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Imaging of EdU, an Alkyne-Tagged Cell Proliferation Probe, by Raman Microscopy

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Supporting Information

ABSTRACT: Click-free imaging of the nuclear localization of an alkyne-tagged cell proliferation probe, EdU, in living cells was achieved for the first time by means of Raman microscopy. The alkyne tag shows an intense Raman band in a cellular Raman-silent region that is free of interference from endogenous molecules. This approach may eliminate the need for click reactions in the detection of alkyne-labeled molecules.

Click chemistry is considered a promising method for the detection of molecules in cells.[1] In particular, Cu(I)-catalyzed [3 + 2] cycloaddition has been used for detection of various small molecules as well as proteins and nucleotides.[2] The utility of click chemistry is based on the bioorthogonality of alkyne and azide tags. However, the toxicity of the Cu catalyst is problematic for applications to living cells (Figure 1A). To address this issue, Cu-free click chemistry was developed by Bertozzi and co-workers.[3] Strain-promoted cyclization of cyclooctyne derivatives avoids the use of Cu and improves the reaction kinetics. Fluorescent detection of an azide-containing sialic acid moiety on the surface of living cells was beautifully demonstrated with this Cu-free approach (Figure 1B). Here, the strained alkyne is used to introduce the fluorophore and not as a tag for bioactive small molecules, because its relatively bulky structure may affect the properties of the molecule. However, another problem of click chemistry is the reaction time. The click reaction is generally fast, but the detection is not done in real time, which is a drawback for live-cell imaging. Therefore, we focused on direct live-cell imaging of an alkyne-tagged molecule (Figure 1C).

Raman microscopy detects specific vibrational signals of molecules and is potentially a powerful tool for cell imaging, but until just a few years ago it was difficult to obtain high-contrast Raman images of cells because of the low intensity of Raman scattering.[4] We recently succeeded in imaging molecules in living HeLa cells by means of a slit-scanning detection technique[5b] utilizing Raman peaks of cytochrome *c*, protein β-sheet, and lipid molecules.[5] However, there are still only a few examples of live-cell imaging of specific small molecules, and high-contrast Raman imaging remains challenging.[6] Essentially, Raman microscopy enables us to visualize molecules without labeling, but the Raman signals of most molecules overlap with and are difficult to distinguish from those of cellular materials. Fortunately, alkynes show strong Raman scattering at ~2120 cm^{-1}, which is in a Raman-silent region of cells (1800−2800 cm^{-1}). Therefore, alkynes are expected to be excellent as small and bioorthogonal Raman tags.[7] Here we show that the alkyne tag can be employed for click-free imaging of molecules in living cells by means of Raman microscopy. Further, we demonstrate live-cell imaging of an alkyne-tagged nucleotide mimic as a proof of concept.

In medicinal chemistry research, many thymidine analogues have been developed (Figure 2).[8] Among them, BrdU (5-bromo-2′-deoxyuridine) is used for the measurement of DNA synthesis in cells.[8] Recently, EdU (5-ethynyl-2′-deoxyuridine) was reported as an alternative for BrdU based on click chemistry.[9] EdU is readily incorporated into cellular DNA during DNA replication and accumulates in the nucleus.[10] The successful incorporation of EdU represents the benefit of the sufficient smallness of the alkyne tag. Thus, we selected EdU for demonstrating the utility of an alkyne as a Raman tag.

Figure 3 shows the Raman spectra of these thymidine analogues. As expected, an intense alkyne peak (2122 cm^{-1}) for EdU is observed in the silent region, where cellular Raman scattering is minimal, confirming the desirable character of the alkyne moiety as a Raman tag (Figure 3, red line). BrdU and dU (2′-deoxyuridine) showed several intense peaks derived from the pyrimidine base, but they are located in the cellular Raman-active region (Figure 3, blue and green lines).

