A Two-Armed Probe for In-Cell DEER Measurements on Proteins**

Qing Miao*,[a] Enrico Zurlo*,[b] Donny de Bruin,[b] Joeri A. J. Wondergem,[b] Monika Timmer,[a] Anneloes Blok,[a] Doris Heinrich,[b, c] Mark Overhand,[a] Martina Huber,*[b] and Marcellus Ubbink*,[a]
Author Contributions

D.H. Investigation: Supporting; Resources: Supporting.
Supporting Information

Table of Contents

Table S1 .................................................................................................................. S2
Table S2 .................................................................................................................. S2
Figure S1 ............................................................................................................... S3
Figure S2 ............................................................................................................... S4
Figure S3 ............................................................................................................... S5
Figure S4 ............................................................................................................... S5
Figure S5 ............................................................................................................... S6
Figure S6 ............................................................................................................... S7
Figure S7 ............................................................................................................... S8
Figure S8 ............................................................................................................... S9
Figure S9 ............................................................................................................... S10
Figure S10 ........................................................................................................... S10
Figure S11 ........................................................................................................... S11
Scheme S1 ........................................................................................................... S12
Material and Methods ........................................................................................ S12
Synthesis ............................................................................................................. S16
Supplementary references ................................................................................... S18
Table S1. Mass spectrometry results (in Da).

| Sample | Mutant 1<sup>a</sup> | Mutant 2<sup>b</sup> | CLaNP13<sup>c</sup> | CLaNP13<sup>b</sup> | CLaNP13<sup>c</sup> |
|--------|------------------|------------------|-------------------|-------------------|-------------------|
|        |                  |                  | Mutant 1<sup>a</sup> | Mutant 2<sup>b</sup> | Mutant 1<sup>a</sup> | Mutant 2<sup>b</sup> | Mutant 1<sup>a</sup> | Mutant 2<sup>b</sup> |
| Calcd. | 18697            | 18426            | 19600             | 20250<sup>d</sup> | 19646<sup>d</sup> | 20306<sup>d</sup> | 19674<sup>d</sup> | 20344             |
| Experm.| 18697±4          | 18428±4          | 19598±4           | 20248±4           | 19642±4           | 20304±4           | 19670±4           | 20341±4           |

<sup>a</sup>15N enriched (96.5%) T4Lys K147C/N151C, <sup>b</sup>T4Lys N55C/V57C/K147C/T151C (natural isotope abundance), <sup>c</sup>CLaNP13 linked to T4Lys mutants, <sup>d</sup>monohydrate results (+18)

Table S2. PCS-based $\Delta \chi$ tensor parameters of Yb(III)-CLaNP5 attached to T4Lys variants.

| Protein | T4Lys |
|---------|-------|
|         | N55C/V57C | K147C/T151C |
| $\Delta \chi_{ax}$<sup>a</sup> | 6.3±0.1 | 9.9±0.1 |
| $\Delta \chi_{rh}$<sup>a</sup> | 3.6±0.1 | 4.3±0.1 |
| Restraints | 91 | 78 |
| $Q_a$<sup>b</sup> | 0.03 | 0.02 |
| PDB entry | 3dke<sup>11</sup> | 3dke<sup>11</sup> |

<sup>a</sup>in 10<sup>-32</sup> m<sup>3</sup>; <sup>b</sup>See eq. S2 in the Materials and Methods section
Figure S1. ESI-TOF MS spectra of CLaNP13a (a), CLaNP13b (b) and CLaNP13c (c) linked to $^{15}$N enriched T4lys T147C/N151C mutant.
Figure S2. Overlay of $^1$H-$^{15}$N HSQC spectra (left, full; right detail) of Lu(III), Gd(III) and Yb(III) loaded CLaNP13a (a, b), CLaNP13b (c, d), or CLaNP13c (e, f) attached to T4Lys K147C/T151C. The NMR spectra were recorded at 14.1 T (600 MHz). Peaks of some residues mentioned in the text are labeled.
Figure S3. Overlay of $^1$H-$^{15}$N HSQC spectra of Yb(III) and Lu(II) loaded CLaNP5 attached to T4Lys N55C/V57C (a) and T4lys K147C/T151C (b). The NMR spectra were recorded at 14.1 T (600 MHz).

