Supporting Information for

Alkyne-tagged Raman probes for local environmental sensing by Hydrogen-Deuterium exchange

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Materials and Methods

Chemicals

All chemicals were purchased from Sigmal-Aldrich unless otherwise specified. 5-Ethynyl-2′-deoxyuridine (EdU, CAS# 61135-33-9) was purchased from TCI America. 5'-Ethynyl-2'-deoxycytidine (EdC, CAS# 69075-47-4) was purchased from Cayman Chemicals. 5-Ethynyl Uridine (EU, CAS# 69075-42-9) was purchased from Sigma-Aldrich. 5-Ethynyl-2'-deoxyuridine 5'-triphosphate (5-EdUTP) was purchased from Jena Bioscience. BCECF, AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) and BCECF, free acid were purchased from ThermoFisher scientific. Pwo DNA polymerase was purchased from Roche. Exonuclease I (ExoI) was purchased from Sigma-Aldrich. Nigericin sodium salt (CAS# 28643-80-3) was purchased from Sigma-Aldrich. Propargylcholine bromide (PCho, CAS# 111755-76-1) was synthesized according to Ref. 3. Briefly, propargyl bromide (80 wt. % solution in toluene, Sigma-Aldrich) was added dropwise to a stirring solution of 2-dimethylaminoethanol (Sigma-Aldrich) in anhydrous THF in ice bath under argon gas protection. The reaction mixture was slowly warmed up to room temperature and stirred overnight. The resulting white solids were filtered and washed extensively with cold anhydrous THF to obtain pure PCho.

Buffer preparation

For solution experiments, we used different buffering systems according to the final pD region to prepare D_2O buffers. (Buffer range: citric acid-Na_2HPO_4, 2.6-7.6; NaH_2PO_4-Na_2HPO_4, 6.2-8.2; CAPSO-CAPSO sodium salt, 8.9-10.3). We prepared D_2O buffers by directly diluting corresponding buffer powders to the same amount

of D_2O as that for the H_2O system. To keep ion strength consistent for all buffers to avoid the influence on the alkyne-HDX kinetics, we added extra NaCl to those buffers with less salt concentrations. The detailed buffer recipes are shown below: pD= 5.3 (46.4 mM citric acid, 107.2 mM Na_2HPO_4); pD= 6.2 (33.9 mM citric acid, 132.2 mM Na_2HPO_4); pD= 6.6 (27.25 mM citric acid, 145.5 mM Na_2HPO_4); pD= 7.0 (17.65 mM citric acid, 164.7 mM Na_2HPO_4); pD= 7.6 (DPBS); pD= 7.9 (3 mM NaH_2PO_4, 30 mM Na_2HPO_4, 60 mM NaCl); pD= 9.4 (28mM CAPSO, 7 mM CAPSO sodium salt, 143 mM NaCl); pD= 10.4 (19 mM CAPSO, 16 mM CAPSO sodium salt, 134 mM NaCl); pD= 10.4 (10 mM CAPSO, 25 mM CAPSO sodium salt, 125 mM NaCl). The final pD values were determined by the pH-meter. For salt concentration experiment, 2 M NaCl was added into the DPBS-D_2O buffer solution.

For BCECF experiments, high K^+ conditions were used to meet the requirement of nigericin. The detailed high K^+ buffer recipes are shown below: High K^+ D_2O buffer, pD = 7.64 (120 mM KCl, 5 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in D_2O); pD=6.88, 7.11, 7.24 buffers were made by adjusting pD of pD=7.64 buffer with drops of 0.1 M HCl in D_2O. pD= 8.01 buffer was made by adjusting pD of pD=7.64 with drops of 0.1 M $NaHCO_3$ in D_2O. High K^+ H_2O buffer, pH = 7.35 (120 mM KCl, 5 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in H_2O); pH = 6.83, 6.95, 7.12 buffers were made by adjusting pH of pH=7.64 buffer using 0.1 M HCl in H_2O.

pH/pD determination

pD/pH reading for all D_2O buffers and H_2O buffers were acquired on a pH meter (Mettler Toledo FiveEasy Plus FP20, with LE 410 sensor) at room temperature (~ 22 °C). The pH meter was calibrated with standard solutions (pH=4.01, 7.00, 10.01, Mettler Toledo) before measuring the custom-made buffers. As we discussed in the main manuscript, to avoid confusion, we show all the pD values in our manuscript from direct pH meter reading (i.e. the $pH_{meter\ reading}$) for all D_2O buffers. As shown below, the calibration factor is small and would not influence any of our conclusions.

The offset between $pH_{meter\ reading}$ and pD values was obtained by two control experiments. First, we compared the $pH_{meter\ reading}$ of DPBS-H_2O solution (7.35, considered as real pD if H_2O is replaced with D_2O) to that of DPBS-D_2O solution (7.6, considered as $pH_{meter\ reading}$). Second, we used the pH-sensitive ratiometric fluorophore BCECF to provide additional calibration between $pH_{meter\ reading}$ and pD values reported through BCECF ratios. In brief, we dissolved BCECF acid in water and made a 2 mM stock solution. We then diluted the stock BCECF solution in the high K^+ H_2O/D_2O buffers with adjusted $pH_{meter\ reading}$ (directly reading from the pH meter) to be in the range 6.8-8.1. The final BCECF concentration is 4 μM. We then

acquired fluorescence images using 445 nm and 488 nm excitation lasers (ZEISS LSM 980). The ratios of fluorescence intensity at 488 nm over that at 445 nm were plotted against respective readings from the pH meter for H_2O buffers and D_2O buffers (Figure S7a). The offset between linearly fitted ratio vs pH/pD curves is 0.25. The reading from the pH meter for D_2O buffers is shown as $pH_{meter\ reading}$ (and are reported as the pD values in our manuscript). The determined relationship is consistent with the above two different experiments: $pD=pH_{meter\ reading} - 0.25$.

DFT calculation

DFT calculations were performed using the Gaussian09 software. Structures were optimized and then characterized using frequency calculations at the B3LYP/6-311(G)++(d,p) level of theory.

Spontaneous Raman Spectroscopy

Spontaneous Raman spectra were acquired using an upright confocal Raman spectrometer (Horiba Raman microscope; Xplora plus). A 532 nm YAG laser was used to illuminate the sample with a power of 12 mW through a 100×, N.A. 0.9 objective (MPLAN N; Olympus). Data acquisition was performed with 10 s integration by the LabSpec6 software. For whole spectra recording, background was subtracted by measuring signal from the same solution without probe molecules. The spectra shown in Figure 1a and S1a are normalized to the alkyne peak.

