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# Deuteration of terminal alkynes realizes simultaneous live cell Raman imaging of similar alkyne-tagged biomolecules†

Syusuke Egoshi, 🝺 <sup>a</sup> Kosuke Dodo, 🕩 \*<sup>a,b</sup> Kenji Ohgane ២ <sup>a</sup> and Mikiko Sodeoka 🕩 \*<sup>a,b</sup>

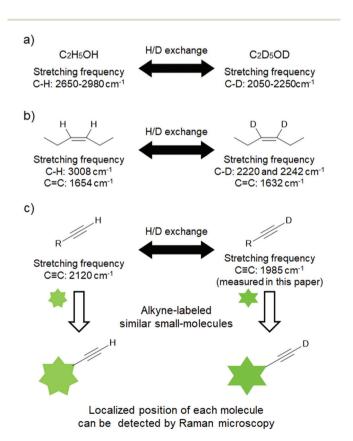
Alkynes were employed as tags to observe small molecules in cells by Raman microscopy. Herein, simple deuteration was found to shift the vibrational frequency of the alkyne by 135 cm<sup>-1</sup>. Twocolor Raman imaging of D-alkynes and H-alkynes made it possible to distinguish between and observe similar small molecules in live cells.

Raman microscopy is a powerful imaging tool for observing endogenous biomolecules, as it can detect molecular vibrations in cells without exogenous labeling.<sup>1</sup> However, cells contain many molecules and display complicated Raman spectra, making label-free Raman imaging of target molecules difficult. To overcome this limitation, several functional groups have been used as Raman tags, including alkynes (C=C), nitriles (C=N), and carbon-deuterium (C-D) bonds.<sup>2-6</sup>

The carbon–carbon triple bond of alkynes exhibits an intense Raman signal in a Raman-silent region  $(1800–2600 \text{ cm}^{-1})$  that is free of interference from natural cellular molecules. Alkynes can be introduced without affecting the biological activities of small molecules and can be selectively detected using a Raman microscope with the technique referred to as alkyne-tag Raman imaging (ATRI).<sup>2,7</sup> Since ATRI was first established by Sodeoka *et al.* in 2011, various alkyne tags (*e.g.*, diynes,<sup>3,8–11</sup> <sup>13</sup>C-labeled alkynes,<sup>12</sup> and *gem*-difluoroalkynes<sup>13</sup>) have been developed to observe biomolecules.

Since D is twice as heavy as H, the Raman scattering of the molecules was significantly affected by deuteration. In fact, C– H bond stretching frequencies of alkanes and alkenes appear in the region of 2650–3050 cm<sup>-1</sup>, while their corresponding C– D stretching frequencies appear in the region of 2050–2250 cm<sup>-1</sup> (*e.g.*, ethanol and ethanol- $d_6$ , Fig. 1a).<sup>14</sup>

Deuteration also affects the vibrational frequency of the C=C bond, which shifts by approximately 20 to 30 cm<sup>-1</sup> (*e.g.*, 1654 cm<sup>-1</sup> for 3-*cis*-hexene and 1632 cm<sup>-1</sup> for 3-*cis*-hexene-3,4 $d_2$ , Fig. 1b).<sup>15</sup> We recently reported Raman imaging of deuterated  $\gamma$ -linolenic acid based on the deuterated C=C stretching signal.<sup>16</sup> Therefore, we anticipated that the C=C vibrational frequency also shifts upon deuteration; deuterated alkynes



**Fig. 1** The effect of deuteration on stretching frequencies detected by Raman microscopy. Comparison of the vibrational frequencies between (a) methanol and methanol- $d_6$  and (b) *cis*-hexene and *cis*-hexene-3,4- $d_2$ . (c) Application of D-labeled alkynes for two-color Raman imaging.

<sup>&</sup>lt;sup>a</sup>Synthetic Organic Chemistry Laboratory, RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. E-mail: dodo@riken.jp,

sodeoka@riken.jp

<sup>&</sup>lt;sup>b</sup>RIKEN Center for Sustainable Resource Science, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan

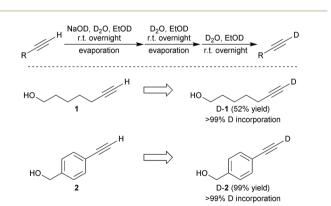
<sup>†</sup>Electronic supplementary information (ESI) available: Experimental procedures, supplemental data, and spectral data. See DOI: 10.1039/d1ob01479j

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(D-alkynes) are expected to offer new tags for live cell Raman imaging (Fig. 1c). Our hypothesis was further supported by the density functional theory (DFT) calculations at the B3LYP/6-31G\* level. The calculated  $C \equiv C$  vibrational frequency of 1-hexyne is red-shifted by 148 cm<sup>-1</sup> upon deuteration (from 2120 cm<sup>-1</sup> to 1972 cm<sup>-1</sup>). This value is much larger than those observed in the replacement of the alkyne carbons with <sup>13</sup>C (*e.g.*, 48 cm<sup>-1</sup> for mono-<sup>13</sup>C-alkyne and 77 cm<sup>-1</sup> for di-<sup>13</sup>C-alkyne).<sup>12</sup> Herein, we demonstrate the potential of D-alkynes as Raman tags for live cell Raman imaging.

