CaCl_2$ PBS, the nuclei were re-suspended in 'DNP juice'. Due to decreased nuclei density and swelling, the DNP rotor was packed in increments of 10 μL and with a higher centrifugation speed (1000 x g, 3 mins, 4 °C). After each successive packing step, the supernatant DNP juice was removed by aspiration. After final packing, a Teflon spacer was placed into the rotor, followed by the cap, and markings on the rotor bottom for MAS detection. Finally, the sample was rapidly plunge frozen in liquid-nitrogen. The total time between DNP juice addition and plunge freezing was 10 minutes. The difference in the number of whole-cells and nuclei packed into rotors was to have a constant sample volume and thereby fully utilize the probe's effective coil volume thus maximizing sensitivity. The *in-vitro* $^{13}C,^{15}N$] Ub DNP sample was prepared by dissolving 1 mg of [$^{13}C,^{15}N$] Ub in 30 μL of modified DNP juice – exactly as above. Rotor filling and plunge freezing was conducted exactly as detailed above.

Confocal and brightfield microscopy

General remarks

All confocal imaging was conducted on a Carl Zeiss LSM700, AxioObserver microscope; with excitation wavelengths of 405, 488, and 555 nm and detection wavelengths of 400-483 nm, 493-550 nm, and 560-800 nm respectively; a Plan-Apochromat 63x/1.40 Oil DIC M27 (WD=0.19mm) objective was used. Z-stacks were acquired with a scaling of 0.2 µm with a 16-bit depth and averaging of 4 scans per z-slice. Final analysis of acquired images was conducted with Zen 3.5 (Zen Lite, Carl Zeiss) software.

Ub retention in nuclei

Following electroporation with 1.2 mM TARMA-Ub and recovery, 4 million nuclei were isolated and resuspended in 200 µL of PBS supplemented with 0.01 M $CaCl_2$ and 1 µg/ml Hoechst. This suspension was gently added onto coated coverslips previously rinsed with 1M HCl-treated, water rinsed, and subsequently treated with 0.01% poly-L-lysine. After 15 minutes, at room temperature, the solution was replaced with 200 µL of 3.7% paraformaldehyde (PFA) and 3% sucrose at pH 7.4. After 30 minutes, at room temperature, the coverslip was washed thrice with type-1 ultra-pure water and allowed to dry overnight. The coverslip was subsequently placed onto a slide and fixed with clear nail polish.

SNAPol-1 entry into whole-cells and isolated nuclei

Whole cells: HeLa cells were seeded at 20% confluency onto a 15 µ-Slide 8 well high ibiTreat and gown until 80% confluency. Following aspiration and washing thrice with PBS, cells were treated with 200 µL of DNP Juice (non-isotope enriched and containing 200 µM of FITC-SNAPol-1) for 10 minutes at room temperature. 200 µM of polarising agent was used so as not to saturate the detectors on the microscope. After 15 minutes at room temperature the slide was washed thrice with PBS and treated with 200 µL of 3.7% paraformaldehyde (PFA) and 3% sucrose at pH 7.4. After 30 minutes, at room temperature, the coverslip was washed thrice with type-1 ultra-pure water and allowed to dry overnight.

Nuclei: 400,000 pelleted isolated nuclei were resuspended in 200 µL of PBS supplemented with 0.01 M $CaCl_2$ and 1 µg/ml Hoechst. This suspension was gently pipetted onto a 15 µ-Slide 8 well high ibiTreat. After 15 minutes, at room temperature, nuclei were treated with 200 µL of DNP Juice (same recipe as above just with non-isotope enriched glycerol) containing 200 µM of FITC-SNAPol-1) for 10 minutes at room temperature. The slide was washed thrice with PBS and treated with 200 µL of 3.7% paraformaldehyde (PFA) and 3% sucrose at pH 7.4. After 30 minutes, at room temperature, the coverslip was washed thrice with type-1 ultra-pure water and allowed to dry overnight.

Assaying nuclei-stability at DNP ssNMR conditions

To assess nuclei viability bright field microscopy was utilized. We note that confocal microscopy was not used because nuclei were observed to undergo morphological changes upon rapid dilution from high glycerol concentration. Initially, freshly isolated nuclei suspended in PBS supplemented with 0.01 mM $CaCl_2$ were stained with 1 µg/ml Hoechst dye and counted by utilizing a hemocytometer. After pelleting by mild centrifugation (800 x g, 3 mins, 4 °C) the nuclei were resuspended in 'DNP juice' and again counted by a hemocytometer. Nuclei were again counted after packing into a sapphire rotor, plunge freezing in liquid nitrogen, and thawing to room temperature. The entire process was repeated but with additional step of inserting the rotor into a pre-cooled DNP probe (100 K) and spinning up to 8 kHz for 30 minutes before thawing to room temperature. In all 4 cases the recovery of nuclei was >90% corrected for recovered volume.