EdU, EdC and EU were dissolved into DMSO to make 100 mM stock solutions. PCho was dissolved into H_2O to make 2M stock solution. For measurement, EdU, EdC and EU are 1:10 diluted into corresponding H_2O buffers or D_2O buffers, while PCho is 1:50 diluted into corresponding H_2O buffers or D_2O buffers.

Model molecules (4-fluorophenylacetylene (CAS# 766-98-3), methyl-4-ethynylbenzoate (CAS# 3034-86-4), 4-ethynylbenzaldehyde (CAS# 63697-96-1), 1-ethynyl-4-nitrobenzene (CAS# 937-31-5), 4-Ethynylanisole (CAS# 768-60-5)) were dissolved into DMSO to make 100 mM stock solutions. For measurement in DMSO-D_2O system, model molecules were diluted into the 1:1 DMSO-D_2O (DPBS- D_2O, pD=7.6) solution to ensure good dissolvability for all model molecules with corresponding dilution factors. For measurement in methanol-OD system, model molecules were 1:10 diluted into methanol-OD (CAS# 1455-13-6).

All data are confirmed by at least three sets of independent experiments.

Stimulated Raman Scattering (SRS) Microscopy

A picoEmerald laser system (Applied Physics and Electronics) was used as the

light source for SRS microscopy. Briefly, it produces 2 ps pump (tunable from 770 nm – 990 nm, bandwidth 0.5 nm, spectral bandwidth ~ 7 cm^{-1}) and Stokes (1031.2 nm, spectral bandwidth 10 cm^{-1}) pulses with 80 MHz repetition rate. Stokes beam is modulated at 20 MHz by an internal electro-optic modulator. The spatially and temporally overlapped Pump and Stokes beams are introduced into an inverted laser-scanning microscope (FV3000, Olympus), and then focused onto the sample by a 25X water objective (XLPLN25XWMP, 1.05 N.A., Olympus). Transmitted Pump and Stokes beams are collected by a high N.A. condenser lens (oil immersion, 1.4 N.A., Olympus) and pass through a bandpass filter (893/209 BrightLine, 25mm, Semrock) to filter out Stokes beam. A large area (10×10 mm) Si photodiode (S3590-09, Hamamatsu) is used to measure the pump beam intensity. A 64 V reverse-biased DC voltage is applied on the photodiode to increase the saturation threshold and reduce response time. The output current is terminated by a 50Ω terminator and pre-filtered by a 19.2-23.6-MHz band-pass filter (BBP-21.4+, MiniCircuits) to reduce laser and scanning noise. The signal is then demodulated by a lock-in amplifier (SR844, Stanford Research Systems) at the modulation frequency. The in-phase X output is fed back to the Olympus IO interface box (FV30-ANALOG) of the microscope. 30 μs time constant is set for the lock-in amplifier. Correspondingly, 80 μs pixel dwell time is used, which gives a speed of 21.3 s/frame for a 512-by-512-pixel image, with two frame-averaging. Laser powers are monitored throughout image acquisition by an internal power meter and power fluctuations are controlled within 1%. The power for the pump and Stocks beam is about 25 mW and 220 mW, respectively. 16-bit greyscale images were acquired by Olympus Fluoview 3000 software. To minimize the line-pattern issue likely due to an interfering Radio frequency (RF) picked up by our lock-in amplifier detection (demodulation at 20 MHz), we have optimized our alignment and replaced a few bandpass filters between our photodiode and lock-in amplifier.

For EdU and PCho measurement, the wavelengths of pump lasers for SRS_H are 845.9 and 844.6 nm, respectively. For ratiometric imaging of EdU and PCho after the exchange, the wavelengths of pump laser for SRS_D are 855.5 and 854.5 nm, respectively. Off-resonance images were taken under 851.3 nm pump wavelength. For D_2O diffusion measurement, the pump wavelength is 820.5 nm. For EdU/EdU dimer spectra recordings, the wavelengths of pump lasers tuned from 843.9 to 847.9 nm with a 0.5 nm interval. For two-color ratiometric imaging of EdU and PCho during the exchange, the wavelengths of pump lasers for SRS_H and SRS_D are 845 and 855 nm, respectively.

Cell culture, sample preparation and alkyne-HDX in cells

For all SRS imaging experiments, cultured HeLa-CCL2 (ATCC) cells were seeded

onto coverslips (12mm, #1.5, Fisher) with a density of 1×10^5/mL in 4 well plate with 0.3 mL DMEM culture medium (DMEM+10%FBS+1% penicillin-streptomycin) for 20 h at 37 °C and 5% CO_2. Prior to imaging, coverslips were collected and attached to a microscope slide (1mm thick, VWR) with an imaging spacer (0.12mm thick, Sigma-Aldrich).

For the EdU experiment, DMEM culture medium was then changed to DMEM medium (FBS-free, Gibco) for 20-22 h for cell cycle synchronization. After synchronization, the medium was replaced back to DMEM culture medium and EdU (10 mM stock in DPBS) was simultaneously added to a concentration of 100 μM for 20-24 h. Then 4% PFA was added for 20 min for fixation. After that, DPBS was used to wash away PFA and fixed cells could be stored in DPBS at 4 °C for several days.

For UV-irradiation on live cells, cells were put inside the biosafety cabinet (BSC) with UV (254 nm) on for one hour. Morphologies were quickly checked with no severe abnormality under a transmission light microscope. The cells were fixed by 4% PFA immediately after the UV irradiation.

For all the fixed cells alkyne-HDX experiments, corresponding D_2O buffers were used to wash the cells three times and then the coverslip was taken out to make an imaging chamber filled with designating D_2O buffers for SRS imaging.

For live-cell BCECF experiments, cells were first loaded with 2 μM BCECF-AM in HBSS for 20 min in the CO_2 incubator and were subsequently treated with 10 μM nigericin in pH=7.35 high K^+ H_2O buffer for 5 min. Cells were switched into high K^+D_2O buffers containing 10 μM nigericin with designated pD and put onto the microscope slide. The image was collected on an inverted confocal microscope (ZEISS LSM 980) using 40x water immersion objective (NA 1.2) with either 445 or 488 nm excitation laser. Both images were acquired using the same PMT which was set to collect photons from 530-570 nm. Control experiments were done under similar conditions yet with the addition of DMSO instead of nigericin in all the buffer solutions.