Figure S4. PCS isosurfaces of Yb(III)-CLaNP5 plotted on the structures of T4Lys (PDB entry 3dke)\cite{1} N55C/V57C (a) and K147C/T151C (b). The protein backbones are drawn in wheat ribbon representation. The iso-surfaces correspond to PCSs of ±0.4 ppm. Positive and negative PCS are indicated by blue and red, respectively. (c, d) The experimental $^1$H$^N$ PCS (ppm) of Yb(III)-CLaNP5 were plotted against the back-calculated values after fitting to eq. S1 for T4Lys N55C/V57C (c) and T4Lys K147C/T151C (d).
Figure S5. ESI-TOF MS spectra of CLaNP13a (a), CLaNP13b (b) and CLaNP13c (c) linked to T4Lys N55C/V57C/T147C/N151C.
Figure S6. Incorporation of T4Lysozyme (T4L) into *D. Discoideum* cells. Different stages of the internalization process of T4L-ATTO-647 in vegetative *D. discoideum* are shown in a), b) and d). a) Just prior (*t* = 0 min) to incubation, cells are adhered to the substrate; b). Incubation and subsequent internalization results in a shock response and cell rounding. Confocal fluorescence image (red) shows cells after *t* = 30 min of incubation. Blue line marks the direction of intensity profile plot; c) Intensity profile of three cells during incubation, the protein is ubiquitous in cell body and surrounding medium; d) After washing with PBS (*t* = 60 min), the cells recover and start spreading. T4L-ATTO-647 remains in the cell after washing with phosphate buffered saline (*t* = 75 min); e) To determine the protein concentration inside cells after incubation, cell edges are recognized (green) and mean fluorescence (red) intensities measured; f) Mean intensity histogram of various concentrations of T4L-ATTO-647 in PBS (grey) versus mean fluorescence inside cells (blue) during and after incubation, measurements were repeated with higher power (inset); g) State of the cells after 3 hours of incubation; h) State of the cells after EPR experiments.
Figure S7. EPR of samples in vitro. Shown is a Field-Swept Electron-Spin Echo (FSESE) spectrum at 95 GHz obtained at 10 K of Gd-CLaNP13b tagged T4Lys N55C/V57C/K147C/T151C (200 μM). The measurement was carried out by a 2-pulse {π/2-τ-π-τ-echo} sequence with t(π/2) = 32 ns, t(π) = 64 ns. and τ = 360 ns. A thorough EPR characterization of the ligands is in preparation.
**Figure S8.** Four-pulse DEER results obtained for Gd13iT4L (with i = a,b,c) at 10 K. On the left: Normalized DEER traces fitted with appropriate background decay (in red). Middle: Same DEER traces after background removal along with the fits obtained by Tikhonov regularization (red). On the right: Distance distribution obtained by Tikhonov regularization ($\alpha = 1000$) in DEER Analysis 2018.[2] a) *In vitro* Gd13aT4L; b) *In vitro* Gd13bT4L; c) *In vitro* Gd13cT4L; d) In-cell Gd13bT4L.
**Figure S9.** Four-pulse DEER traces and distance distributions for the Gd-CLaN13a tagged T4Lys N55C/V57C/ K147C/T151C samples *in vitro* (black), incubated in *E. coli* lysate for 1 hour (blue) and for 18 hours (violet). Measurements were done at 10 K. a) Normalized DEER traces fitted with appropriate background decay (red); b) Background corrected DEER traces. Red lines, fits obtained with the distance-distribution calculations shown in (c) obtained after Tikhonov regularization (α = 1000) in DEER Analysis 2018.[2] Peaks marked with an asterisk do not contribute significantly to the data.

