Electronic Supplementary Material (ESI)

Observation of the changes in the chemical composition of lipid droplets using Raman microscopy

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Contents

1. Experiments

Cell culture. HeLa cells were cultured in 2 mL of Dulbecco's modified Eagle's medium (D1145, Sigma) supplemented with 10% fetal bovine serum (10437-028, Sigma), 5×10^4 U/L penicillin G and 50 mg/L streptomycin sulfate (15070-063, Gibco). Cells were incubated without or with 1 mM of oleic acid (159-00246, Wako) and/or elaidic acid (050-04101, Wako) for 1, 2, 4, 8, 12, and 18 hours. 2 wt% bovine serum albumin (08587-84, Nacalai tesque) was added to culture medium in order to improve the introduction of fatty acids into cells.

Fluorescence staining. Nile red (N0659, Tokyo Chemical Industry) (0.1 μg/mL) in Hanks' balanced salt solution (HBSS) (H8264, Sigma) was prepared just before the fluorescence measurement by adding the stock solution (1 mg/mL Nile red in dimethyl sulfoxide (13435-75, Nacalai tesque)) to HBSS. After treatment of HeLa cells with free fatty acid in a glass-bottomed dish (3960-035, Iwaki), the culture medium was removed from the dish, and HeLa cells were washed with HBSS. Thereafter, cells were stained with 2 mL of Nile red (0.1 μg/mL) in HBSS for 10 min at room temperature; subsequently, HeLa cells were washed with HBSS, and the dish was filled with 2 mL of HBSS.

Fluorescence images. Fluorescence measurements were performed by a fluorescence microscope (ECLIPSE Ti-U/B, Nikon) with an objective lens (50x/0.80NA, TU Plan Flour, Nikon). Images were obtained with CMOS camera (WRAYCAM-SR300, WRAYMER).

Raman images. Raman images were obtained using a multi-confocal Raman microscope (Phalanx-R, Tokyo instruments) with an inverted microscope (ECLIPSE Ti, Nikon), as previously described.[1] The output from a frequency-doubled Nd:YVO_4 CW laser (Millennia Vs, Spectra-Physics) at 532 nm was used as the excitation source. The laser beam was split into 10×10 beams (beamlets), which were focused on a sample with an objective lens (60x/1.49NA oil immersion, plan Apo 60X, Nikon). The Raman signals from all the spots were collected to a fibre bundle arranged in a 10×10 square matrix

and subsequently converted into a 1×100 single line output. The single line of Raman signals was introduced into a spectrograph and detected by a thermoelectric cooled CCD camera (iKon-L, ANDOR). Using this system, therefore, 100 Raman spectra from all the spots were simultaneously measured without the scan of the excitation light or the sample stage. Raman images were obtained by integrating the Raman band of interest at each point. During the measurement, temperature around the glass-based dish was maintained at ~ 37°C using a stage top heater (Tokai Hit) on the microscope.

Before the measurement of a Raman image, the culture medium in a glass-based dish was removed, and HeLa cells were washed with HBSS. Thereafter, the glass-based dish was filled with 2 mL of HBSS to avoid fluorescence from the medium. A single Raman spectral image was constructed using the Raman spectra from 900 points (30×30 points with an interval of 1.1 μm). The irradiated laser intensity and exposure time at each point were 0.5–1 mW and 10 s, respectively. Thus, the measurement time for obtaining a Raman image was ~ 90 s because 100 Raman spectra were simultaneously measured using the present multi-confocal Raman microscope.

2. Analyses

The amount of lipid droplets. The amount of lipid droplets (LDs) in HeLa cells was estimated by analyzing the fluorescence images using the image processing program ImageJ. First, the influence of fluorescence from cytoplasm was removed, and then the images were binarized. Finally, LDs in the images were detected as particles, and their number of pixels was automatically counted.

Noise reduction of Raman spectra. All the analyses of Raman spectra were performed using Igor Pro program package (WaveMetrics, Inc.). As previously reported[1], the inhomogeneity of the laser intensity among beamlets was compensated by a Raman image of buffer solution, and singular value decomposition (SVD) analysis was performed for all the spectra. In the SVD analysis, a raw matrix that configures a Raman image (spatial × spatial × spectral) was decomposed into three matrices containing matrices of singular vectors and a diagonal matrix representing singular values in decreasing order. The Raman spectra were reconstructed using 10 major spectral components (corresponding to the first ten singular values), resulting in the reduction of noise having low singular values.