For live-cells alkyne-HDX experiment for pD sensing, HeLa cells were first incubated with 10 μM nigericin in high K^+ H_2O buffer for 5 min at 37 ˚C. The high K^+ H_2O buffer was then removed, and cells were washed with high K^+ D_2O buffer with 10 μM nigericin of different pD values. We started timing as the cells were washed with high K^+ D_2O buffers. The coverslip with cells was placed onto a microscope slide with spacer filled with 10 μM nigericin high K^+ D_2O buffers. Control experiments were done under similar conditions but with the addition of

DMSO instead of nigericin in all the buffer solutions.

For the PCho and EdU experiment, DMEM culture medium was changed to DMEM medium (FBS-free, Gibco) for synchronization. After synchronization, medium was replaced back to DMEM culture medium by simultaneously adding both propargylcholine (100 mM stock in DPBS) and EdU (10 mM stock in DPBS) to the culture medium with a final concentration of 1 mM and 100 μM, respectively, for 20-24 h. For the live cells alkyne-HDX experiments, home-made DMEM-D_2O buffer through dissolving DMEM powder into D_2O, was used to wash the cells three times. Then the coverslip was taken out to make an imaging chamber filled with the DMEM-D_2O buffer for SRS imaging.

Synthesis and purification of dsDNA and ssDNA

Pwo DNA polymerase, an enzyme showing good performance to accept modified nucleotides, was used to incorporate EdUTP into DNA through PCR process. A typical PCR reaction contained ~100 ng plasmid template, 0.05 mM each of the forward and reverse primers, 2.5 U polymerase and 10x polymerase buffer with magnesium, 100μM of dNTPs (dATP, dCTP and dGTP, NEB) instead of dTTP and 100μM modified EdUTP. The reactions were done in an overall volume of 50 μL with the addition of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen). For ssDNA synthesis, asymmetric PCR was used. The concentration of the forward primer and the reverse primer is 0.001 mM and 0.05 mM, respectively, while other conditions are the same with that indicated above. PCR experiments were performed on a Bio-Rad C1000 thermal cycler.

Primers and the template employed for PCR experiments:
Forward primer: 5´- GGAAATCGGTACTGGCTTTCCATTCGAC
reverse primer: 3´- GTGAGTTAAAGTTGTACTCGAGTTTGTGTCCG
Template (sequence of 746 bp, contains 382 T):
GGAAATCGGTACTGGCTTTCCATTCGACCCCCATGATGGTTCCGTTCAACTA
GCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACC
AGACAACCATTACCTGTCGACACAATCTGTCCTTTCGAAAGATCCCAACGAA
AAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACAC
ATGGCATGGATGAGCTCTACAAAGGCGGTGGGTCGGGCGGGGGCTCCCCC
GGGGGTGGCGGTTCATGATCAGGTGGAGGGTCAGGGGGCGGATCAATGAG
CAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATG
GTGATGTTAATGGGCACAAATTTTCTGTCCGTGGAGAGGGTGAAGGTGATGC
TACAAACGGAAAACTCACCCTTAAATTTATTTGCACTACTGGAAAACTACCTG
TTCCATGGCCAACACTTGTCACTACTCTGACCTATGGTGTTCAATGCTTTTCC
CGTTATCCGGATCACATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCG
AAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGACCTACAAG

ACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTT
AAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGACACAAACTCGAGT
ACAACTTTAACTCAC

PCR products were purified with a Monarch PCR & DNA Cleanup Kit (NEB) and confirmed by gel electrophoresis and Sanger sequencing. The products of PCR reaction were separated by 1% agarose (UltraPure™ Low Melting Point Agarose, Invitrogen) gel electrophoresis. dsDNA and ssDNA were purified with a Monarch Gel Extraction Kit (NEB). Exonuclease I (Thermo Scientific, 20 U/μL) was used to digest ssDNA in 37℃, which was confirmed by gel electrophoresis as well. Before enzyme digestion, 5 min 95℃ heat shock was used for denaturation. The images were recorded with Bio-Rad Gel and Blot Imaging Systems. The combined PCR products (~50 tubes per sample) were loaded into Microcon-10kDa Centrifugal Filter (Millipore Sigma) and washed with UltraPure™ DNase/RNase-Free Distilled Water three times to remove the remaining salts and EDTA in the elusion buffer through buffer exchange. Then the PCR products in pure water were concentrated into ~1 uL through vacufuge (Vacufuge Plus Concentrator, Eppendorf). The concentrated PCR products were diluted into DPBS-D_2O / DPBS-D_2O + 2 M NaCl buffer solutions for alkyne-HDX kinetics measurements.

Formation and purification of EdU dimers

For solution samples, EdU (or thymine) was dissolved into water to make saturated solutions. After frozen into ice, the EdU (or thymine) solution was put into a homemade dry ice chamber and was irradiated with a UV lamp (254nm, UVLS-24 EL Series UV Lamp, 4 Watt) for 6 hours. Analytical HPLC coupled with mass spectrometry (LC-MS) for the UV-irradiated EdU product was performed on Agilent 1290 infinity LC system using ZORBAX RRHD Eclipse Plus C18, 95Å, 2.1 x 50 mm, 1.8 μm column with Agilent 6140 Series Quadrupole LCMS/LC-MS/MSD/Mass Spectrometer System. The mobile phase is water (0.1% AcOH) and acetonitrile with a running method of gradient 40% - 95% acetonitrile (1 ml/min, 10 min for total running time). The data shown in the supplementary figures (Figure S6e) are the absorption (254 nm) intensity traces.

The aqueous reaction mixture (100 mg) post UV-radiation was first concentrated under reduced pressure at 40 ˚C and was then load on a reverse phase Biotage cartridge (12g SNAP Ultra C18). The flash column chromatography was automated by the Biotage Isolera System, with acetonitrile and water as the mobile phases. The flushing gradient was set to 5% to 40% acetonitrile over 10 column volume (CV) and the EdU dimer should be the second UV active portion eluted around the second to third CV. The collect portion was confirmed by normal phase TLC. EdU dimer would have an R_f value of 0.15 and EdU has an R_f of 0.6 when

running with pure Ethyl Acetate. The purified EdU dimer was confirmed through high-resolution mass spectrometry (calculated exact mass: 505.1571, detected mass: 505.1575) and dissolved into H_2O to prepare a stock solution for further alkyne-HDX measurements.