First, to determine the potential of D-alkynes as Raman tags, we examined their physical and chemical properties. D-alkynes were synthesized by the treatment of H-alkynes in heavy water (D<sub>2</sub>O) containing strong bases.<sup>17,18</sup> We synthesized deuterated 6-heptyn-1-ol (D-1) from 1 as a non-conjugated terminal alkyne and deuterated 4-ethynylbenzyl alcohol (D-2) from 2 as a conjugated terminal alkyne using the procedure shown in Scheme 1. High incorporation of D (>99%, determined by <sup>1</sup>H-NMR spectroscopy) was achieved by repeating the same reaction three times (Scheme 1). 1 exhibited an alkynyl C-H stretch at 3304 cm<sup>-1</sup> and D-1 displayed an alkynyl C-D stretch at 2595 cm<sup>-1</sup> (Fig. S1a<sup>†</sup>). In addition, 2 exhibited alkynyl C-H stretches at 3268 cm<sup>-1</sup> and 3289 cm<sup>-1</sup>, and D-2 displayed alkynyl C–D stretches at 2575 cm<sup>-1</sup> and 2585 cm<sup>-1</sup> (Fig. S1b<sup>†</sup>). However, the Raman intensity of the C-H/C-D stretching frequencies was too weak to be observed in cells. In addition, the C-H stretching frequency was difficult to detect in aqueous solutions because the Raman signal of water appears as a broad peak from 2800 cm<sup>-1</sup> to 3800 cm<sup>-1</sup>.<sup>19</sup>

On the other hand, the effect of deuteration on the C=C vibrational frequency was very interesting. Upon deuteration, the C=C vibrational frequency of **1** shifted from 2119 cm<sup>-1</sup> to 1985 cm<sup>-1</sup> (D-1), while that of **2** also shifted similarly from 2110 cm<sup>-1</sup> to 1974 cm<sup>-1</sup> for D-2 (Fig. S1†). These changes are more pronounced than those from the deuteration of alkenes (~20 cm<sup>-1</sup>). The Raman peak area ratio corresponding to deuterated and protonated C=C (<0.01) was in good agreement with the ratio estimated by <sup>1</sup>H-NMR analysis (Fig. S1† and <sup>1</sup>H-NMR). Furthermore, strong correlations between the molar ratios of the H-/D-alkynes and the Raman peak area



Scheme 1 Synthesis of D-alkynes (D-1 and D-2) with high D incorporation.

ratios of the H-/D-alkynes were confirmed (Fig. S2†). These results indicate that the real-time H/D exchange of terminal alkynes can be quantitatively evaluated by Raman microscopy. Moreover, the difference in vibrational frequencies was approximately 135 cm<sup>-1</sup> between the H-/D-alkynes, even though they are nearly identical in structure. We therefore speculated that H-/D-alkynes could be employed as Raman tags to distinguish between and observe similar alkyne-tagged small molecules in cells.

In order to pursue the use of H-/D-alkynes in cells, we first verified the stability of the D-alkynes by measuring the timecourse Raman spectra in phosphate buffer (pH 6.0, 7.0, or 8.0) containing 20 mM D-1 or D-2 at 37 °C. Surprisingly, D/H exchange of the D-alkynes was observed at every pH. Moreover, conjugated D-2 reacted faster than non-conjugated D-1 (Fig. 2). The C≡C Raman peak of D-1 disappeared under slightly alkaline conditions after 8 h at pH 8.0. After 12 h at neutral pH, more than 40% of the C≡C signal of D-1 remained. At slightly acidic pH (pH 6.0), more than 90% of the original C $\equiv$ C signal was observed after 12 h (Fig. 2a). Conversely, the C=C Raman peak of D-2 was not detected after only 1 h at pH 8.0 and after 4 h at pH 7.0. At pH 6.0, only 20% of the original C≡C signal remained after 12 h (Fig. 2b). Furthermore, we found that D/H exchange of the D-alkynes at pH 7.0 depends on the temperature of the solution (Fig. S3<sup>†</sup>). These results suggest that nonconjugated D-alkynes under neutral or acidic conditions can be employed as Raman tags in cells.