Raman imaging of living HeLa cells treated with EdU (Figure 4A) or without EdU (Figure 4B) was investigated. HeLa cells were treated with 20 μM EdU for 6 h, and Raman images (excitation at 532 nm) were obtained after excess EdU was washed out. Raman scattering images of the living HeLa cells reconstructed from the distribution of Raman signals at 749, 2123, and 2849 cm^{-1} are shown in Figure 4. As reported previously, the Raman signals at 749 and 2849 cm^{-1} can be assigned to cytochrome *c* and lipid molecules (CH_2 stretching), respectively.[5b] The signal at 2123 cm^{-1} for HeLa cells treated with EdU is obviously derived from the nucleus. In contrast, HeLa cells without EdU treatment

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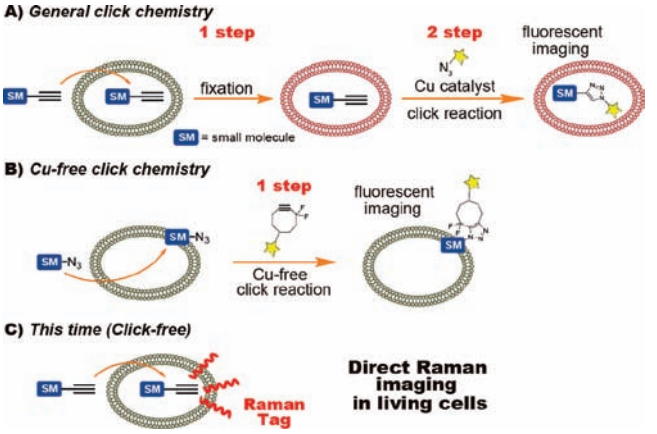

Figure 1. Concept of click-free imaging.

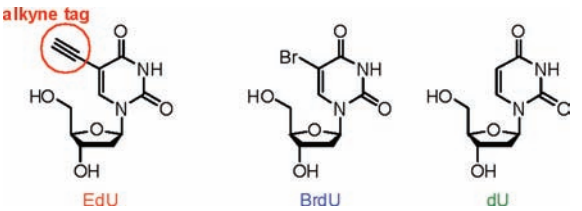

Figure 2. Structures of thymidine analogues.

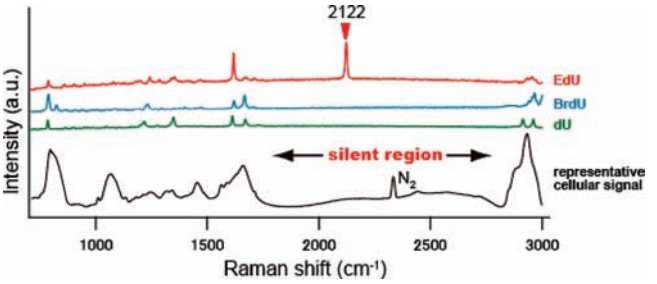

Figure 3. Raman spectra of thymidine analogues and a representative cellular spectrum. The band in the silent region of the cellular signal (2334 cm^{-1}) is derived from N_2.

showed no clear signal at 2123 cm^{-1}. Finally, we confirmed the localization of EdU in fixed HeLa cells under the standard conditions of click chemistry using AlexaFluor azide. EdU was confirmed to be localized in the nucleus, as previously reported (see the Supporting Information).[10] These results indicate that the Raman signal at 2123 cm^{-1} localized in the nucleus is derived from EdU.

Figure 5 shows the average Raman spectra obtained from 10 × 10 pixels in the cytoplasm and nucleus. The alkyne peak (2123 cm^{-1}) was detected in the nucleus but not in the cytoplasm of HeLa cells treated with EdU (Figure 5). As noted above, the alkyne peak at 2123 cm^{-1} is in the silent region where the cytoplasm and nucleus show no endogenous peaks. In contrast, peaks derived from pyrimidine bases cannot be discriminated from other peaks derived from HeLa cells. Thus, the advantage of the alkyne tag for Raman imaging is clear.