**Figure S10.** Four pulse DEER traces and distance distributions for the Gd-CLaN13b tagged T4Lys N55C/V57C/ K147C/T151C samples in *D. discoideum* cell lysate + milliQ H₂O (black), in medium (violet), in lysate + medium (blue). Measurements were done at 10 K. a) Normalized DEER traces fitted with appropriate background decay (red). b) The corresponding four pulse DEER traces along with the fits obtained by Tikhonov regularization (red). Traces are shifted vertically for clarity. c) Distance distributions obtained by Tikhonov regularization (α = 1000) in DEER Analysis 2018.[2] Peaks marked with the asterisk do not contribute significantly to the data.
Figure S11. Results of validation of DEER curves for a) In vitro Gd13aT4L, b) In vitro Gd13bT4L, c) In vitro Gd13cT4L, d) In-cell Gd13bT4L. In-cell Distance = 3.9 ± 0.3 nm, width d.d. = 0.55 ± 0.4 nm. DEER data analysis and validation: All DEER time traces where truncated before processing, by eliminating the last 800 ns from the traces. Black line: Highest confidence line. Grey: Range of distance distributions for validation trials. Noise added: level 1.5, 10 points, background start time: 50 points, range 300-800 ns. The background start for the analysis in Figure 3 in the main text was 320 ns. e) Normalized DEER trace of Gd13aT4L, real part (black) and the imaginary part (red curve) was shifted vertically by + 0.78 for clarity. For details see text.
Materials and Methods

General: 1-(2-aminoethyl)-1H-pyrrole-2,5-dione, 1-(3-aminoethyl)-1H-pyrrole-2,5-dione, 1-(4-aminoethyl)-1H-pyrrole-2,5-dione and cyclen were purchased from Abosyn Chemical Inc. and CheMatech. Gd(OAc)_3 \cdot 4H_2O, Yb(OAc)_3 \cdot 4H_2O, N-(tert-butoxycarbonyloxy)succinimide and methyl 2-bromoacetate were purchased and used without further purification. Solvents were purchased from Honeywell, BIOSOLVE or Aldrich and directly used for synthesis. ATTO-647 maleimide was obtained from ATTO-TEC GmbH. Axenic D. discoideum (AX2) was obtained from Dr. Günther Gerisch (MPI for Biochemistry, Martinsried, Germany). Superdex 75 columns and Sephadex G-25 PD10 desalting columns were purchased from GE Healthcare. Reactions were followed by liquid chromatography-mass spectrometry (LC-MS), TLC analysis on silica gel (F 1500 LS 254 Schleicher and Schuell, Dassel, Germany) in which compounds were visualized by UV and/or ninhydrin, KMnO_4. Flash chromatography was performed on Screening Devices silica gel 60 (0.04-0.063 mm). A Waters preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 \mu m OBD (30 x 150 mm) column and an Äkta Basic FPLC (GE Healthcare Inc.) were used for purification. NMR spectra were recorded on a Bruker AV-400 (400/100 MHz), AV-500 (500/125 MHz) or AV-600 (600/150 MHz) spectrometer. A LCQ liquid chromatography mass spectrometry system and a Finnigan LTQ Orbitrap system were used for high-resolution mass spectrometry and protein conjugation analysis. A Thermo Scientific™ NanoDrop 2000 spectrophotometer was used for protein concentration measurements.

Protein expression and purification: T4Lys mutant K147C/T151C was produced as described in previous work. The tetra-cysteine mutant N55C/V57C/K147C/T151C was generated by the Quikchange method (Agilent). After confirming the successful mutations by DNA sequencing, the gene was expressed in Escherichia coli BL21 (DE3). Transformed cells were incubated overnight at 37°C on LB agar plates with kanamycin and chloramphenicol (100

---

Scheme S1. Synthesis route of Gd-CLaNP13. a) methyl 2-bromoacetate, K_2CO_3, ACN, rt, 16 h; b) i) TFA/DCM (4:1, v:v); ii) 2-(chloromethyl)pyridine 1-oxide, K_2CO_3, ACN, rt, 16 h; c) N-hydroxysuccinimide, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, 1-(2-aminopropyl)-1H-pyrrole-2,5-dione, DMF, rt, 24 h. d) Gd(OAc)_3 \cdot 4H_2O, DMF, rt, overnight.
µl/mL and 34 µl/mL, respectively). Single colonies were transferred to 2 mL LB medium with kanamycin and chloramphenicol and incubated at 37°C, 250 rpm for 6 h. The precultures were used to inoculate 50 mL (1:1000) for overnight incubation in minimal medium (M9, 37°C, 250 rpm). The 50 mL culture was diluted 100 times and incubated at 37 °C, 250 rpm. When the OD<sub>600</sub> value reached 1, gene expression was induced by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). The temperature was reduced to 30 °C and the cells were harvested 18 h after induction by centrifugation. The T4Lys N55C/V57C/K147C/T151C was purified according to the method described by Georgieva et al. with some modifications. Cells were lysed using a French press and cleared by centrifugation (45 min, 2500 rpm). The supernatant was loaded on a 5 mL HiTrap CM column equilibrated with 25 mM Tris pH 7.5. The column was washed with 0-40% linear gradient of buffer B (25 mM Tris pH 7.5, 500 mM NaCl). T4Lys N55C/V57C/K147C/T151C was eluted in one step with 100% buffer B.