Raman images and spectra of lipid droplets. Raman images of the C-H stretching bands were constructed by the integration of the intensity from 2810 to 3018 cm^{-1}. To extract the Raman spectra of LDs from a Raman image, the integration intensities of the C–H stretching band ($I_{C\text{-}Hstr}$, 2810-3018 cm^{-1}) and the O–H stretching band ($I_{O\text{-}Hstr}$, 3147-3568 cm^{-1}) were calculated for each point. The points where the ratio between $I_{C\text{-}Hstr}$ and $I_{O\text{-}Hstr}$ ($I_{C\text{-}Hstr}$ / $I_{O\text{-}Hstr}$) was larger than 0.22 were evaluated as LDs, and the average of their spectra was calculated. Baseline correction was conducted for the Raman spectra of LDs in the fingerprint region (1100-1800 cm^{-1}), and each spectrum was normalised with the integration intensity of the C–H bending band (1396-1487 cm^{-1}).

Contribution of exogeneous fatty acids in individual lipid droplets. The baseline correction and the intensity normalisation for all the Raman spectra of LDs were carried out in advance as mentioned above. We assumed that the average Raman spectra of LDs in control cells (S_{0h}) and 18-hour fatty-acid-treated cells (S_{18h}) were consist only of endogenous and exogenous lipids, respectively. The spectral region containing CH_2 twisting and =C-H deformation bands (1168-1388 cm^{-1}) in each Raman spectrum (S_{single}) was fitted by a linear combination of S_{0h} and S_{18h}:

$$S_{\mathrm{single}} = B((1 - A) \cdot S_{0\mathrm{h}} + A \cdot S_{18\mathrm{h}}) \quad (1)$$

where A and B are fitting coefficients. Coefficient A corresponds to a contribution rate of the exogeneous fatty acid in each LD.

3. Figures

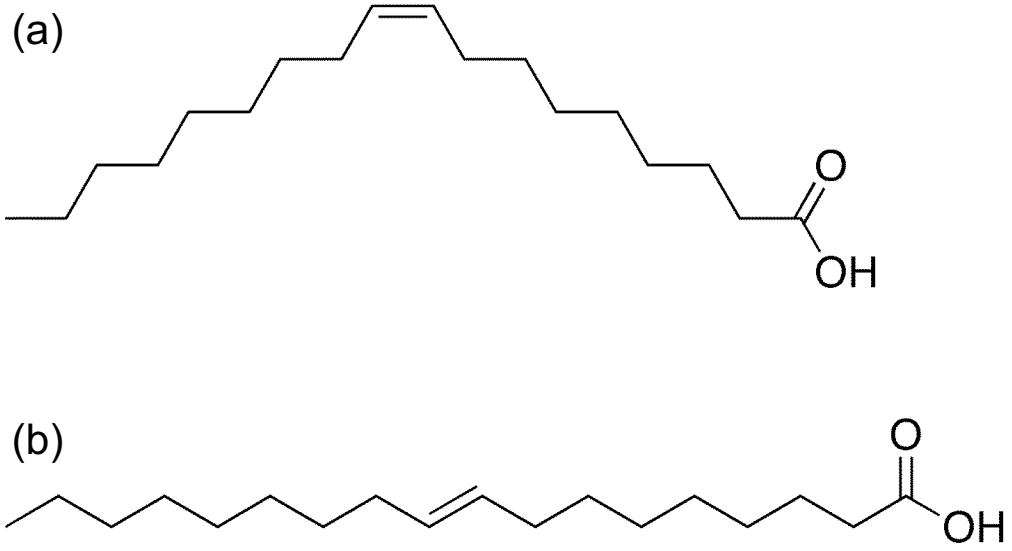

Fig. S1 Molecular structures of (a) oleic acid (OA) and (b) elaidic acid (EA).