We also investigated the H/D exchange from the H-alkynes to the D-alkynes in deuterated buffer. The buffer pD was

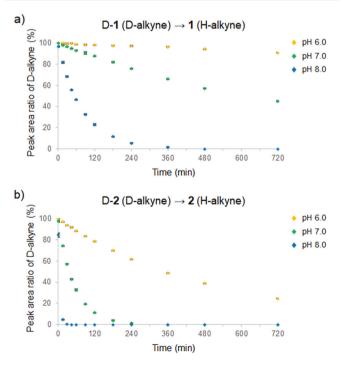


Fig. 2 Real-time analysis of D/H exchange from D-alkynes to H-alkynes at various pH (6.0–8.0) in phosphate buffer at 37 °C of (a) D-1 and (b) D-2. Data are presented as mean  $\pm$  SD (n = 3).

 Table 1
 Apparent D/H exchange rate constants (min<sup>-1</sup>) from D-alkyne to H-alkyne in phosphate buffer<sup>a</sup>

	pН	°C	OH <sup>-b</sup>	D-1 to 1	D-2 to 2
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	7.0	4 20 37	$\begin{array}{c} 1.86 \times 10^{-8} \\ 6.50 \times 10^{-8} \\ 2.32 \times 10^{-7} \end{array}$	$(1.49 \pm 0.03) \times 10^{-5}$ $(1.05 \pm 0.005) \times 10^{-4}$ $(1.16 \pm 0.01) \times 10^{-3}$	$\begin{array}{c} (1.97\pm0.005)\times10^{-3}\\ (1.26\pm0.007)\times10^{-4}\\ (1.69\pm0.003)\times10^{-3}\\ (1.84\pm0.01)\times10^{-2}\\ (1.94\pm0.02)\times10^{-1} \end{array}$

 $^a$  Obtained from data in Fig. 2 and S3.†  $^b$  The value (mol  $\rm L^{-1})$  of [OH<sup>-</sup>] was calculated according to ref. 21.

adjusted according to pD = pH + 0.41<sup>20</sup> As a result, H/D exchange of the H-alkynes was also observed at all pD, with 2 reacting faster than 1 (Fig. S4<sup>†</sup>) in the same manner as D-1 and D-2. We then calculated the apparent rate constants for D/H and H/D exchange of the terminal alkynes (Tables 1 and 2). As the pH/pD value increases by 1.0 or the temperature increases by ~15 °C, the rate constants increase by an order of magnitude. Furthermore, D/H exchange in the pH buffer was faster than H/D exchange in pD buffer of the same value. These results indicate that the D/H or H/D exchange rate strongly depends on the amount of [OH<sup>-</sup>] or [OD<sup>-</sup>] in the buffer. A proportional relationship exists between the D/H or H/D exchange rate constants and the amount of [OH<sup>-</sup>] or  $[OD^{-}]$  in each buffer, which were calculated from the  $pK_w$ values at several temperatures.<sup>21</sup> Theoretically, the calculated amount of [OH<sup>-</sup>] should be 10 times higher than the amount of [OD<sup>-</sup>] for the same pH and pD values, and thus expected D/H exchange rate constants could be 10 times higher than that of H/D exchange. However, the reaction rate constants of D/H exchange differed from the rate constants of H/D exchange by a factor of approximately three in our experiments (Tables 1 and 2). This difference can be attributed to the slower D/H exchange of the D-alkynes than H/D exchange of the H-alkynes, presumably due to the kinetic isotope effect (KIE) of deuterium.

To demonstrate Raman imaging of D-alkynes in live cells, we next turned our attention to long-chain fatty acids (FAs). Saturated FAs (SFAs) exhibit different intracellular metabolism/distribution from unsaturated FAs (UFAs), even with chains of the same carbon number. For example, the C18 SFA octadecynoic acid (3) exhibits a cytoplasmic distribution after accumulating in lipid droplets (LDs),<sup>12</sup> whereas the C18 UFA oleic acid (4) remains accumulating in LDs.<sup>22</sup> In order to

