Electronic Supplementary Material (ESI) for Chemical Communications

Simultaneous imaging of protonated and deprotonated carbonylcyanide *p*-trifluoromethoxyphenylhydrazone in live cells by Raman microscopy

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Supporting Information

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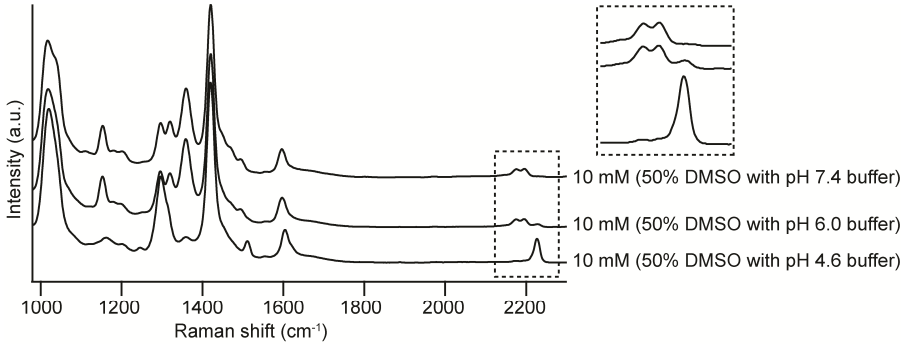
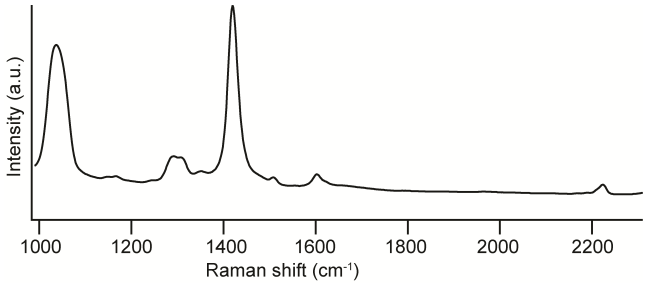
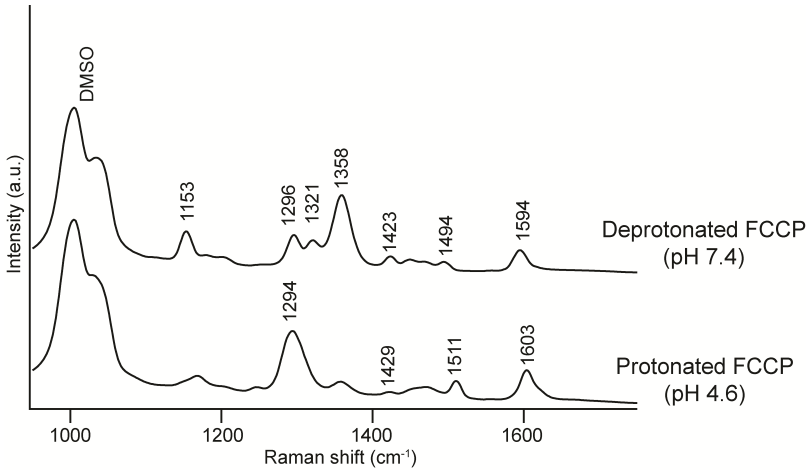