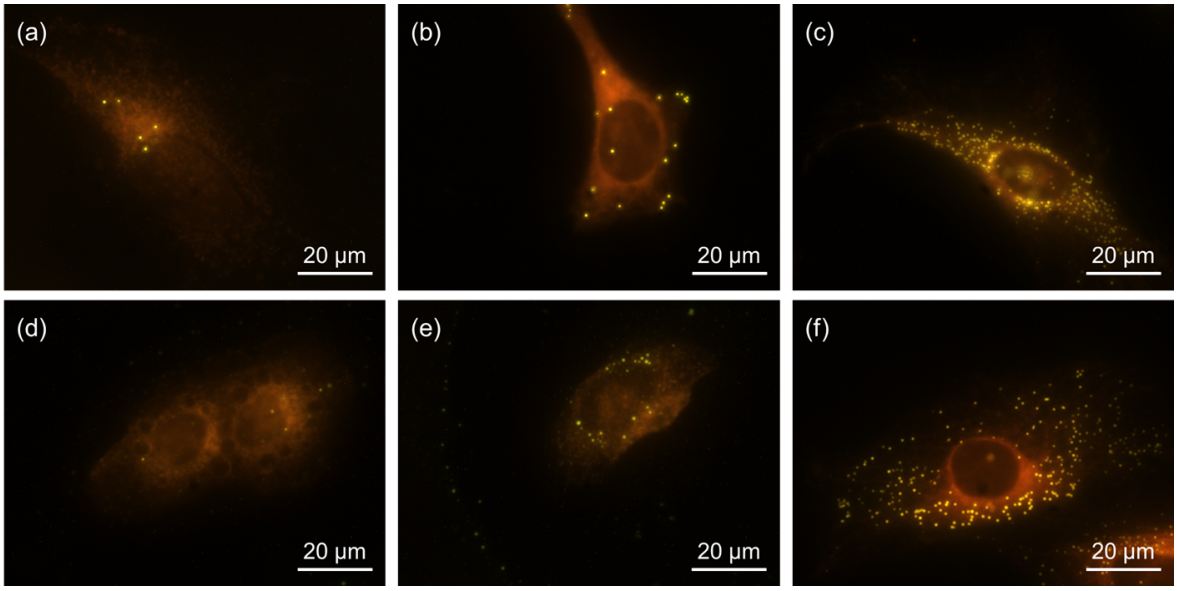

Fig. S2 Fluorescence images of HeLa cells stained with Nile red incubated for 2 h with (a) OA 10 μM, (b) OA 100 μM, (c) OA 1000 μM, (d) EA 10 μM, (e) EA 100 μM and (f) EA 1000 μM.

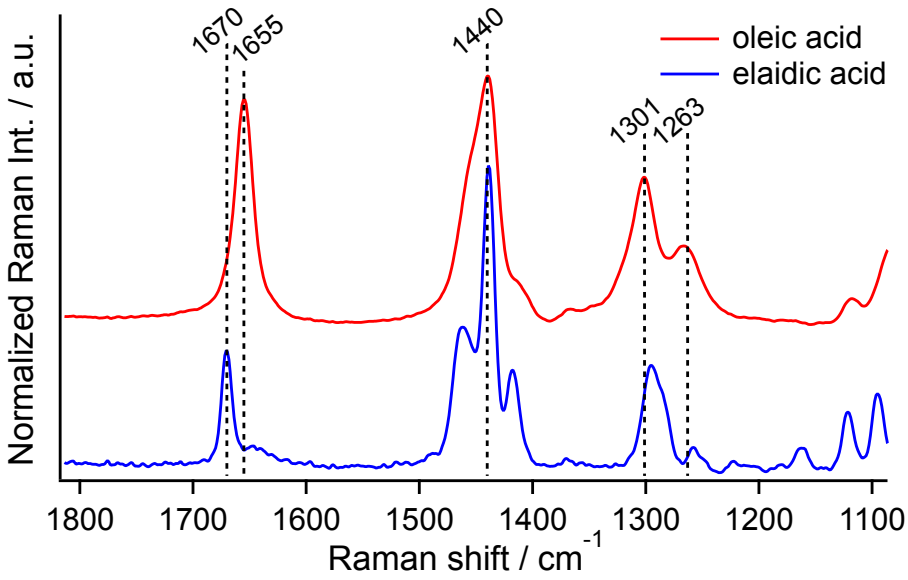

Fig. S3 Raman spectra of pure liquid OA (red) and solid EA (blue).