Solution-state NMR studies

50 million HeLa cells were electroporated with 1.2 mM ^{13}C, ^{15}N-enriched Ub and allowed to recover for 5 hours in pre-incubated cell culture medium. After being washed thrice with PBS, cells were pelleted by centrifugation (400 x g, 3 mins, 4 oC) and re-suspended in 450 µL of Leibowitz medium (L-15) supplemented with 10% FCS. This mixture was added to a 5 mm Wilmad tube, mixed with 50 µL of D_2O, and expediently centrifuged (400 x g, 1 min). After acquisition of whole cell data, cells were fractionated with NP-40, as described above. The cytoplasmic fraction was obtained as described above – the 2 mL of cytoplasmic fraction were concentrated to 450 µL in a 3 kDA cut-off Amicron and supplemented with 50 µL of D_2O for subsequent measurement. The nuclear pellet was also resuspended in 450 µL of PBS + 50 µL of D_2O and subjected to NMR experiments. The nuclear lysate was obtained by mechanical shearing with a Dounce homogenizer as per the protocol in Ref. [13].

Acquisition of solution-state NMR data was conducted by utilizing a spectrometer with a 21.1 T static magnet field, corresponding to a Larmor frequency of 900 MHz for 1H, equipped with a TCI cryo-probe. SOFAST-HSQC experiments were recorded with 1024 scans and a recycle delay of 0.25 seconds.[6] Spectral width and acquisition parameters were as follows: 1H – 20.035 ppm/ 2048 points/56.8 ms 15N – 26 ppm/256 points/53.9ms. BEST-TROSY experiments were recorded with 480 scans and a recycle delay of 0.30 seconds.[14] Spectral width and acquisition parameters were as follows: 1H – 14.0311 ppm/ 3072 points/121.7 ms 15N – 26.4 ppm/128 points/26.6 ms. Both spectrum types were processed in Topspin 4.1.1 with SSB 2 in both spectra dimensions.

DNP-ssNMR and conventional ssNMR experiments

DNP MAS supported-solid-state measurements were conducted at a static magnetic field strength of 18.8 T (800 MHz - ^{1}H frequency); the spectrometer was further equipped with a 9.7 T gyrotron (527 GHz). Measurements were conducted at cryogenic conditions (100 K), 8 kHz MAS rate, and utilized a 3.2 mm double channel (H-X) DNP MAS probe (Bruker, USA). A permeant sweep coil was used to modulate the static magnetic field to obtain maximal enhancement from doped SNAPol-1 (values matched those found in the literature).[1] Spectra requiring proton-decoupling utilized SPINAL-64[15] at a strength of 91 kHz.

1D ^{13}C CP, enhancement and T_B measurements and error Analysis

^{13}C-enhancements were determined indirectly for all samples by utilizing ^{1}H-^{13}C adiabatic cross polarization (CP) with a 60 µsec contact time, 2 s recycle delay, 1458 points (400 ppm spectral window) and an 8 step-phase cycle. CP spin locking was optimized for each sample individually but hovered around 63 kHz for ^{1}H and 72 for kHz ^{13}C. On/off spectra utilized 2048

scans whereas buildups were acquired with 32 scans per delay. 1D spectra were processed in Topspin 4.1.1 with line broadening (150 Hz) being applied prior to Fourier transformation (FT).[16] T_B was determined indirectly through ^{1}H-^{13}C saturation recovery experiments; resulting curves were fit with up to 3 exponentials in Prism 9.

$$I[t] = I[0]\left(1 - e^{-t/T_1}\right)$$

The enhancement error ($\Delta\varepsilon$) was calculated as described in [17,18]according to the following formula:

$$\boldsymbol{\Delta\varepsilon = \varepsilon(\Delta I_{\mu w,on}/I_{\mu w,on} + \Delta I_{\mu w,off}/I_{\mu w,off})}$$

where I is the intensity and ΔI is the signal to noise in DNP experiments with and without microwaves on.

For Table 1:

$\varepsilon_{on/off}$ = Signal DNP ON/OFF $\varepsilon_{abs} = \varepsilon_{on/off}\chi_{bleach}$ $\Sigma = \varepsilon_{abs}\sqrt{(T_{1,undoped}/T_B)}$ as per convention.[1,19,20]

For T_1 and T_B buildup curves, the error is reported as standard deviation (s.d.) of the signal to noise (S/N). The S/N was determined using the following formula:

$$signal\ to\ noise\ = \frac{maxval}{2 \times noise}$$

$$noise\ = \sqrt{\frac{\Sigma_{i=-n}^{n} y(i)^2 - \frac{1}{n}\left(\Sigma_{i=-n}^{n} y(i)^2 + \frac{\Sigma_{i=-n}^{n} i(y(i) - y(-i))^2}{N^2 - 1}\right)}{N - 1}}$$

where $maxval$ is the highest intensity within the designated region, N is the total number of points within the noise,

$n = \frac{(N - 1)}{2}$, and $y(i)$ in the nth point in the noise region – adapted Bruker Topspin 4.1.1.

Sy.x denotes the standard deviation of the residuals. It is computed as follows:

$$Sy.x\ = \sqrt{\frac{\Sigma(residual)^2}{n - K}}$$

where n-k is the degrees of freedom of the regression – adapted from PRISM.