Protein labeling: To label T4Lys mutants with Gd-CLaNP13/ATTO-647-maleimide, the protein sample (1 mL, 200–300 μM) was treated with DTT (final concentration 10 mM) at 0 °C. After 1 h, the protein buffer solution was loaded on a PD-10 column pre-equilibrated with labeling buffer (25 mM sodium phosphate, 100 mM NaCl, pH 7.8, argon degassed) to remove DTT. The eluted protein was mixed immediately with labeling buffer containing Gd-CLaNP13 (6 equiv.) or ATTO-647-maleimide (4 equiv.) under an argon atmosphere. The mixture was stirred at 4 °C for 6 h. Then the sample volume was reduced to 500 μL by ultrafiltration and purified by using a Superdex 75 gel filtration column, respectively. The yield of labeling for Gd-CLaNP13 labeled T4Lys sample estimated from the LC-MS and NMR, was more than 95%. Labeling with Yb(III)/Lu(III)-CLaNP5 followed a similar procedure except that the labeling buffer was 20 mM sodium phosphate, 100 mM NaCl, pH 7.5 and Yb(III)/Lu(III)-CLaNP5-labeled protein was purified from excess CLaNP-5 with a HiTrap SP column using 30 column volumes for a 0-500 mM NaCl gradient.

The best result for double maleimide reactions with the cysteines was using a buffer with pH 7.8, at 4 °C for 6 h. To test whether free thiolates remained after the reaction, iodoacetamide was added. The LC-MS results, yielded masses that match those expected for probes attached via two arms, assuming that one or two water molecules remained bound to the protein-probe complexes (Figure S1 and Table S1). No peaks with additional mass of 58 Da were detected, which would be expected for protein with one-arm attached probe and an additional acetamide group linked to the second cysteine sulfur atom, and also no free protein was detected, suggesting that the protein labelling efficiency was more than 95%. In the MS spectrum of the CLaNP13a, a very small peak (20552 Da) is observed that represents T4Lys with two probes bound, each via a single arm. After attachment of the first maleimide group, the reaction with the second will generally be efficient because it is intramolecular. The limited length of the linker in CLaNP13a may allow for some competition with the second-order reaction of the protein-probe complex with a second probe molecule. However, according to the MS spectrum, the fraction of this species is very small.

Protein LC-MS sample preparation: Gd(III)-CLaNP13 labelled T4Lys (10-15 μM) in labelling buffer was incubated with DTT (final concentration 10 mM) on ice for 1h. Iodoacetamide (final concentration 5 mM) was added and the solution was incubated for
another 1 h under protection from light. A Bio-rad 6 desalting column was used for buffer exchange before loading the sample on a C4 polymeric reversed phase UPLC column and analyzing it using either an LTQ-Orbitrap mass spectrometer (ThermoScientific) or Synapt G2-Si mass spectrometer (Waters), 10–25 min after thawing. The data was deconvoluted for mass/charge peaks using the Thermo Xcalibur software.

**DEER sample preparation:** The samples for in vitro and in lysate DEER measurements contained 100-150 µM of Gd(III)-CLaNP13 labeled N55C/V57C/K77C/T151C T4Lys in 20 mM sodium phosphate, pH = 5.5, 150 mM NaCl, 20% (v/v) glycerol, *E. coli* cell lysate (20% (v/v) glycol) and *D. discoideum* cell lysate (20% (v/v) glycol). The sample for in-cell DEER measurement was obtained by incubating *D. discoideum* medium with the protein labelled with Gd(III)-CLaNP13b at a concentration of 115 µM for 90 mins. The cells were recovered, and washed three times in medium to remove excess protein, and to concentrate the cell suspension. The sample was cooled to approx. 4 °C on ice, and 10% (v/v) DMSO was added to the medium to prepare the cells for freezing and EPR measurement.