Table 2 Apparent H/D exchange rate constants (min<sup>-1</sup>) from H-alkyne to D-alkyne in deuterated phosphate buffer at 37  $^{\circ}C^{a}$ 

pD	$OD^{-b}$	1 to D-1	2 to D-2
6.0 7.0 8.0	$2.66 \times 10^{-9}$ $2.66 \times 10^{-8}$ $2.66 \times 10^{-7}$	$\begin{array}{c} (3.14\pm0.08)\times10^{-5} \\ (3.78\pm0.1)\times10^{-4} \\ (3.79\pm0.1)\times10^{-3} \end{array}$	$\begin{array}{c} (5.88\pm0.07)\times10^{-4} \\ (6.30\pm0.03)\times10^{-3} \\ (6.14\pm0.06)\times10^{-2} \end{array}$

 $^a$  Obtained from data in Fig. S4.†  $^b$  The value (mol  $\rm L^{-1})$  of [OD<sup>-</sup>] was calculated according to ref. 21.

perform simultaneous live cell Raman imaging of C18 SFAs and UFAs labeled with H-/D-alkyne, the appropriate FAs were prepared. Deuterated 17-octadecynoic acid (D-3, 99% D incorporation by <sup>1</sup>H NMR spectroscopy) was synthesized from 3 following a procedure similar to that shown in Scheme 1 (see Scheme S1<sup>†</sup>). Moreover, the C=C vibrational frequency shifted from 2115 cm<sup>-1</sup> to 1980 cm<sup>-1</sup> upon deuteration of 3 to obtain D-3, and the Raman peak area ratio between the C=C of D-3 and the C=C of 3 resulted in 1:0.013, which is in good agreement with the NMR analysis (Fig. S5a and b<sup>†</sup> and <sup>1</sup>H-NMR).

Next, we performed ATRI of living HeLa cells treated with 3, D-3, and alkyne-tagged oleic acid (5; Raman spectra are shown in Fig. S5c†). SFA 3 and D-3 initially accumulated in LDs during the first 6 h of treatment, and then spread throughout the cells during the remaining treatment time (Fig. 3b and c). Conversely, UFA 5 remained within the LDs during the 24 h treatment time (Fig. 3d). Since time-course Raman imaging of D-3 and 3 showed almost the same images (Fig. 3b and c), we concluded that deuteration has little effect on the intracellular metabolism of these labeled compounds. D/H exchange of D-3 in the lipid region was calculated; only 10% of D-3 was converted to 3 after treatment with HeLa cells for 6 h (Fig. 4). These results clearly indicate that D-alkynes are viable as Raman tags, exhibiting Raman vibrational frequencies that are distinct from the H-alkynes. Simultaneous Raman imaging of

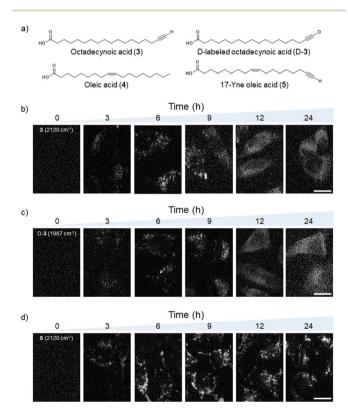
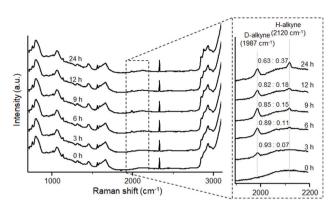


Fig. 3 (a) Structures of the C18 fatty acids. (b–d) Raman images of H-alkynes and D-alkynes in HeLa cells treated with 100  $\mu$ M C18 fatty acids at various times (0–24 h). Raman images obtained from the intensity of the C=C signal of (b) 3 detected at 2120 cm<sup>-1</sup>, (c) D-3 detected at 1987 cm<sup>-1</sup>, and (d) 5 detected at 2120 cm<sup>-1</sup>. Scale bars are 10  $\mu$ m.



**Fig. 4** Average Raman spectra of the lipid region ( $20 \times 20$  pixels) of ten HeLa cells treated with 100  $\mu$ M D-3. Raman spectra after various incubation times (0–24 h). The Raman peak area ratios between D-alkyne and H-alkyne signals at each treatment time are shown.

D-3 and EdU (an alkyne-tagged dT analog that is known to incorporate in nuclear DNA)<sup>23</sup> was performed by treating HeLa cells with D-3 for 6 h after 22 h cultivation with EdU to obtain two-color Raman images (Fig. S6†).