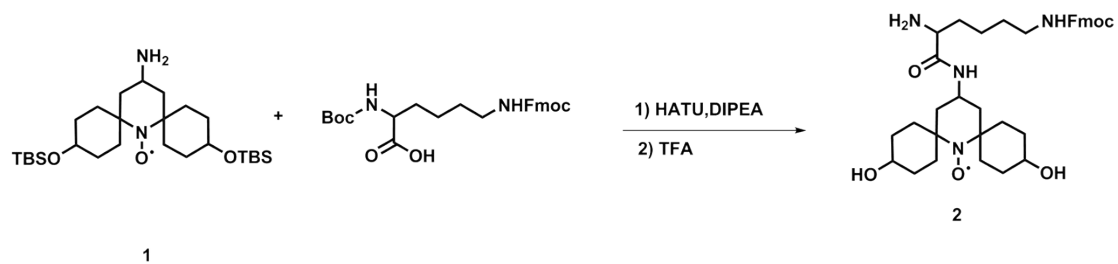
3D 2Q-1Q-1Q ^{13}C-^{13}C-^{13}C Correlation Experiments

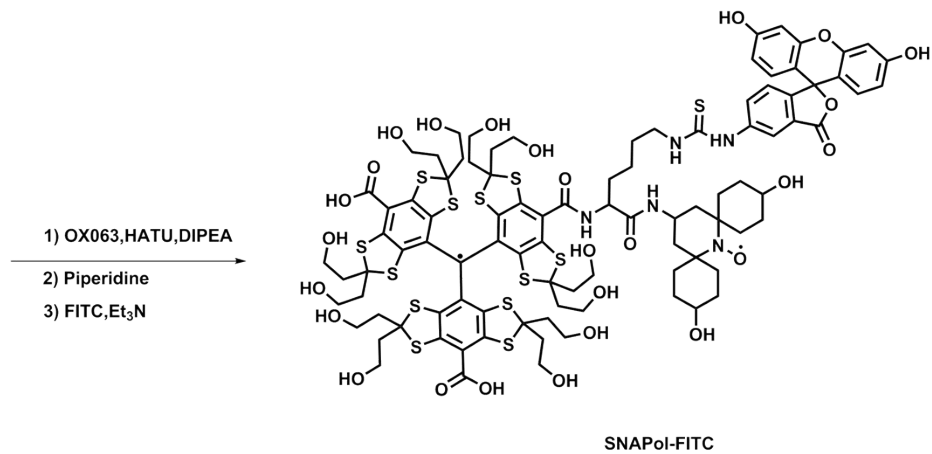
Both cellular and microcrystalline ^{13}C-^{13}C-^{13}C 3D spectra were recorded with double-quantum filtering to suppress DNP juice depleted glycerol signals. A ^{1}H-^{13}C CP contact time of 60 μsec (63 kHz ^{1}H and 72 kHz ^{13}C) was used. Double-quantum excitation and reconversion was achieved by utilizing SPC5[21] blocks (0.5 msec total mixing time at ^{13}C 40 kHz) with continuous ^{1}H decoupling at 94.3 kHz. Spectral width and acquisition parameters were as follows: F3: 354.9 ppm/ 1132 points /7.9 ms F2: 180.02 ppm/70 points/9.6 ms F1: 230 ppm/54 points/5.8ms. PARIS[22] with a 30 ms mixing time. 64 scans were acquired with a 2s recycle delay. Data was processed in Topspin 4.1.1 with qsine SSB 2 for all spectral dimensions. Literature assignments from a Ub residue were considered resolved if 2 or more correlations were observed.

Solid-state NMR Spectral Analysis

Spectra were analyzed using POKY.[23] Assignments were obtained from a combination of ssNMR experiments on microcrystalline ubiquitin (BMRB ID: 7111) and solution-state experiments on the free monomer (BMRB ID: 15410). Solution-state assignments were used for ubiquitin loop regions that were missing in the ssNMR data. Predicted ^{13}C DQ chemical shifts were prepared with FANDAS 2.0.[24]