Immunofluorescence staining

The UV-irradiated cells and control cells were first fixed and treated with 0.2% Triton-X-100 in 1x DPBS (no calcium, no magnesium, Gibco) at room temperature for 30 min. Then the Triton-X-100 was removed, and cells were washed with 1x DPBST (DPBS + 0.1% Tween 20) three times. Cells were incubated with 1% BSA, 22.52 mg/mL glycine in PBST at room temperature for 60 min for blocking. After washed with PBST, cells were incubated with 1:300 diluted anti-thymine dimer antibody (Mouse monoclonal, ~2 mg/mL, MilliporeSigma) in 0.1% BSA, PBST overnight at 4°C. The primary antibody solution was removed, and cells were washed three times with DPBST. Then cells were incubated with 3% BSA in PBST at room temperature for 60 min for blocking. After washed with PBST, cells were incubated with 1:400 diluted Goat anti-Mouse IgG (H+L), (Invitrogen, Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 647, 1 mg/mL) in 0.1% BSA, PBST for 2 hours at room temperature. The secondary antibody solution was removed, and cells were washed three times with DPBST. Fluorescent imaging was conducted immediately after sample preparation through the same Olympus FV3000 confocal microscope with CW laser excitation (640 nm, Coherent OBIS LX laser) and standard bandpass filter sets.

Data processing

All spectra were processed using LabSpec6 software. Spectral baselines were subtracted. Peak centers and intensity were read out by Gaussian peak fitting. All images were processed using ImageJ software. Corresponding off-resonance images were subtracted.

For precise $t_{1/2}$ fitting, we obtained the time-zero intensity (i.e. I_0(R-C≡C-H)) by the normalization of I_1(R-C≡C-H) and I_1(R-C≡C-D), the intensity measured from the first exchange data point at the alkyne-H and the alkyne-D channels, respectively. I_0(R-C≡C-H) is defined as I_1(R-C≡C-H)+I_1(R-C≡C-D)/r, in which r is the intensity correction ratio, defined as dividing I(R-C≡C-D), the alkyne-probe solution after equilibrium in D_2O (i.e. finished with exchange), by I(R-C≡C-H), the corresponding alkyne-probe solution in H_2O with the same concentration without any exchange. This strategy was used for both the spontaneous Raman measurement of solution samples and the SRS imaging recording of cell samples. I(R-C≡C-H) and I(R-C≡C-D) from spontaneous Raman measurements were read out by Gaussian peak fitting. I(R-C≡C-H) and I(R-C≡C-D) from SRS measurements (also refer as SRS_H

and SRS_D) were read out from all the nucleus regions in one field of view based on SRS images.

Derivation of alkyne-HDX kinetics

The rate-determining step (RDS) in HDX between alkyne-tagged probes (R-C≡C-H) and catalytic base OD^- is shown in Eq. (1) below. It includes three elementary steps: a) diffusional collision, b) equilibrium redistribution of the hydrogen in the intermediate state, and c) dissociation.

$$R-C\equiv C-H+OD^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R-C\equiv C-H\ldots O-D^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R-C\equiv C^-\ldots H-O-D \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} R-C\equiv C^-+HOD \quad (1)$$

We can assume that: 1) the temperature remains constant; 2) the reaction is diffusion-limited; 3) the intermediates are in steady states. So the concentrations of the two intermediates remain the same in the reaction, shown in Eq. (2).

$$\frac{d[(R-C\equiv C-H\cdots OD)^-]}{dt}=\frac{d[(R-C\equiv C\cdots HOD)^-]}{dt}=0 \quad (2)$$

i.e.

$$k_1[R-C\equiv C-H][OD^-]-k_{-1}[(R-C\equiv C-H\cdots OD)^-]+k_{-2}[(R-C\equiv C\cdots HOD)^-]-k_2[(R-C\equiv C-H\cdots OD)^-]=0$$

$$k_2[(R-C\equiv C-H\cdots OD)^-]-k_{-2}[(R-C\equiv C\cdots HOD)^-]-k_3[(R-C\equiv C\cdots HOD)^-]=0$$

So we can get the concentration of the intermediate shown in Eq. (3).

$$[(R-C\equiv C\cdots HOD)^-]=\frac{k_1k_2}{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}[R-C\equiv C-H][OD^-] \quad (3)$$

The overall rate (k) for transferring a proton from R-C≡C-H to OD^- then becomes:

$$k=\frac{d[R-C\equiv C^-]}{dt}=k_3[(R-C\equiv C\cdots HOD)^-]=\frac{k_1k_2k_3}{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}[R-C\equiv C-H][OD^-] \quad (4)$$

Since the reaction is diffusion-limited, there are some restrictions on rate constants:

$$k_{-1}\ll k_2 \text{ or } k_3\ll k_{-2}, \qquad k_{-1}\approx k_3$$

So the overall rate can be simplified as Eq. (5).

$$k=\frac{k_1}{\frac{k_{-2}}{k_2}+1}[R-C\equiv C-H][OD^-] \quad (5)$$

In addition, $\frac{k_{-2}}{k_2}=10^{pK_a(R-C\equiv C-H)-pK_a(HOD)}\gg 1$, so Eq. (5) can be further simplified as Eq. (6).

$$k=k_1\cdot(10^{pK_a(donor,\ R-C\equiv C-H)-pK_a(acceptor,\ HOD)}+1)^{-1}\cdot[OD^-]\cdot[R-C\equiv C-H] \quad (6)$$

k_1 is the diffusion-limited collision constant, upper-bounded by 10^{10} $M^{-1}s^{-1}$. As

fluctuation of OD^- concentration is negligible during HDX, the RDS is considered as a pseudo-first-order reaction. Since the acceptor pK_a is much smaller than the donor pK_a, the corresponding exchange half-life ($t_{1/2}$) is reduced to Eq. (7),

$$\lg(t_{1/2}) = pK_a - pD + lg\,(ln2) - 10 - pK_a\,(HOD) + pK_a(D_2O) \qquad (7)$$

Here pK_a designates the donor pK_a, i.e. $pK_a(R - C \equiv C - H)$. Taking the pK_a of alkynyl hydrogen as 20-25 and the pK_a of HOD as ~15, we estimated the $t_{1/2}$ of alkyne-HDX in physiological pD (7.6) to be on the order of minutes. For example, if we used 20 for pK_a of alkynyl hydrogen, the calculated $t_{1/2}$ could be 174 s.