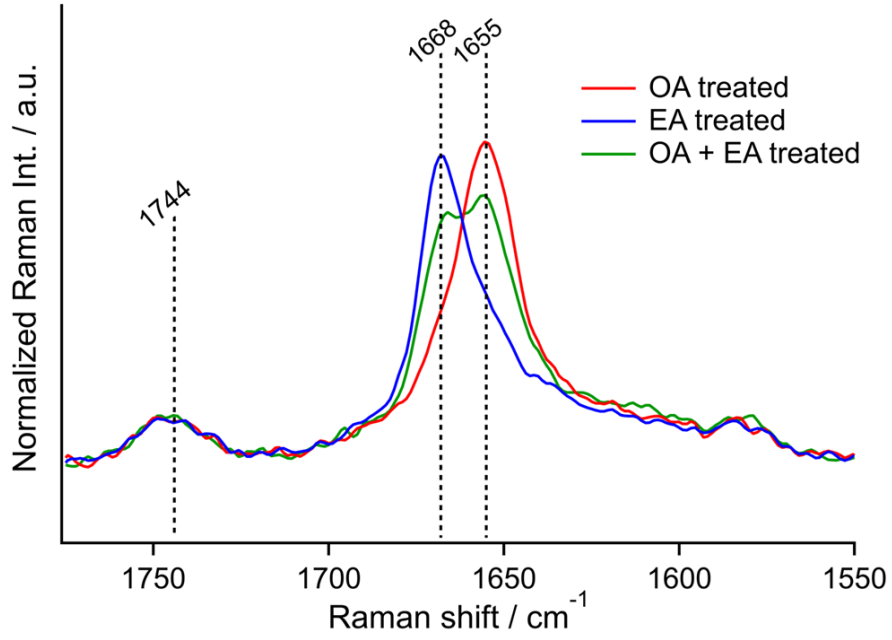

Fig. S4 The average Raman spectra of LDs in HeLa cells: (red) OA-treated, (blue) EA-treated and (green) treated with EA and OA with almost the same concentration.