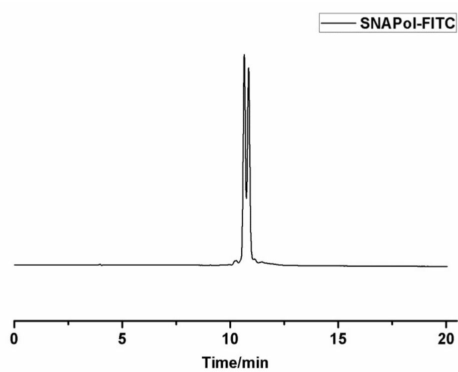
Supplementary Figures

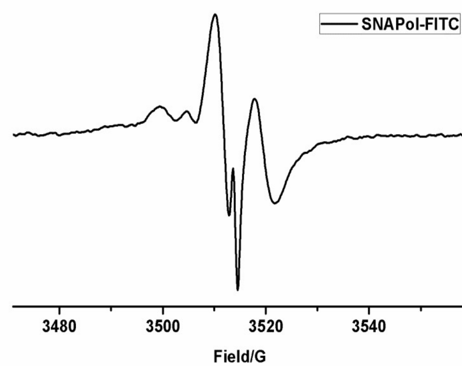




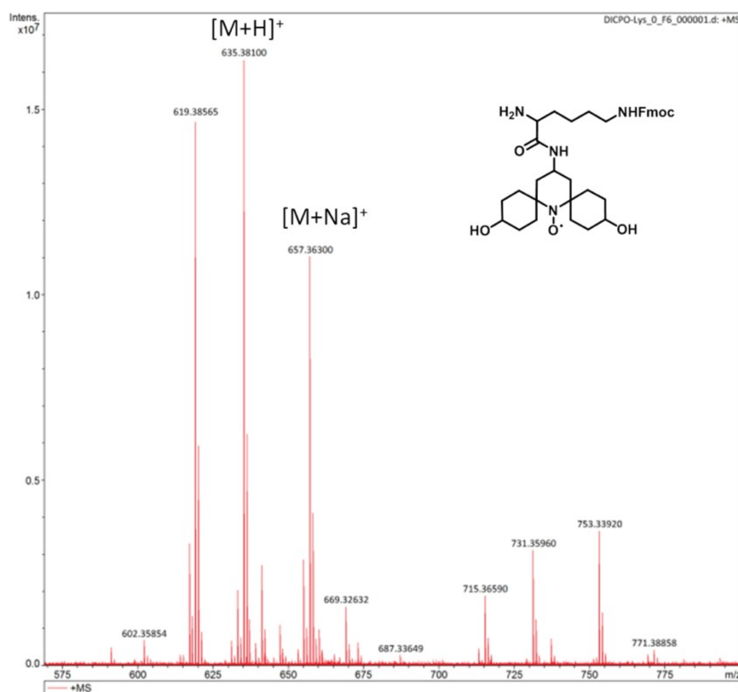
(A)

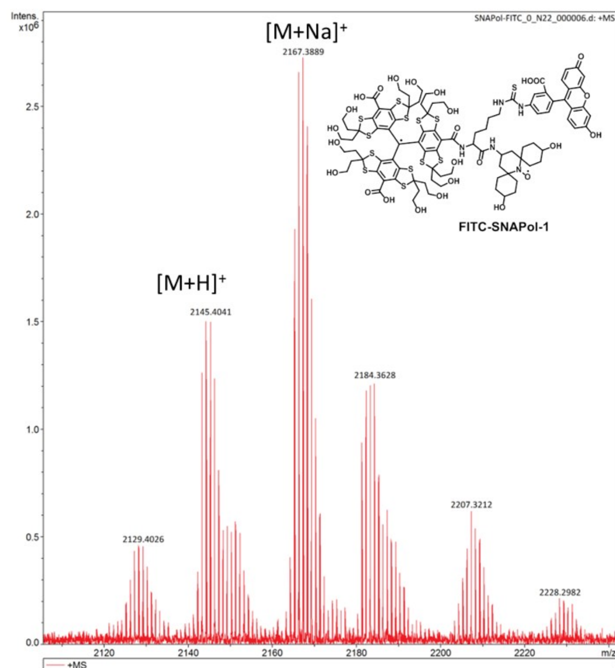
(B)





(C)





(D)

Figure S1: Preparation & Characterization of FITC SNAPol-1. Panel A. Synthetic scheme for preparation of FITC SNAPol-1. Panel B. Left- HPLC trace of FITC SNAPol-1; Right – EPR spectrum of FITC SNAPOl-1. Panel C. HRMS spectrum of Compound 2. Panel D. HRMS spectrum of FITC-SNAPol-1.

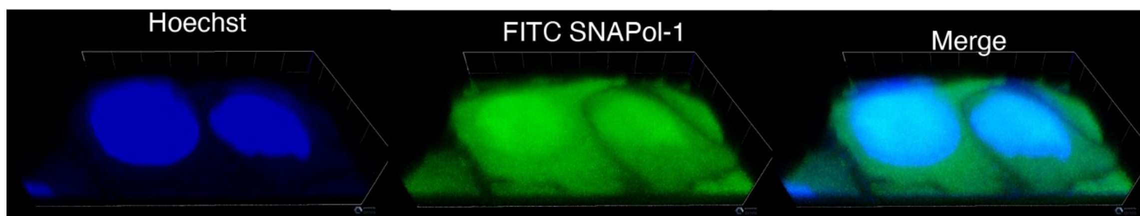


Figure S2: 3D renderings of confocal microscopy conducted on HeLa cells treated with 200 μM FITC-SNAPol-1. The 3D rendering further confirm the homogenous distribution of SNAPol-1 (green) within the cellular lumen (nucleus-blue).