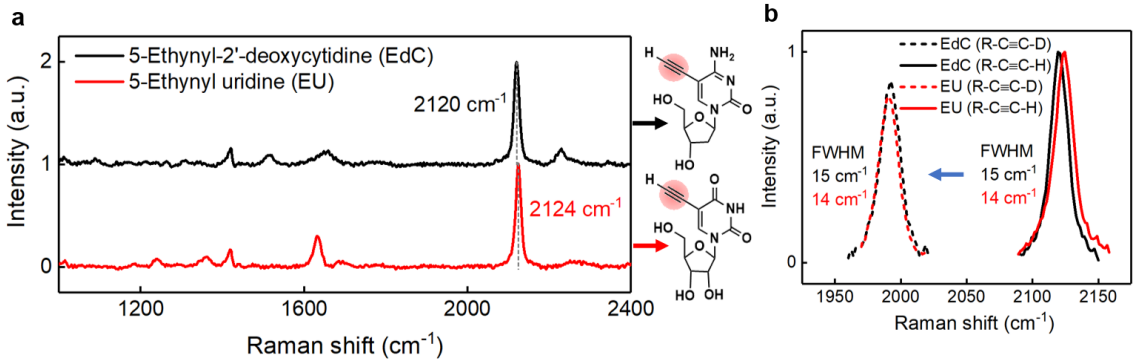

Figure S1 Characterizations of hydrogen-deuterium exchange on terminal alkyne groups (alkyne-HDX) by spontaneous Raman spectroscopy. a) Chemical structures and the corresponding spontaneous-Raman spectra of EdC (10 mM) and EU (10 mM) solutions. Pink-shade highlight the terminal alkynes. b) Spontaneous-Raman peaks of alkyne in EU (10 mM, red) and EdC (10 mM, black) solutions before (solid lines) and after (dashed lines) HDX.

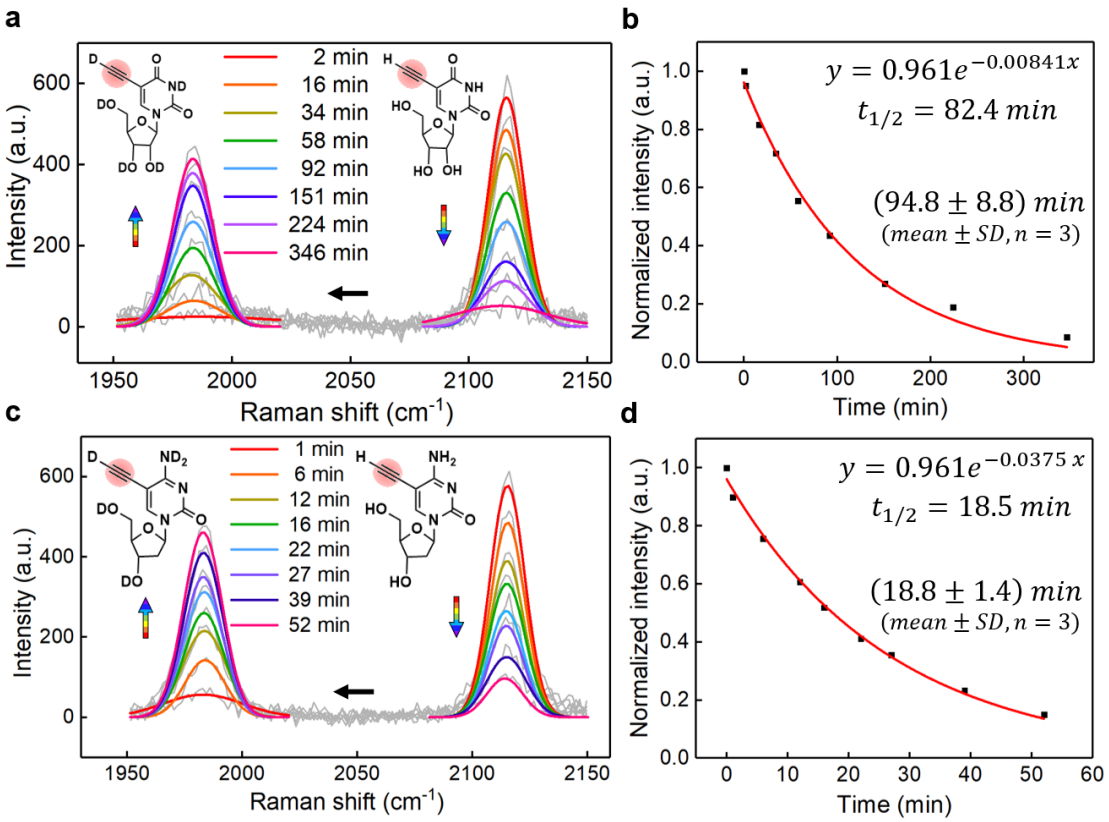

Figure S2 The alkyne-HDX rates for EdC and EU solutions. a) The Kinetics trace of the spontaneous Raman spectra for the decrease of alkyne-H peaks and the increase of alkyne-D peaks from EU (10 mM) during alkyne-HDX in the pD=7.6 D_2O buffer solution. b) Exponential curve fitting of normalized alkyne peak intensities for EU (10 mM) from (a). The average of $t_{1/2}$ over three independent measurements is also shown. c) The kinetics trace of the spontaneous Raman spectra for the decrease of alkyne-H peaks and the increase of alkyne-D peaks from EdC (20 mM) during alkyne-HDX in the pD=7.6 D_2O buffer solution. d) Exponential curve fitting of normalized alkyne peak intensities for EdC (20 mM) in (c). The average of $t_{1/2}$ over three independent measurements is also shown.