**Protein NMR spectroscopy:** All protein NMR spectra were recorded at 298 K on a 600 MHz Bruker Avance III spectrometer. A $^1$H,$^15$N HSQC spectrum was acquired for each sample. The final NMR samples contained 100-200 µM of labeled T4Lys in 20 mM sodium phosphate, pH = 5.5, 150 mM NaCl and 5% D$_2$O for lock.

**PCS data analysis:** The $\Delta \chi$-tensors were calculated using Numbat software.$^{[5]}$ Crystal structures of T4Lys (PDB entries3dke)$^{[1]}$, to which hydrogens had been added, were used. The experimental PCS were fitted to **equation S1**. The $Q_a$ factor provides a normalized measure for the agreement between a set of observed and calculated data according to **equation S2**

$$
\text{PCS} = \frac{1}{12 \pi \rho_{IM}^2} \left[\Delta \chi_{ax}(3 \cos^2 \theta - 1) + \frac{3}{2} \Delta \chi_{rh}(\sin^2 \theta \cos 2\Omega)\right]
$$

$$
Q_a = \sqrt{\sum_i (\delta_{PCS,i} - \delta_{PCS,i})^2 / \sum_i (\delta_{PCS,i} + \delta_{PCS,i})^2}
$$

where $\rho_{IM}$ is the distance between the unpaired electron and nucleus, $\theta$, and $\Omega$ are the polar coordinates of the nucleus with respect to the principal axes of the $\Delta \chi$-tensor, $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ are the axial and rhombic components of the $\Delta \chi$ tensor, $\delta_{PCS,i}^{\text{cal}}$ and $\delta_{PCS,i}^{\text{exp}}$ are the back-calculated and experimentally observed PCS for each residue.

**EPR measurement conditions:** The 95 GHz DEER measurements were recorded using an ELEXSYS E680 spectrometer (Bruker, Rheinstetten, Germany) with a home-built probe head. The measurements were done at a temperature of 10 K. The DEER spectra were recorded using the standard four pulse DEER sequence.$^{[6]}$ The pump-pulse duration was 40 ns and the observer-pulse durations were 32 and 64 ns, respectively. The pump-pulse power was adjusted to invert the echo maximally. The separation between the pump and observer frequencies ($\Delta \nu$) was 60 MHz, with the pump pulse adjusted to irradiate at the maximum of the EPR spectrum. To make optimum use of the resonator bandwidth, the pump and observer frequencies were set to 30 MHz higher and lower frequencies respectively with respect to the resonance frequency of the loaded resonator. The full sequence for the observer was $\pi/2 - \tau - \pi - \Delta \nu - \pi - \text{echo}$ with a
delay time $\tau$ of 360 ns and time steps for DEER evolution of 8 ns. The DEER data were analyzed with the program DeerAnalysis 2018.[2]

Since the modulation depth depends strongly on the precise setup of the experiment, e.g. choice of pump and observer frequency relative to the resonance frequency of the EPR resonator, this parameter can differ from experiment to experiment on the order of 0.5 % in absolute modulation depth, which, given a total modulation depth of 2 %, makes for a relative error of the modulation depth in the order of 25 %. The in-cell DEER experiment had been setup for a higher signal to noise ratio rather than for higher modulation depth due to instrumental issues. Also it must be noted that the experiment lasted less than 8 hours, instead in most cases in-cell measurements are taken for a longer period of time.[7–9]

**DEER data analysis and validation:** All DEER time traces where truncated before processing, by eliminating the last 800 ns from the traces. The artefact observed towards the end of the traces, see times larger than 2300 ns in Figure S11e, is attributed to overlap of pump and observer pulse. DEER traces were validated using the DeerAnalysis validation tool using the following parameters: White noise level: 1.5, 10 points. Background start range: 300 to 800 ns, 50 points.

**Preparation of samples in E. coli lysate:** *Escherichia coli* BL21 (DE3) cells were incubated overnight at 37°C on LB agar plates with kanamycin and chloramphenicol (100 µL/mL and 34 µL/mL, respectively). Single colonies were transferred to 4 mL LB medium with kanamycin and chloramphenicol and incubated at 37°C, 250 rpm for 12 h. Then the cells were harvested by centrifugation. Cells were lysed using sonication (10 min, 4 °C) and cleared by centrifugation (5 min, 2500 rpm). The supernatant (20 µL) was mixed with Gd-CLaNP13 labeled T4Lys N55C/V57C/K147C/T151C (20 µL (215 µM, in 20 mM sodium phosphate, pH = 5.5, 150 mM NaCl)). To the above mixture glycol was added (20%, v/v) and incubated under room temperature for 1 h and 18 h before DEER measurements.