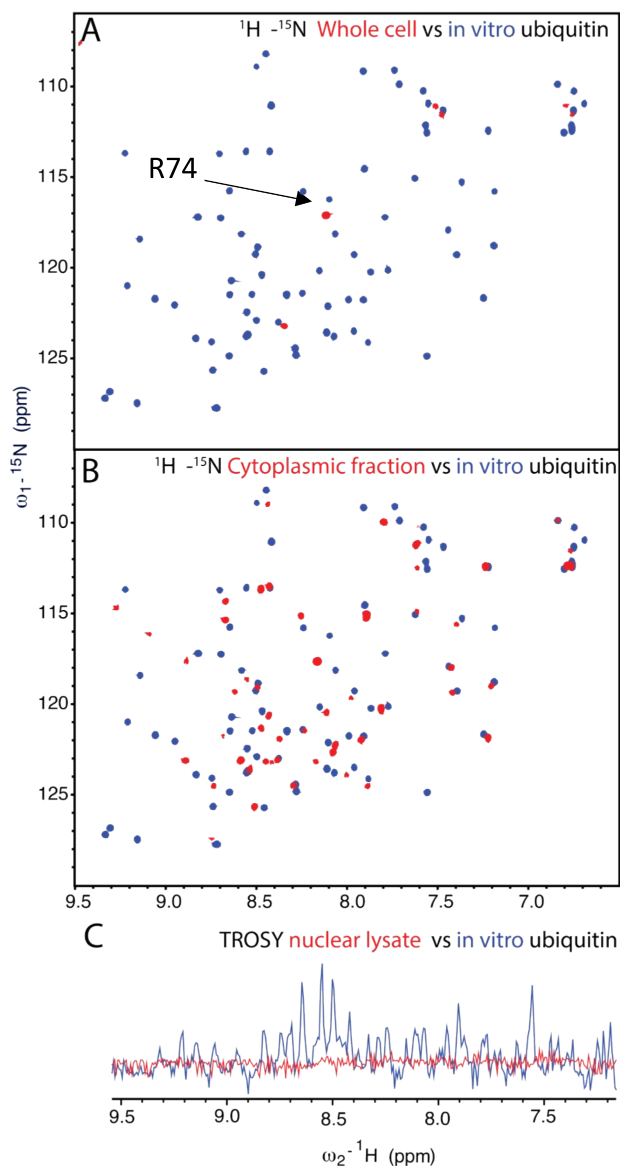


Figure S3: Solution-state NMR of fractionated HeLa cells electroporated with ^{13}C, ^{15}N ubiquitin. Panel A. SOFAST-HSQC of whole cells electroporated with ^{13}C, ^{15}N ubiquitin (red) overlayed with *in vitro* ubiquitin (blue). Only residues located in the highly dynamic C-terminus or sidechains are visible. Panel B. SOFAST-HMBC of the cytoplasmic fraction isolated from whole cells electroporated with ^{13}C, ^{15}N ubiquitin (red) overlayed with *in vitro* ubiquitin (blue). The disruption of the cell

decreases viscosity giving rise to more Ub correlations. Panel C. 1D-BEST-TROSY slices for *in vitro* ubiquitin (blue) and *in situ* cell nucleus isolated from whole cells electroporated with ^{13}C, ^{15}N ubiquitin. With the same measurement times we did not observe solution-state NMR correlations for Ub within isolated nuclei. We attribute spectroscopic changes between NMR spectra of Ub in buffer vs. whole-cell and cytoplasmic lysates to specific and non-specific interactions between different labeled Ub species (incl. covalent and non-covalent interactions) in presence of other cellular components as shown previously.[25]