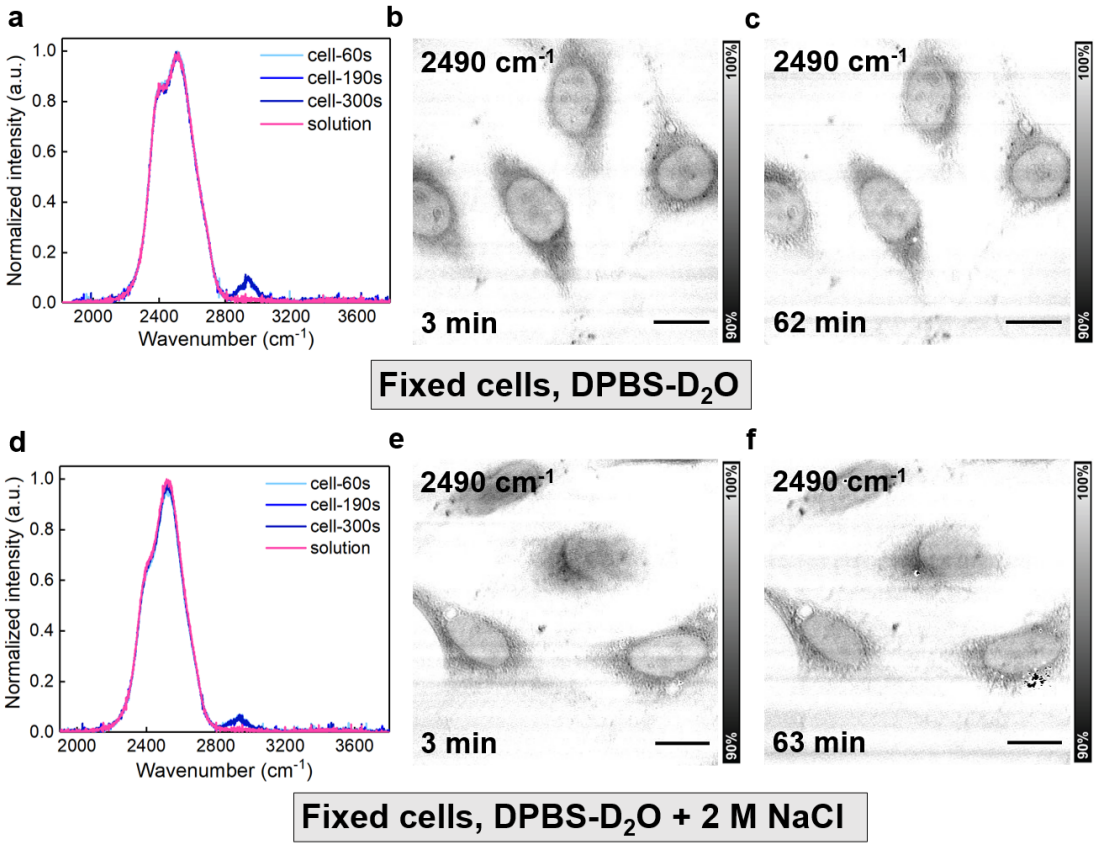

Figure S3 Fast D_2O diffusion across cells in solutions with different osmolarities. a) Time-trace spontaneous Raman spectra in the nuclear region of a fixed cell in the DPBS-D_2O solution. The solution spectrum is taken in the surrounding region without cells from the same sample. b-c) SRS image of O-D vibrational peak (2490 cm^{-1}) for the same set of fixed cells in DPBS-D_2O buffer solution after 3 min (b) and 62 min (c) incubation. d) Spontaneous Raman spectra in the nuclear region of a fixed cell in the high osmolarity solution (DPBS-D_2O + 2 M NaCl). The solution spectrum is taken in the surrounding region without cells from the same sample. e-f) SRS image of O-D vibrational peak (2490 cm^{-1}) for the same set of fixed cells in DPBS-D_2O + 2 M NaCl buffer solution after 3 min (e) and 63 min (f) incubation. Scale bar: 20 μm.

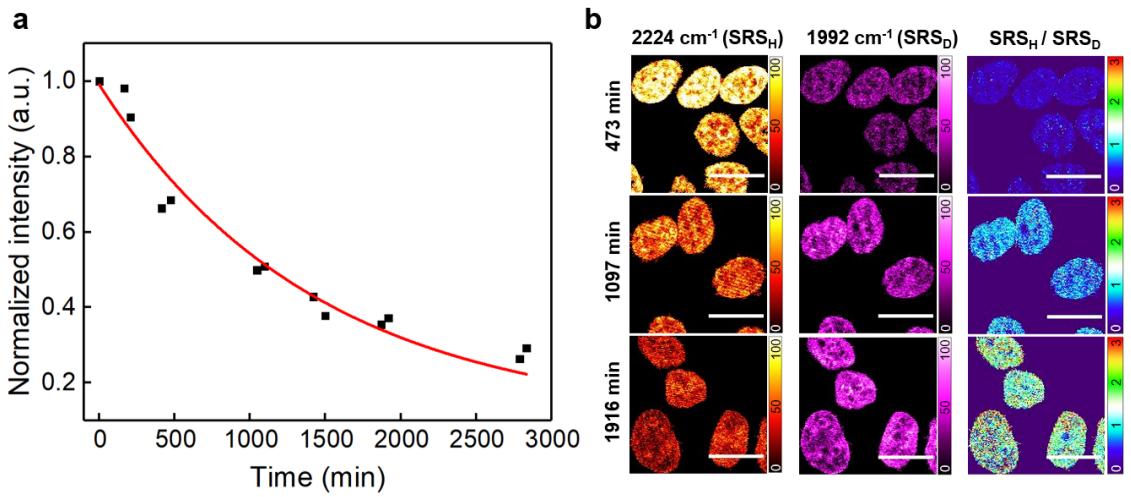

Figure S4 Representative data sets for SRS imaging-based alkyne-HDX kinetics on EdU-labeled cells. a) Exponential curve fitting of normalized alkyne peak intensities for EdU-incorporated cells in a pD=7.6 D_2O buffer solution. b) Representative ratiometric imaging of SRS_D / SRS_H (right) generated by dividing the SRS images at the alkyne-H channel (2224 cm^{-1}) (left) by those at the alkyne-D channel (1992 cm^{-1}) (middle) for cells immersed in DPBS-D_2O from a series of alkyne-HDX time points (473 min, 1097 min, 1916 min).