**Cell culture and live cell imaging:** Axenic *D. discoideum* (AX2) was obtained from Dr. Günther Gerisch (MPI for Biochemistry, Martinsried, Germany). Cells were grown at 20 °C in HL5 medium and cultured in 100 mm Petridishes (TC-treated culture dish, Corning, USA) and confluency was kept below 70%. For microscopy experiments, cells were harvested and centrifuged at 1500 rpm for 3 min followed by three successive washing steps of the cellular pellet with 17 mM K-Na-phosphate buffered saline (PBS), adjusted to pH = 6.0. After resuspension in PBS, the cells were pipetted into a 70 µL well inside a 35 mm imaging dish (Insert and dish, Ibidi GmbH, Martinsried, Germany) and left to adhere for 30 min. While imaging, T4Lys K147C/T151C linked to ATTO-647 (ATTO-TEC GmbH) was added to a final concentration of 100 µM. Cells were left to incubate for 60 min and were imaged every 30 seconds in two channels (BF and 647), through a 60x objective (Plan Apo VC 60x, Nikon, Japan) with a Nikon Eclipse Ti microscope, equipped with a Yokogawa confocal spinning disk unit operated at 10,000 rpm (Nikon, Tokyo, Japan). ATTO-647 was excited by a 647 nm solid state diode laser (Coherent, Santa Clara, U.S.A.), supported in an Agilent MLC4 unit (Agilent Technologies, Santa Clara, U.S.A.). Images were captured (50 ms) by an Andor iXon Ultra 897 High Speed EM-CCD (Andor Technology, Belfast, Northern Ireland) through a Cy5 HYQ
filter (Nikon, Tokyo, Japan). After incubation, the well was carefully washed with PBS five times (50 µL each step) and imaged for another 15 min.

**Quantification of the in-cell T4L-ATTO-647 concentration:** Known concentrations of T4Lys K147C/T151C linked to ATTO-647 in PBS were imaged in the same condition as described for cells. The data were averaged over all images and pixels to find mean intensities per concentration. For the cell data, all pixels belonging to each cell were determined using an in-house Matlab (The Mathworks, Inc., Natick, MA, U.S.A.) script for cell edge recognition (see Figure S6 e). In-cell pixels were averaged and subsequently compared to the concentration calibration measurements (see Figure S6f) for two laser powers. The high- and low laser powers were set to 6% and 20% of 1.4mW respectively, controlled by an acousto-optic tunable filter. All images were pre-processed by flat-field correction and dark-field subtraction and intensities were normalized to the saturation intensity (16-bit). Through these results, we estimate the average concentration of the protein in the cells to be 5 µM.

**Uptake of T4L-ATTO-647 into cells:** *D. discoideum* was chosen for our experiments due to its known high uptake of extracellular components. After a 30 min incubation, almost all cells have taken up the fluorescent protein (Figure S6b). After 60 mins, the excess protein is removed from the medium by washing, leaving clearly detected fluorescent protein present in the cells (Figure S6d). The protein can still be detected within the cells 3 h after the start of the incubation (Figure S6g).

To quantify the concentration of the protein in the cells, known concentrations of T4Lys K147C/T151C linked to ATTO-647 in PBS were imaged in the same conditions as described for cells. The data was averaged over all images and pixels to find mean intensities per concentration.

**Preparation of the in-cell DEER samples and tests of cell viability:** For DEER experiments, the cells were incubated with the double CLaNP13b tagged T4Lys. After incubation, the cells were washed, concentrated, and cooled on ice. Before the cells were frozen for the EPR measurements, 10% (v/v) DMSO was added to the medium. DEER experiments were performed at 10 K for 48 h. After the DEER experiments, the samples were thawed until liquid, transferred to an 8-well chamber slide with a tissue culture treated surface (Ibidi GmbH, Martinsried, Germany), and 200 µL culture medium was added. The cells were allowed to recover in this state overnight. Live cell imaging was then performed in the brightfield to confirm the presence of cells and observe their active mobility.