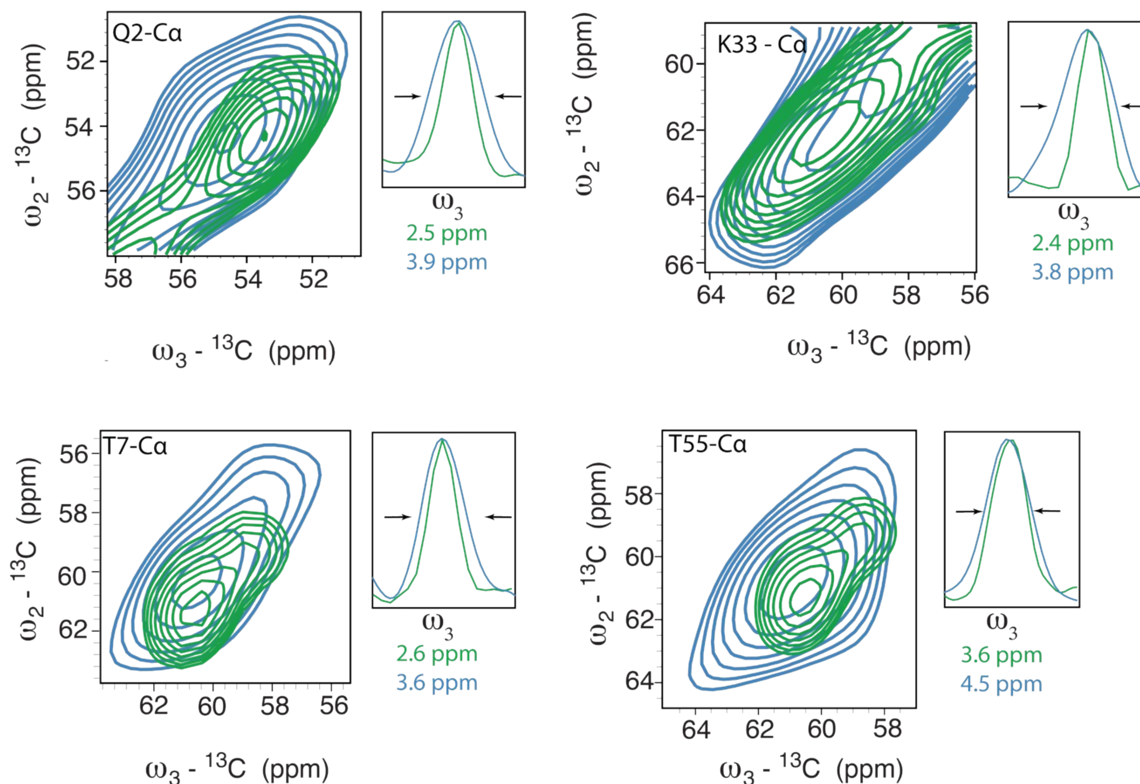


Figure S4: Comparison of microcrystalline with in-cell Ub ssNMR linewidths at 800 MHz. Comparison of in-cell 800 MHz linewidths with those obtained on microcrystalline Ub (also recorded at 800 MHz) as 2D projections and 1D projections.

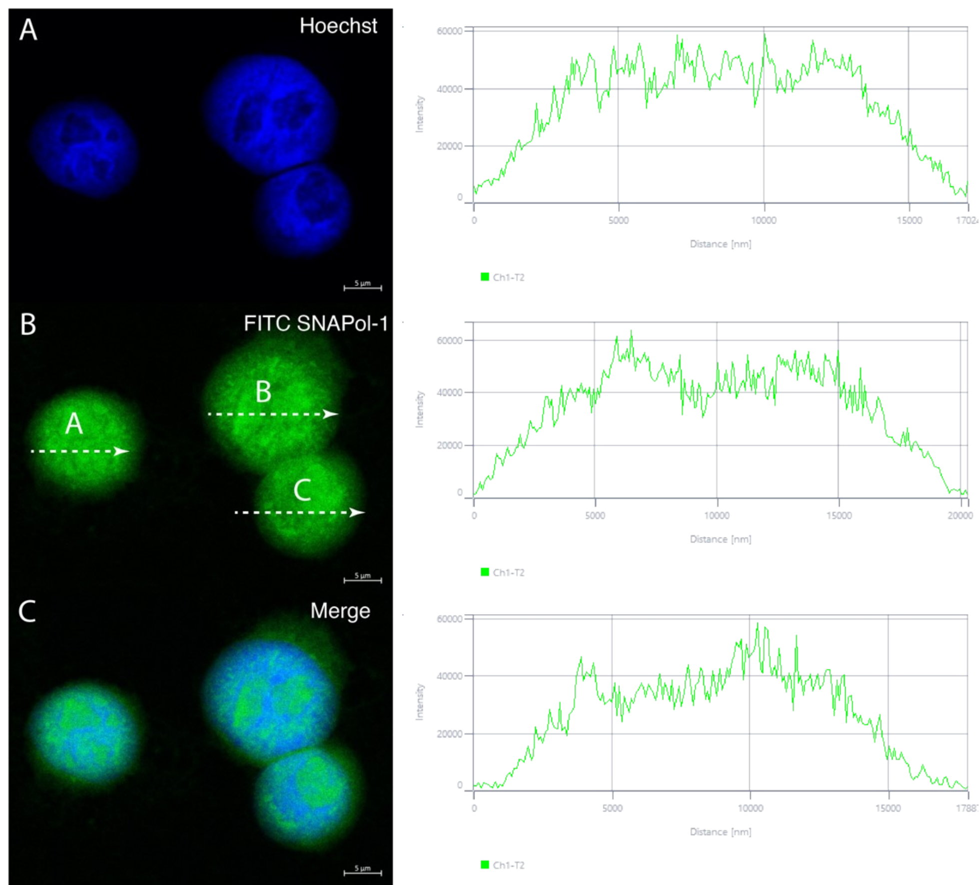


Figure S5: Z-stack confocal microscopy of isolated-nuclei treated with 200 µM FITC-SNAPol-1. Intensity profiles, Hoechst (Panel A), FITC-SNAPol-1 (Panel B), Merge (Panel C) taken as indicated in the FITC-SNAPol-1 (green) Panel B with corresponding intensity plots which show homogenous distribution of SNAPol-1. Depicted z-stacks were chosen to display largest nuclei surface area.