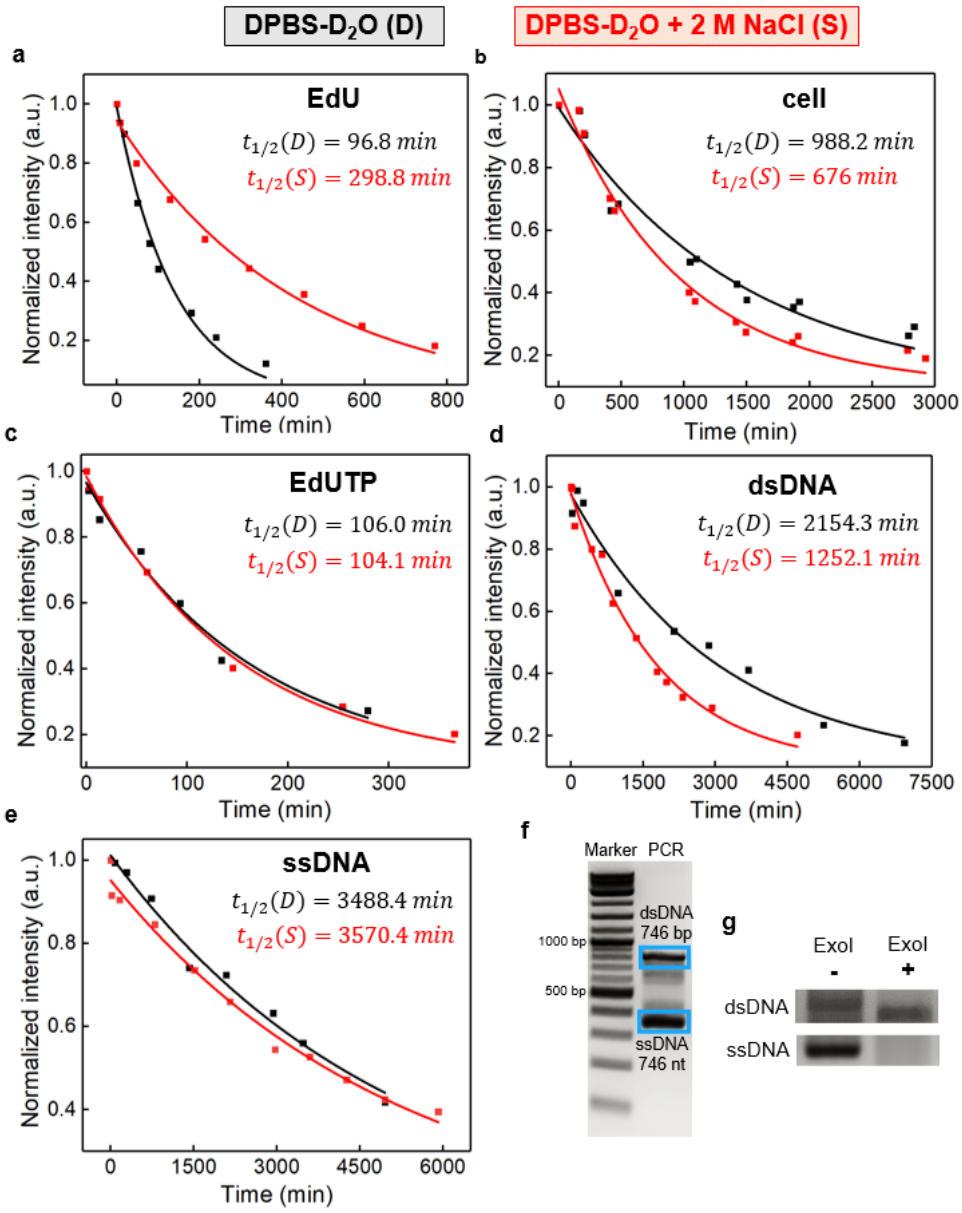

Figure S5 The alkyne-HDX kinetics for EdU and EdU-labeled structures at the regular (DPBS-D_2O, D, black) and high (DPBS-D_2O+2M NaCl, S, red) salt concentration. a-e) Exponential curve fitting of normalized alkyne peak intensities for EdU (10 mM) (a); EdU-incorporated cells (b); EdUTP (10 mM) (c); EdU-labeled dsDNA (d); and EdU-labeled ssDNA (e) in a pD=7.6 D_2O buffer solution (black) and pD=7.6 D_2O buffer solution with 2M NaCl (red), respectively. f) DNA gel electrophoresis for confirming the EdU-incorporated products of dsDNA and ssDNA. Left: 1 kb DNA ladder; right: EdU-labeled PCR products, containing 746 bp dsDNA and 746 nt ssDNA. g) DNA gel electrophoresis confirming the presence of EdU-dsDNA and EdU-ssDNA with Exonuclease I (ExoI) digestion. Left: control PCR products; right: PCR products after Exonuclease I (ExoI) digestion.

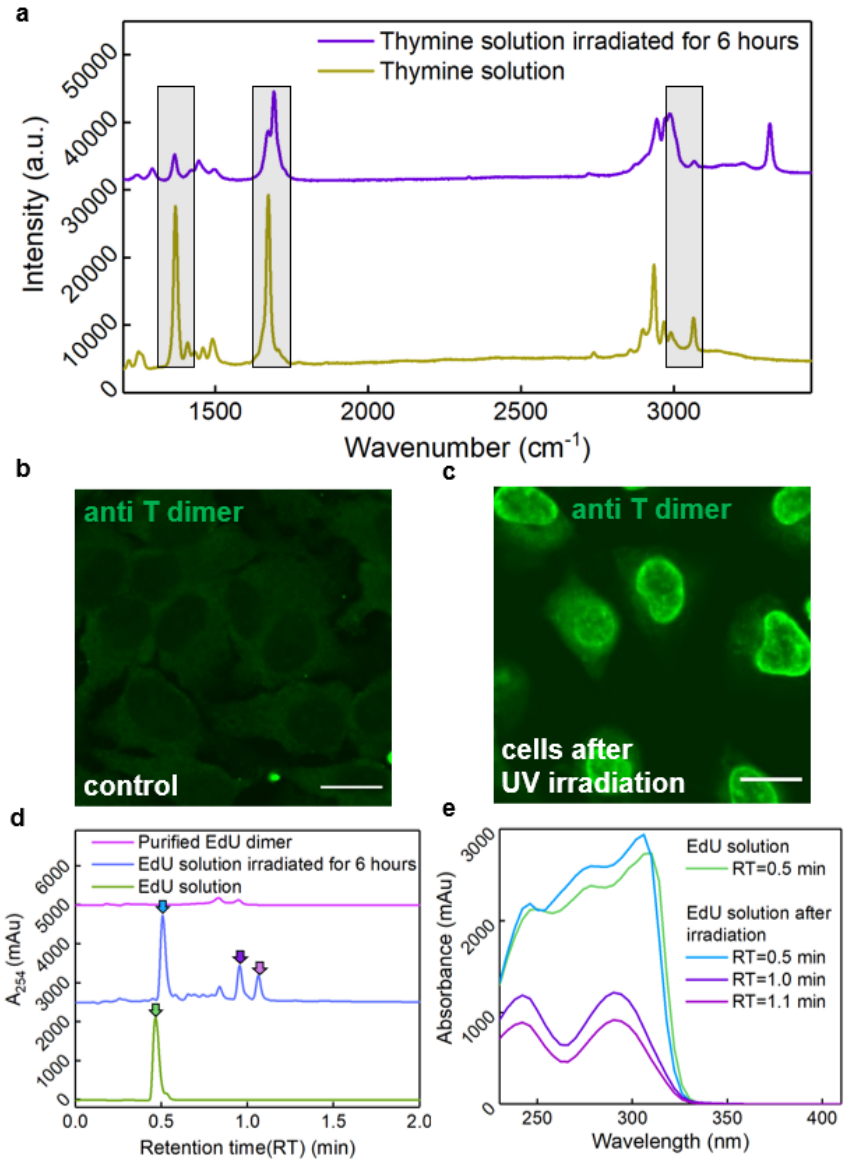

Figure S6 Confirmation for the UV-induced dimer formation. a) Spontaneous Raman spectra of thymine solution (yellow) and thymine solution irradiated for 6 hours (purple). The gray-boxed regions show the featured Raman spectra changes for thymine dimer formation, consistent with what was reported in Ref. 62. b-c) Immunofluorescence staining images with anti-T dimer-Alexa Fluor 647 for control cells without UV irradiation (control, b) and UV-irradiated cells (c). d) HPLC trace of EdU solution (light green), EdU solution irradiated for 6 hours (light purple) and purified EdU dimer (magenta). Arrowed peaks indicate the corresponding color-coded UV absorption traces in (e). e) UV absorption traces of peaks indicated by color-coded arrows in the HPLC trace (d) of the EdU solution before and after UV irradiation.