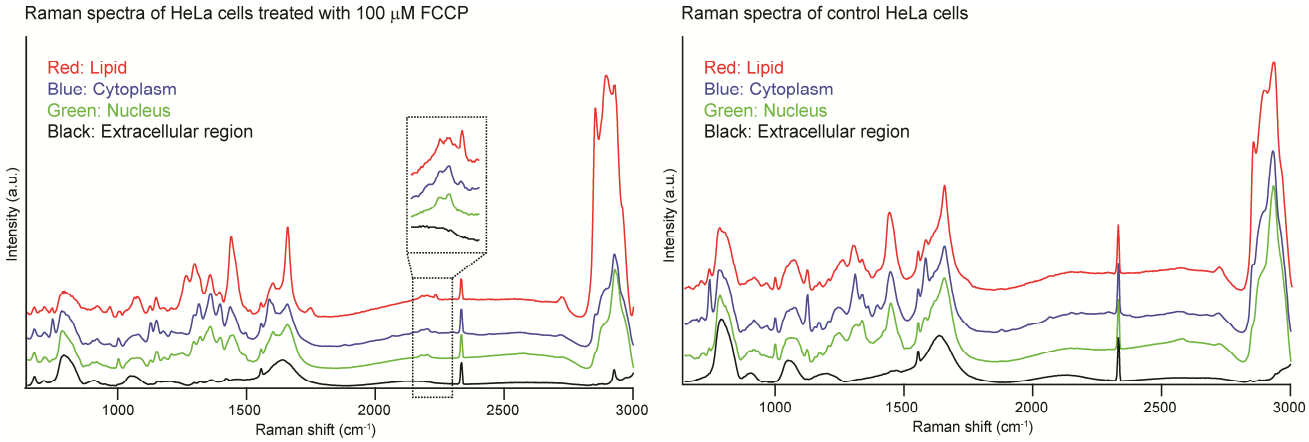
Supplementary Figure 1. Raman spectra of 10 mM FCCP in 50 % DMSO with buffer
Raman spectra were obtained by excitation at 532 nm. The light intensity at the sample plane was 3 mW/μm^2, and the exposure time for each line was 10 sec. Spectra are vertically offset for easy viewing.



Supplementary Figure 2. Raman spectrum of 10 mM FCCP in DMSO
Raman spectra were obtained by excitation at 532 nm. The light intensity at the sample plane was 3 mW/μm^2, and the exposure time for each line was 10 sec.



Supplementary Figure 3. Raman spectra of 10 mM FCCP in 50 % DMSO-*d*6 with buffer
Raman spectra were obtained by excitation at 532 nm. The light intensity at the sample plane was 3 mW/μm^2, and the exposure time for each line was 10 sec. Spectra are vertically offset for easy viewing.



Supplementary Figure 4. Average Raman spectra obtained from HeLa cells
Spectra were obtained from a lipid-rich region (10 points), a cytoplasmic region (10 x 10 pixels), a nuclear region (10 x 10 pixels), and an extracellular region (10 x 10 pixels) of HeLa cells. Spectra are vertically offset for easy viewing.

Experimental Procedure

Cell culture

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (BSA) and antibiotics (penicillin / streptomycin).

Raman Microscopy

Raman spectra of compounds: Raman spectra of compounds were obtained using a slit-scanning Raman microscope (RAMAN-11; Nanophoton, Osaka, Japan) with excitation at 532 nm. The laser output was focused into the sample by a 60X/1.2 NA water-immersion objective lens (UPLSAPO 60XW, Olympus, Tokyo, Japan). The slit width of the spectrograph was 50 μm. The laser power was 3 mW/μm^2.

Raman imaging: All Raman images of live HeLa cells were obtained using a slit-scanning Raman microscope (built in our laboratory by modifying a Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan) with 532 nm excitation (Verdi V-18, Coherent, Santa Clara, CA) and slit-scanning excitation and detection. The laser output is shaped into a line by a cylindrical lens and focused into the sample by a 60X/1.27 NA objective lens (CFI Plan Apo IR 60x WI, Nikon, Tokyo, Japan). Backward Raman scattering signals from the illuminated line are collected by the same objective lens, filtered through a Raman edge filter (Semrock, Rochester, NY) and then imaged at the entrance slit of a dispersive spectrograph (Bunkoh Keiki, MK-300, Tokyo, Japan). Raman light from each position in the illuminated line is dispersed by a grating and detected with a cooled CCD camera (Pixis 400, Princeton Instruments, Trenton, NJ) to obtain the Raman spectra.