**Synthesis**

**Di-tert-butyl4,10-bis(2-methoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,7-dicarboxylate, compound 2**

Compound 2 was prepared according to ref. NMR data are in agreement with the literature results. HR-MS: m/z 517.3232 [M+H]⁺, calcd. [C₂₄H₄₆N₄O₈] 517.3237.
2,2'-(4,10-bis(carboxymethyl)-1,4,7,10-tetraazacyclododecane-1,7-diy1)bis(methylene))bis(pyridine 1-oxide), compound 3

Compound 2 (0.5 g, 0.96 mmol) was dissolved in a mixture of DCM and TFA (10 mL, DCM:TFA= 1:4, v:v) and stirring was continued for 16 h. The reaction mixture was concentrated by co-evaporation with toluene to obtain a yellow oily residue. The residue was dissolved in ACN (10 mL) and 2-(chloromethyl)pyridine 1-oxide [12] (0.35 g, 2.4 mmol) and K$_2$CO$_3$ (0.4 g, 2.9 mmol) were added and stirring was continued at rt for 16 h. The salt was removed by filtration over a workman filtrating paper and the organic layer was concentrated under reduced pressure on a rotary evaporator to yield a brown oily residue. This brown oily product was dissolved in a dioxane water mixture (v/v= 1:1, 10 mL) where containing 0.25 M of NaOH to obtain compound 3. The crude product purified by reverse phase HPLC (0.2% TFA and a 15-25% acetonitrile gradient on C18 preparative column), yielding 40% of compound 4 (0.19 g, 0.38 mmol). $^1$H NMR (500 MHz, D$_2$O, 343K): $\delta$ = 3.78-3.90 (m, 18H), 5.14 (s, 4H), 8.18-8.21 (t, 2H), 8.24-8.27 (t, 2H), 8.35 (d, 2H), 8.96 (d, 2H). $^{13}$C NMR (500 MHz, D$_2$O, 343K): $\delta$ = 49.5 (CH$_2$N), 51.69 (CH$_2$N), 53.76 (NCH$_2$COOH), 54.4 (CH$_2$C), 129.11 (CH) 130.61 (CH) 131.83 (CH), 140.79 (CH), 166.54 (COOH), 166.52 (CCH$_2$).

HR-MS: m/z 503.2604 [M+H]$^+$, calcd. [C$_{24}$H$_{34}$N$_6$O$_6$] 503.2618.

FTIR (cm$^{-1}$): 3089.4, 2358.2, 2341.0, 1684.2, 1458.1, 1312.2, 1196.3, 1130.5, 799.9, 772.7, 719.8, 668.3.

2,2'-(4,10-bis(2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,7-diy1)bis(methylene))bis(pyridine 1-oxide), compound 4a

Compound 3 (0.1 g, 0.2 mmol) was dissolved in dried DMF (3 mL) and treated with N-hydroxysuccinimide (0.09 g, 0.8 mmol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (0.15 g, 0.8 mmol). After 10 mins with continues stirring was added 1-(2-aminoethyl)-1H-pyrrole-2,5-dione (0.12 g, 0.8 mmol) to the reaction mixture and stirring was continued at rt. The reaction was followed by LC-MS. After 24 h, no more compound 3 was detected by LC-MS and the reaction mixture was concentrated under reduced pressure using a rotary evaporator. For purification, HPLC (0.2% TFA and a 15-25% acetonitrile gradient on C18 preparative column) was employed to yield 70% of compound 4 (0.1 g, 0.14 mmol). A similar procedure was used for synthesis compound 4b and 4c with 1-(3-aminopropyl)-1H-pyrrole-2,5-dione (0.1 mg, 0.53 mmol) and 1-(4-aminobutyl)-1H-pyrrole-2,5-dione (0.1 mg, 0.5 mmol), respectively. $^1$H NMR (500 MHz, MeOD, 323K): compound 4a $\delta$ = 3.27 (s, 8H), 3.33-3.35 (t, 4H), 3.46 (s, 8H), 3.54 (s, 4H), 3.61-3.63 (t, 4H), 4.45 (s, 4H), 6.80 (s, 4H), 7.60-7.66 (m, 4H), 7.88-7.90 (t, 2H), 8.45-8.46 (t, 2H). $^{13}$C NMR (500 MHz, MeOD, 323K): $\delta$ = 38.10 (CH$_2$NHCO), 39.47 (CH$_2$NCOCO), 51.95 (CH$_2$N), 53.96 (NCH$_2$C) 55.81 (CH$_2$CONH), 128.65 (CH) 130.09 (CH) 130.71 (CH), 135.56 (CHCON), 141.78 (CH), 172.69 (CO). Compound 4b $^1$H NMR (500 MHz, MeOD, 333K) $\delta$ = 1.77-1.82 (m, 4H), 3.16-3.17 (t, 4H), 3.25 (br, 8H), 3.47-3.53 (br, 12H), 3.66 (s, 4H), 4.39 (s, 4H), 6.78 (s, 4H), 7.56-7.63 (m, 4H),
7.84-7.86 (dd, 2H), 8.41-8.42 (d, 2H). $^{13}$C NMR (500 MHz, MeOD, 323K): δ = 29.11 (CH$_2$CH$_2$NH), 36.19 (CH$_2$NHCOCO), 37.98 (CH$_2$NCO), 51.29 (CH$_2$CH$_2$N), 52.24 (CH$_2$CH$_2$N), 53.88 (CH$_2$C), 56.12 (NCH$_2$CONH), 128.48 (CH) 129.88 (CH) 130.60 (CH), 135.46 (CHCON), 141.76 (CH), 172.60 (CO). Compound 4c showing the same spectra as 4a.