Since EdU is commonly used as a cell proliferation probe, we employed our click-free imaging method for monitoring incorporation of EdU into living cells. Time-course Raman

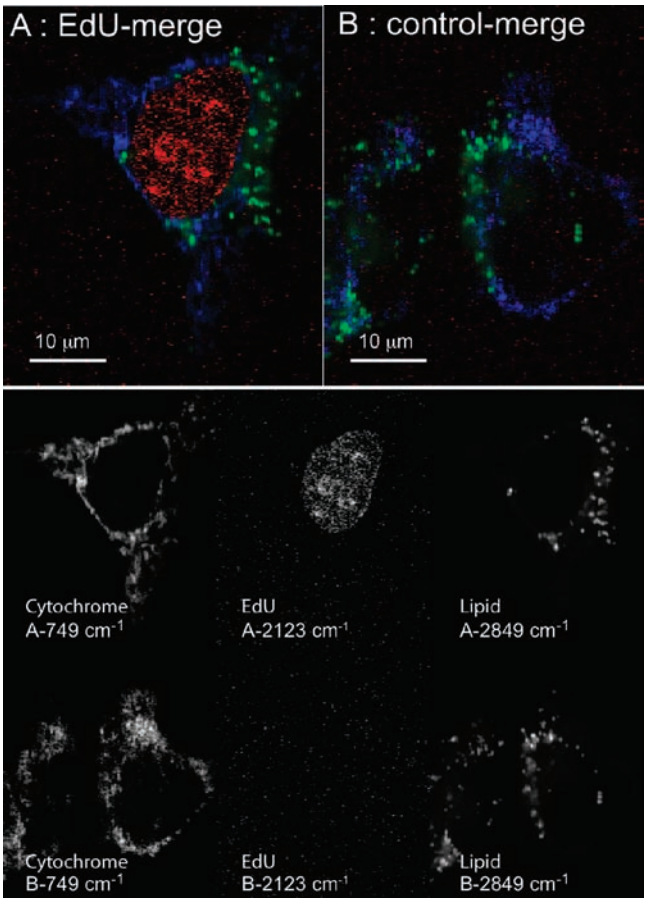

Figure 4. Click-free Raman imaging of EdU in HeLa cells. (A) Raman image obtained from HeLa cells treated with EdU. (B) Raman image obtained from control HeLa cells. Images at 749, 2123, and 2849 cm^{-1} are shown at the bottom. The overlay images at the top were constructed by merging the images at 749, 2123, and 2849 cm^{-1}, which were assigned to the blue, red, and green channels, respectively. The light intensity at the sample plane was 3.52 mW/μm^2. The exposure time for each line was 20 s. The total number of lines of exposure was 127. The image acquisition time was 49 min.

images of living HeLa cells treated with EdU were obtained (Figure 6; also see the Supporting Information). Before the treatment, no cell showed a Raman signal at 2123 cm^{-1} (Figure 6A). Depending on the cell proliferation, the number of EdU-positive cells increased with time (Figure 6B−E). After 21 h, almost all of the cells incorporated EdU (Figure 6F). These results are in agreement with the reported doubling time of HeLa cells (~20 h).[11] Therefore, we have demonstrated the real-time monitoring of active DNA synthesis in living HeLa cells by using an alkyne tag with Raman microscopy.

To further show the potential utility of our technique for live-cell imaging applications, we investigated the detection limit for the loading concentration of EdU and the fastest image acquisition time possible with our current Raman microscope. We found that the threshold concentration for detecting EdU was ~5 μM. On the other hand, an image acquisition time of ~10 min was obtainable (see the Supporting Information). These results show that the current spontaneous Raman technique has sufficient sensitivity and imaging speed to make it useful for real-time detection of small molecules in living cells.