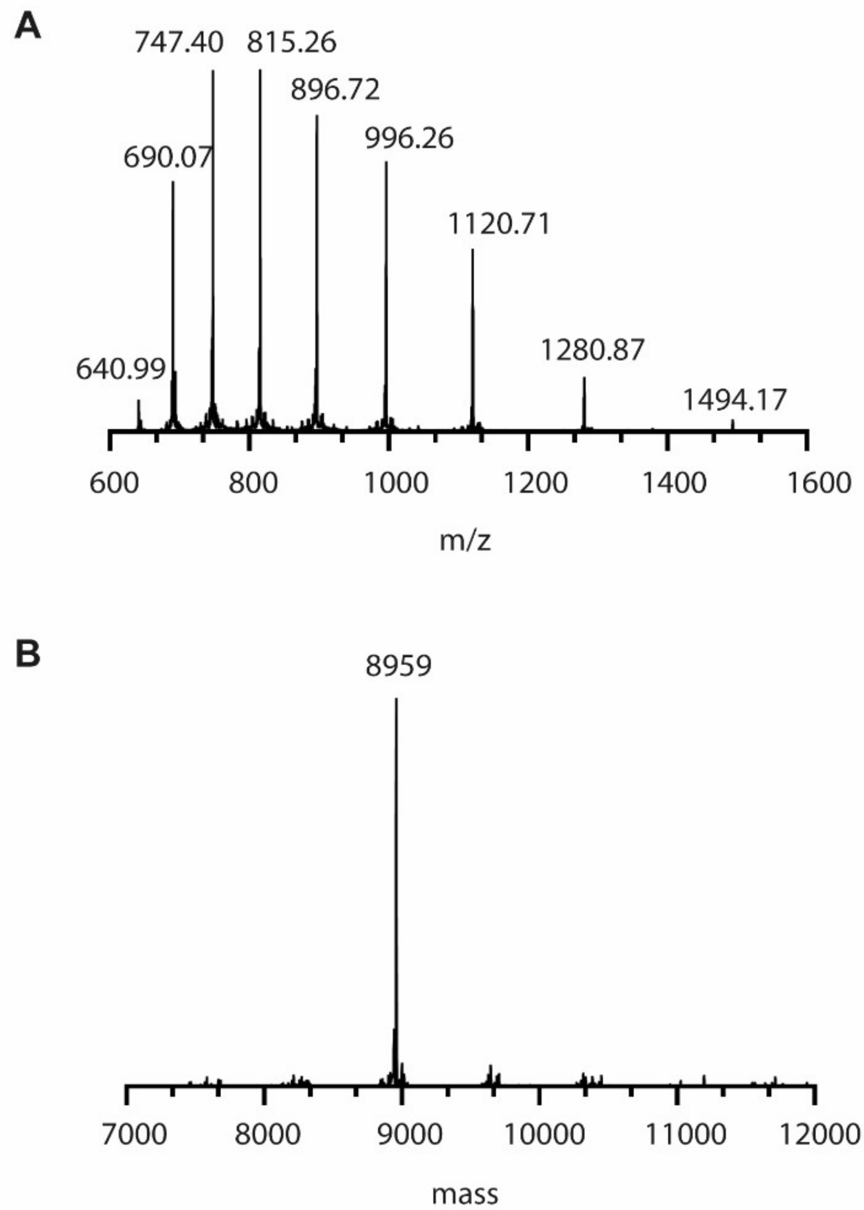


Figure S6: Characterization of TAMRA-Ub. Panel A. HRMS of TAMRA-Ub. Panel. B deconvoluted mass of TAMRA ubiquitin – 8959 m/z.