Metal ion complex: To a solution of compound 4a (30 mg, 40 μmol) in 400 μL dried DMF, 1.2 equiv. of Gd(OAc)$_3$·4H$_2$O was added. The mixture was stirred at rt for 16 h and the formation of the metal ion complex was checked by LC-MS. Without further purification, Gd-CLaN13a was used for proteinlabeling. Gd-CLaN13a/b/c were prepared following the same procedure. HR-MS: Gd-CLaN13a m/z 3080.8, 2359.6, 2332.4, 1705.7, 1684.2, 1443.8, 1410.9, 1200.6, 1173.4, 1130.5, 1054.61, 1033.1, 830.0, 799.9, 777.0, 719.8, 696.9, 668.3. Compound 4b and 4c showing the same spectra as 4a.

Supplementary References

[1] L. Liu, M. L. Quillin, B. W. Matthews, Proc. Natl. Acad. Sci. 2008, 105, 14406–14411.
[2] G. Jeschke, V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. Banham, C. R. Timmel, D. Hilger, H. Jung, Appl. Magn. Reson. 2006, 30, 473–498.
[3] W.-M. Liu, S. P. Skinner, D. V. Filippov, A. Blok, M. Ubbink, M. Timmer, M. Overhand, M. A. S. Hass, Chem. Eur. J. 2014, 20, 6256–6258.
[4] E. R. Georgieva, A. S. Roy, V. M. Grigoryants, P. P. Borbat, K. A. Earle, C. P. Scholes, J. H. Freed, J. Magn. Reson. 2012, 216, 69–77.
[5] C. Schmitz, M. J. Stanton-Cook, X.-C. Su, G. Otting, T. Huber, J. Biomol. NMR 2008, 41, 179.
[6] M. Pannier, S. Veit, A. Godt, G. Jeschke, H. . Spiess, J. Magn. Reson. 2000, 142, 331–340.
[7] J. A. Clayton, M. Qi, A. Godt, D. Goldfarb, S. Han, M. S. Sherwin, Phys. Chem. Chem. Phys. 2017, 19, 5127–5136.
[8] E. Matalon, T. Huber, G. Hagelueken, B. Graham, V. Frydman, A. Feintuch, G. Otting, D. Goldfarb, Angew. Chemie. Int. Ed. 2013, 52, 11831–11834.
[9] A. Feintuch, G. Otting, D. Goldfarb, Methods Enzymol. 2015, 563, 415–457.
[10] S. J. Annesley, P. R. Fisher, Mol. Cell. Biochem. 2009, 329, 73–91.
[11] E. M. Neuhaus, W. Almers, T. Soldati, Mol. Biol. Cell 2002, 13, 1390–1407.
[12] P. H. J. Keizers, A. Saragliadis, Y. Hiruma, M. Overhand, M. Ubbink, J. Am. Chem. Soc. 2008, 130, 14802–14812.