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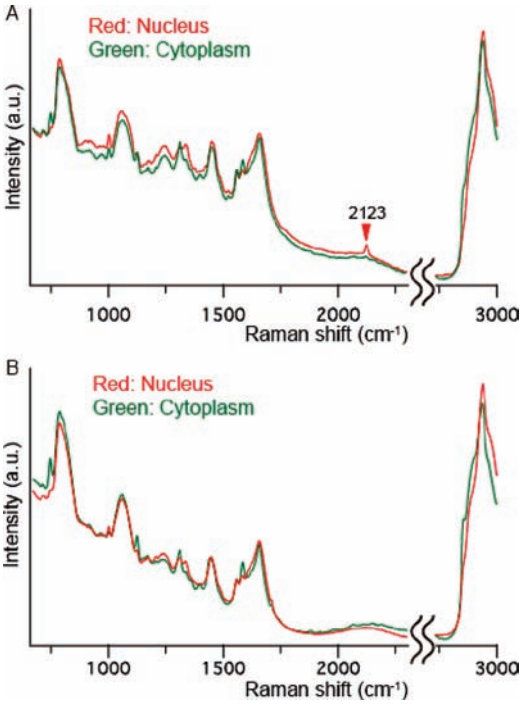

Figure 5. Average Raman spectra obtained from the nucleus (red) and cytoplasm (green) regions (10 × 10 pixels) of HeLa cells (A) treated with EdU and (B) not treated with EdU.

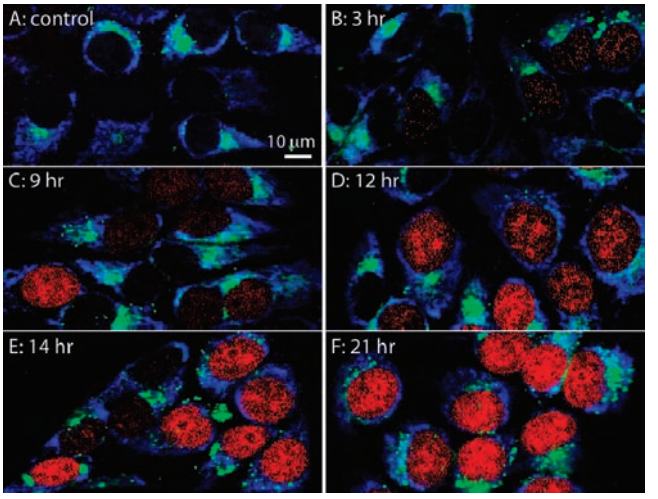

Figure 6. Time-course Raman images of HeLa cells cultured with EdU. The overlay images were constructed by merging images at 749, 2123, and 2849 cm^{-1}, assigned to the blue, red, and green channels, respectively. (A) Raman image obtained from HeLa cells treated without EdU (control). (B−F) Images obtained after treatment with EdU for (B) 3, (C) 9, (D), 12, (E) 14, and (F) 21 h. The light intensity at the sample plane was 6.0 mW/μm^2. The exposure time of each line was 10 s. The total number of lines of exposure for each image was 120, resulting in an image acquisition time of 26 min.

In conclusion, we have achieved click-free visualization of the alkyne-tagged cell proliferation probe EdU by means of Raman microscopy. To our knowledge, this is the first example of direct imaging of an alkyne-modified molecule in living cells. The results demonstrate the potential of the alkyne moiety as an excellent Raman tag for live-cell imaging of small molecules. At least in the case of EdU, the ethynyl group is small enough not to affect the biological activity of the parent small molecule, and this result indicates that click-free imaging using Raman microscopy has the potential to be a powerful methodology for chemical biology research. It should be mentioned that the imaging speed of our approach could be further improved by application of nonlinear Raman techniques such as coherent anti-Stokes Raman scattering (CARS)[12] and stimulated Raman scattering (SRS)[13] microscopy. These efforts are just beginning, and Raman imaging of other small, alkyne-tagged molecules as well as the development of improved instrumentation is under way.

■ ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures and fluorescent images of EdU. This material is available free of charge via the Internet at http://pubs.acs.org.

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