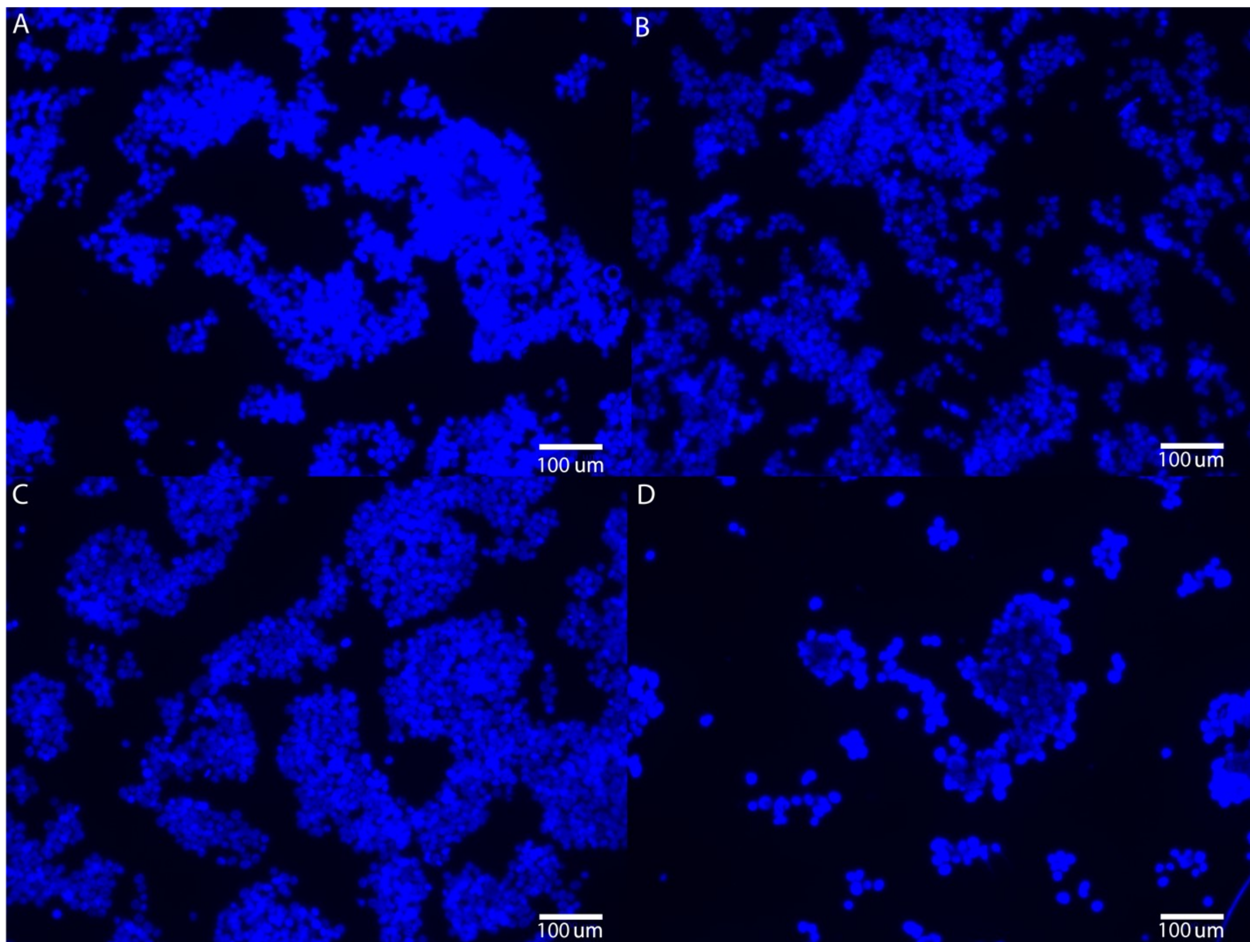


Figure S7: Isolated nuclei stability under DNP-conditions. Panel A. An illustrative example of isolated nuclei suspended in PBS, stained with Hoechst, prior to mild-centrifugation and resuspension in DNP juice - 60% - ^{13}C depleted and ^{2}H-enriched glycerol and 40% D_2O containing 1 x Hanks buffered saline salts (HBSS). Panel B. Hoechst-stained nuclei after suspension in DNP juice prior to plunge freezing in liquid nitrogen. Panel C. Hoechst-stained nuclei after plunge freezing and thawing to room temperature. Panel D. Thawed Hoechst-stained nuclei after plunge freezing, radical treatment, and 8 kHz magic-angle spinning – due to the high viscosity a 4-fold dilution with PBS was used for imaging; based on counts, in all cases, recovery of nuclei was quantitative.

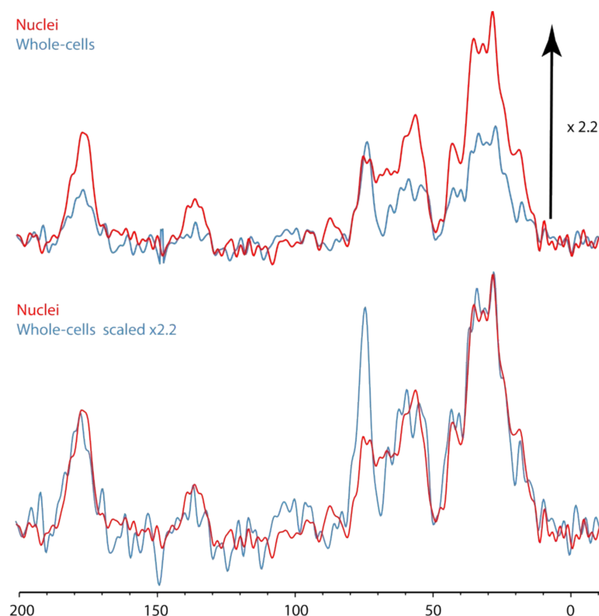


Figure S8: Sensitivity comparison for nuclei and whole cells electroporated with 1.2 mM [^{13}C,^{15}N] Ub. Cumulative 1D projection extracted from 3D DQSQSQ experiments for [^{13}C,^{15}N] Ub in nuclei (red) and whole cells (blue).

Supplementary Tables

Table S1. T_B error analysis for Fig. 3D. Due to a high signal to noise, not all noise signals were visible on the plot.

Time (secs)	Relative Integral	Error +/-
0.001	0.00	0.00
0.05	0.02	0.01
0.1	0.04	0.02
0.5	0.32	0.02
1	0.51	0.04
1.5	0.65	0.06
2	0.74	0.06
2.5	0.81	0.06
5	0.94	0.06
10	0.98	0.04
20	0.99	0.02
30	1.00	0.03
50	1.00	0.03
75	1.00	0.02
100	0.97	0.02

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Author Contributions

D.B., G.E.F., and M.B. designed experiments, analyzed data, and wrote the manuscript. D.B. produced protein, samples, conducted confocal microscopy, and NMR experiments. A.G. maintained DNP instrumentation and A.S. assisted in Ub production. M.K assisted in confocal microscopy acquisition and analysis. R.Y., X.C. and Y.L. produced synthetic SNAPol-1 and FITC-SNAPol-1. F.D., and M.P.C.M. produced TAMRA-Ub. All authors reviewed the manuscript and agreed to its publication.