To acquire a Raman image, the laser line is scanned in one direction across the sample using a single-axis galvano mirror (GSI Lumonics, Billerica, MA) with a step size of ~0.33 μm. The Raman spectra (700-3000 cm^{-1}) from the pixels in the line are then collected in parallel. The slit width of the spectrograph was 40 μm. The irradiated laser intensity at the sample plane and the exposure time for each line are indicated in the figure captions. The laser intensity was calculated from the ratio of the measured laser power at the sample position and the area of the illumination line. The width of the line is given by 0.61λ/NA, while the length is measured from the brightfield image. All reported image acquisition times take into account the spectral data transfer rate of ~3 s/line from the CCD camera to the PC.

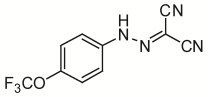
The Raman hyperspectral data set was further processed using the singular value decomposition (SVD) technique for noise reduction.[1] Because of the different amounts of autofluorescence background signal present at each point in the Raman spectrum, a modified polyfit technique was used to determine the autofluorescence baseline signal, which was subtracted from the original Raman spectrum.[2] Finally, the Raman image was constructed by displaying the Raman intensity of the vibrational band of interest at each spatial position. All nitrile images are difference images obtained by subtracting the image at the bottom of the nitrile peak from the image at the center of the nitrile peak.

For Raman imaging, all cell samples were grown on a quartz substrate. Loading concentration of FCCP and incubation times are indicated in the figure captions. Prior to Raman imaging, the medium of the cell sample was replaced with a HEPES-buffered Tyrode's solution composed of (in mM) NaCl, 150; glucose, 10; HEPES, 10; KCl, 4.0; $MgCl_2$, 1.0; $CaCl_2$ 1.0; and NaOH, 4.0.

Chemical Syntheses (General Experimental Procedures)

Nuclear magnetic resonance (NMR) spectra were recorded on JEOL 400; ^{1}H-NMR data are reported with chemical shifts quoted in parts per million (δ p.p.m.) downfield relative to tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Multiplicities are reported using the following abbreviations: s, singlet; m, multiplet. Mass spectra were recorded on a Bruker microTOF-QII-RSL. Flash column chromatography was conducted with standard silica gel (KANTO Co. Ltd., Silica gel 60N, spherical, neutral, 100 ~ 210 μm). All staining for monitoring and visualizing TLC was done with Anis (2.5 % anisaldehyde in ethanol) as the visualizing agent. Anhydrous Et_2O was purchased from KANTO Chemical Co. Ltd. and used directly, and other reagents were used without any purification. Reactions were performed at ambient temperature unless otherwise stated.

Chemical Syntheses (Experimental Procedure and Compound Data)



FCCP

The synthesis of FCCP was based on the report by Hartley et al.[3] Briefly, to a solution of 4-(trifluoromethoxy)aniline (177 mg, 1.0 mmol) in aqueous HCl (1.6 M, 7.0 mL) was added dropwise $NaNO_2$ (85 mg, 1.0 mmol) in H_2O (1.0 mL) at 0 °C. The mixture was stirred for 5 min at 0 °C, and then added dropwise into a stirred solution of malononitrile (99 mg, 1.5 mmol) and NaOAc (2.5 g, 30 mmol) in H_2O (10 mL) at 0 °C. Stirring was continued for 5 min at 0 °C, and Et_2O and H_2O were added to the mixture. The organic layer was separated, and the aqueous layer was extracted with Et_2O twice. The combined layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography ($CHCl_3$ / MeOH = 97 / 3) to give FCCP (225 mg, 89%) as a yellow solid.
Yellow solid; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 9.99 (1H, s), 7.34 (4H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 147.2, 138.2, 122.7, 120.3 (q, *J* = 256 Hz), 117.3, 87.4; MS (ESI) m/z: 253 [$(M-H)^-$]; HRMS (ESI) Calcd. for $C_{10}H_4F_3N_4O$: 253.0343. Found: 253.0366.

References

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2 C. A. Lieber and A. Mahadevan-Jansen, *Appl. Spectrosc*., 2003, **57**, 1363.
3 C. Quin, L. Robertson, S. J. McQuaker, N. C. Price, M. D. Brand and R. C. Hartley, *Tetrahedron*, 2010, **66**, 2384.