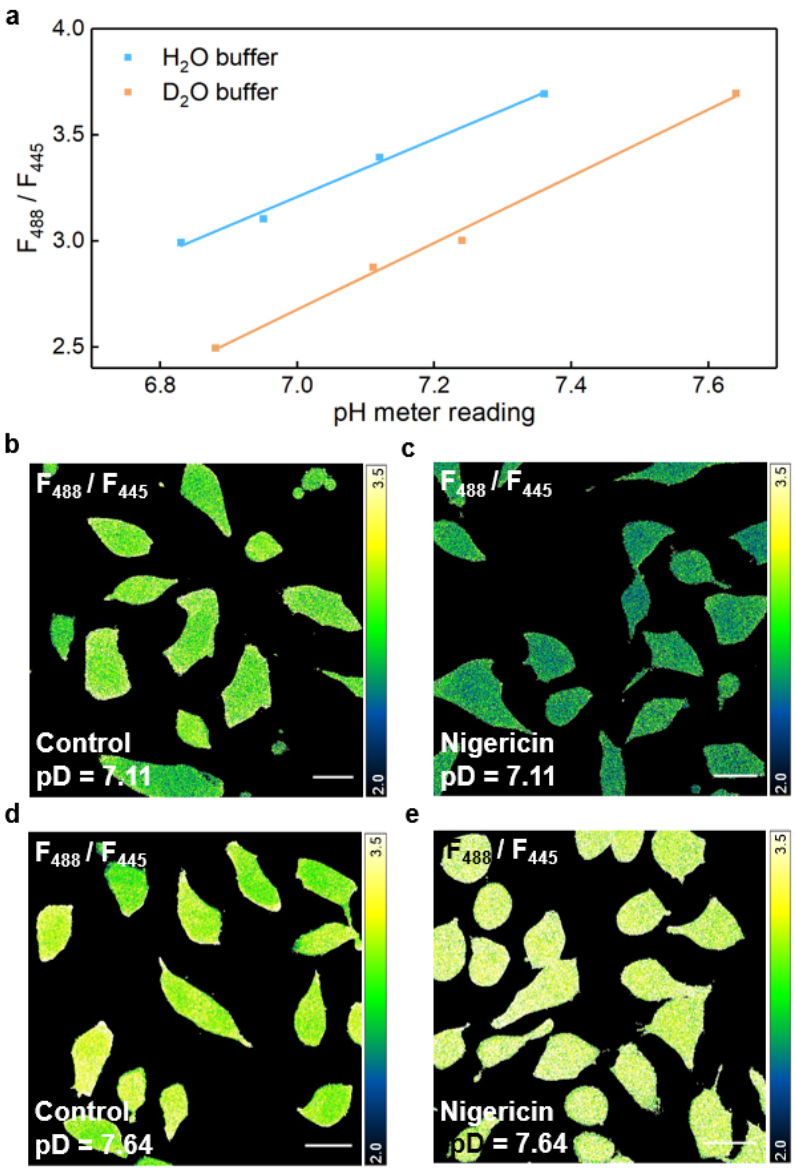

Figure S7 Calibration of solution and intracellular pD by a ratiometric fluorescent pH sensor BCECF. a) Linear relationship between ratios (F_{488} / F_{445}) taken at the 488 nm excitation and 445 nm excitation of BCECF in both H_2O (light blue) and D_2O (orange) buffers with varying reading results from the pH meter over the physiological-relevant range. b-c) Representative ratiometric images (F_{488} / F_{445}) for BCECF in live cells in pD=7.11 DPBS-D_2O buffer without (b, control, average ratio 2.93±0.38) and with nigericin (c, average ratio 2.55±0.31). d-e) Representative ratiometric images (F_{488} / F_{445}) for BCECF in live cells in pD=7.64 DPBS-D_2O buffer, without (d, control, average ratio 3.01±0.31) and with nigericin (e, average ratio 3.34±0.44). Scale bar: 20 μm.

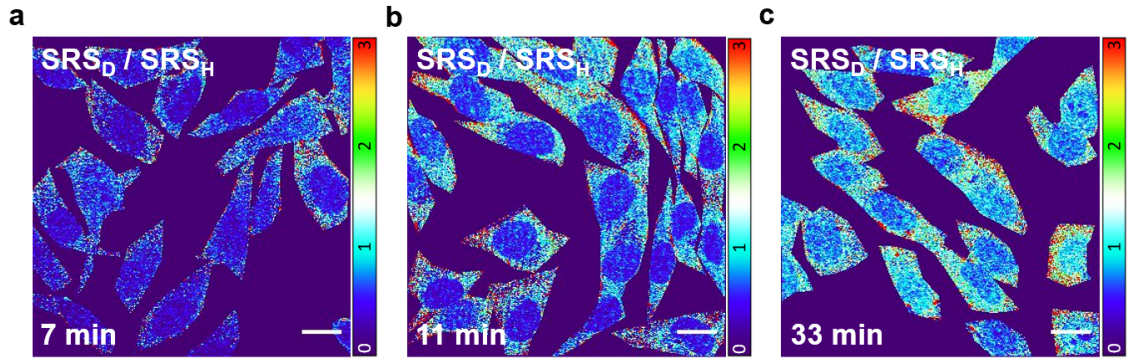

Figure S8 Ratiometric imaging for "two-color" identification of EdU-labeled DNA and PCho-labeled membranes by distinct alkyne-HDX kinetics between the two labeled species. a-c) Ratiometric imaging (SRS_D (1998 cm^{-1})/SRS_H (2137 cm^{-1})) for both EdU and PCho in live cells at three exchange time points. Scale bar: 20 μm.