To further demonstrate the utility of D-alkynes as Raman tags, we performed simultaneous live cell Raman imaging of D-3 and 5 at several concentration ratios (Fig. 5). At higher concentrations, both 3 and D-3 spread faster throughout the cells (Fig. S7 $\dagger$ ). Raman imaging of D-3 and 5 at a 4 : 1 molar ratio displayed clear differences in HeLa cell distribution: 5 localized in the LDs, whereas D-3 was observed in both LDs and the cytoplasm. However, at molar ratios of 1:1 and 2:1, the Raman images of D-3 and 5 appeared the same, with both FAs only detected in the LDs. Interestingly, despite the same con-

 D-3 : 5
 D-3 : 5
 D-3 : 5

 100 μM : 100 μM
 200 μM : 100 μM
 400 μM : 100 μM

 D-alkyne (1987 cm<sup>-1</sup>)
 D-alkyne (1987 cm<sup>-1</sup>)
 D-alkyne (1987 cm<sup>-1</sup>)
 D-alkyne (1987 cm<sup>-1</sup>)

 H-alkyne (2120 cm<sup>-1</sup>)
 H-alkyne (2120 cm<sup>-1</sup>)
 H-alkyne (2120 cm<sup>-1</sup>)
 H-alkyne (2120 cm<sup>-1</sup>)

Fig. 5 Raman images of H-alkyne and D-alkyne signals in HeLa cells treated with D-3 and 5 at different dose ratios (1:1, 2:1, or 4:1) for 6 h. Images were obtained from the Raman intensity of the D-alkyne detected at 1987 cm<sup>-1</sup> and H-alkyne detected at 2120 cm<sup>-1</sup>. Two-color Raman images are the overlapped Raman images of the D-alkyne (red) and H-alkyne (green). Scale bars are 10  $\mu$ m.

ditions for D-3 alone and D-3 co-treatment with 5 (200  $\mu$ M treatment for 6 h), D-3 alone began to spread in cells (Fig. S7b†), whereas D-3 with 5 resulted in both remaining in the LDs (Fig. 5). Previous metabolic studies demonstrated that 4 aided palmitic acid (a C16 SFA) distribution to LDs,<sup>24,25</sup> and we surmised that 5 could also function as a guide to facilitate D-3 delivery to LDs in this experiment.

To confirm the consistency of these results (Fig. 5), Raman imaging of HeLa cells treated with SFA 3 and UFA 4 was performed under the same conditions (Fig. S8<sup>†</sup>). Since previously SFA palmitate and 4 were reported to be metabolized into triglycerides (TGs) in LDs after 6 h,<sup>24</sup> most of the C-H Raman signal for the olefin detected at 3015 cm<sup>-1</sup> was considered to be TGs derived from 4 in this experiment.<sup>26</sup> Raman images derived from the alkyne of 3 and vinylic protons of 4 appeared the same in HeLa cells treated with 1:1 and 2:1 molar ratios, with both 3 and 4 localized in the LDs. However, at a 4:1 molar ratio, the Raman images differed slightly: 3 diffused into the cytosol, whereas 4 was detected only in the LDs. These results confirm that the two-color Raman images of D-3 and 5 are not artifacts. Taken together, we successfully demonstrated the utility of D-alkynes and H-alkynes as Raman tags for simultaneous Raman imaging of similar small molecules in live cells.

### Conclusions

In this study, we investigated the effect of deuteration on the alkyne signal in Raman microscopy. We quantitatively measured the H/D or D/H exchange rate of terminal alkynes in aqueous solutions using spontaneous Raman microscopy. The H/D or D/H exchange of terminal alkynes was dependent on its structure (whether conjugated or non-conjugated), pH (pD), and temperature. Furthermore, deuterated compounds are widely used in various research fields (*e.g.*, synthetic chemistry, pharmaceutical science, and materials science) and the D-alkyne functional group is valuable for the synthesis of other deuterated functional groups, such as D-alkenes or D-alkanes,<sup>13,27–29</sup> D-ketones,<sup>30</sup> and D-aromatics.<sup>31</sup> Our findings related to D/H exchange of terminal alkynes provide further insights for the synthesis of D-labeled compounds using D-alkynes.

Furthermore, we demonstrated the utility of a deuterated non-conjugated alkyne as a Raman tag for live cell imaging, and we obtained simultaneous Raman imaging of a C18 SFA and C18 UFA in HeLa cells by monitoring the D-alkyne and H-alkyne signals. D-alkynes and H-alkynes can serve as Raman tags to distinguish between and observe similar small molecules in cells. Live cell Raman imaging of the non-conjugated D-alkyne tag makes it possible to detect short-term accumulation and localization at slightly acidic or neutral pH, or in the hydrophobic environment. D-alkynes are therefore promising for various applications – such as for markers of acidic organelles, as pH sensors, and as indicators to distinguish between hydrophobicity and hydrophilicity – and we expect that they will be more widely employed in diverse scientific fields in the future.

# Conflicts of interest

There are no conflicts to declare.

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