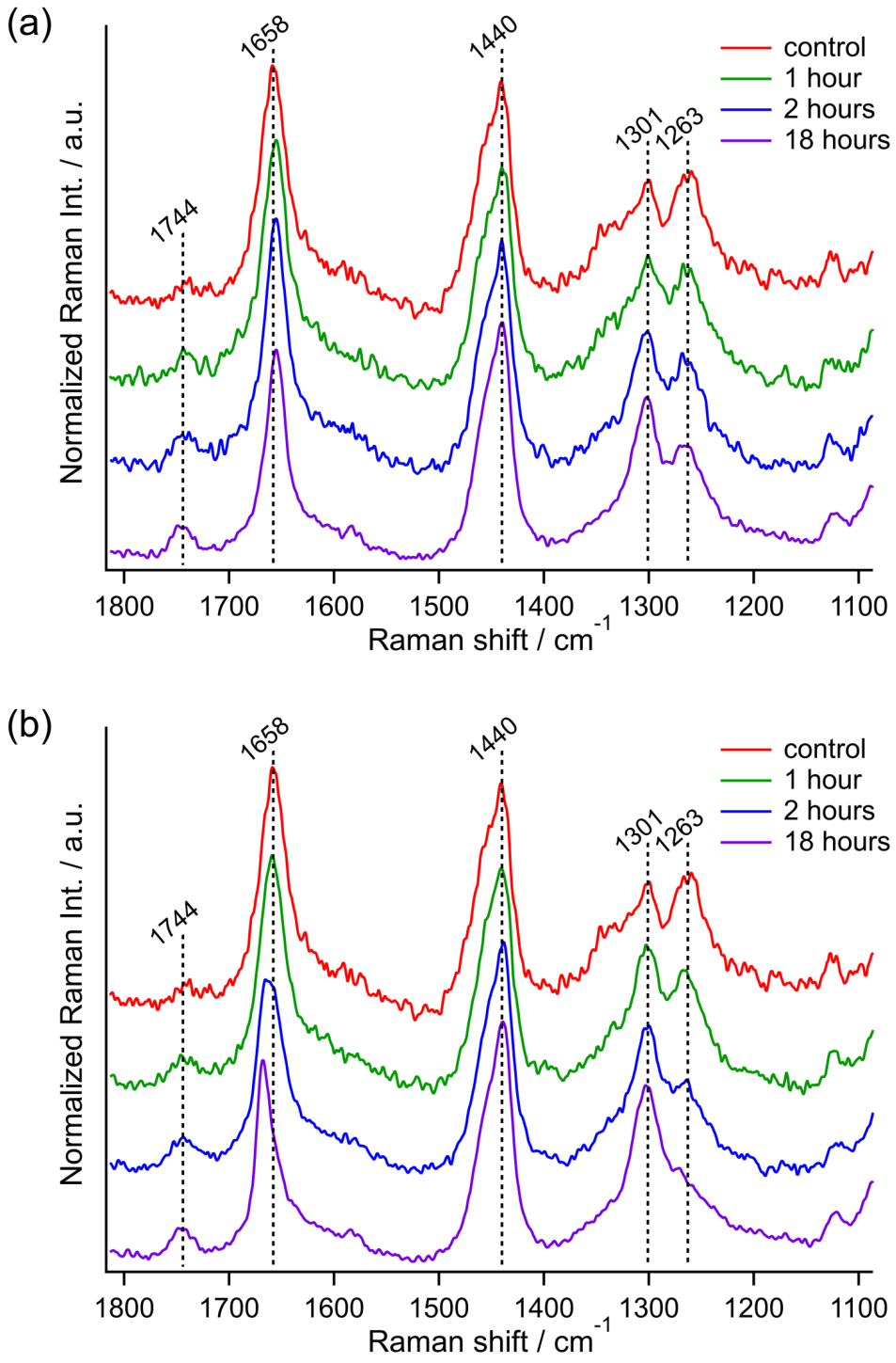

Fig. S5 Average Raman spectra of LDs in (a) OA-treated and (b) EA-treated HeLa cells with different incubation times.

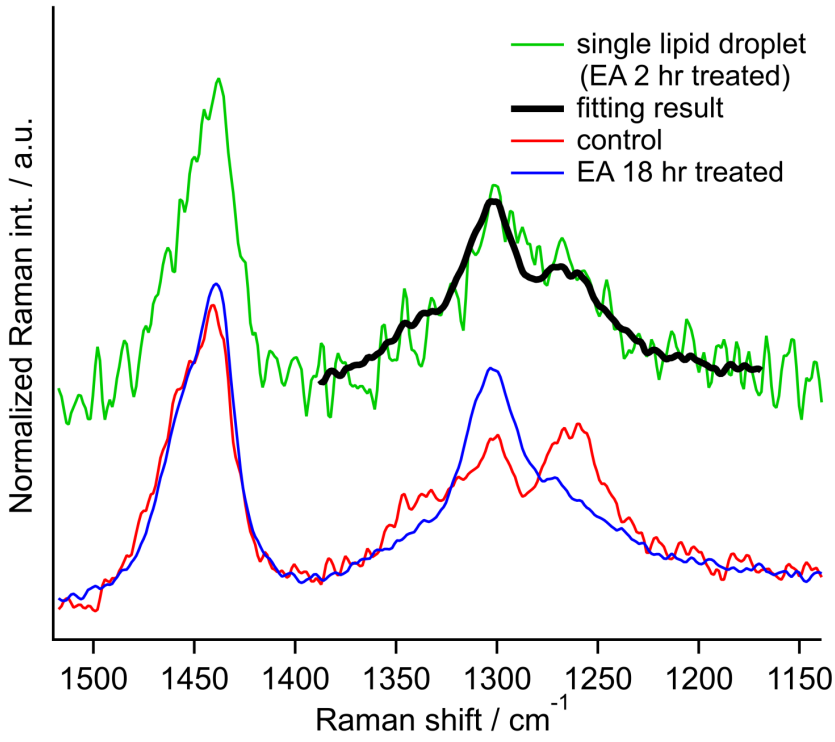

Fig. S6 Example of the fitting result (black) of the observed Raman bands of a single LD (green) with the Raman bands of control (endogenous) (red) and EA-treated LDs for 18 h (exogenous) (blue). The result of this LD was exogeneous 59.6% and endogenous 40.4%.

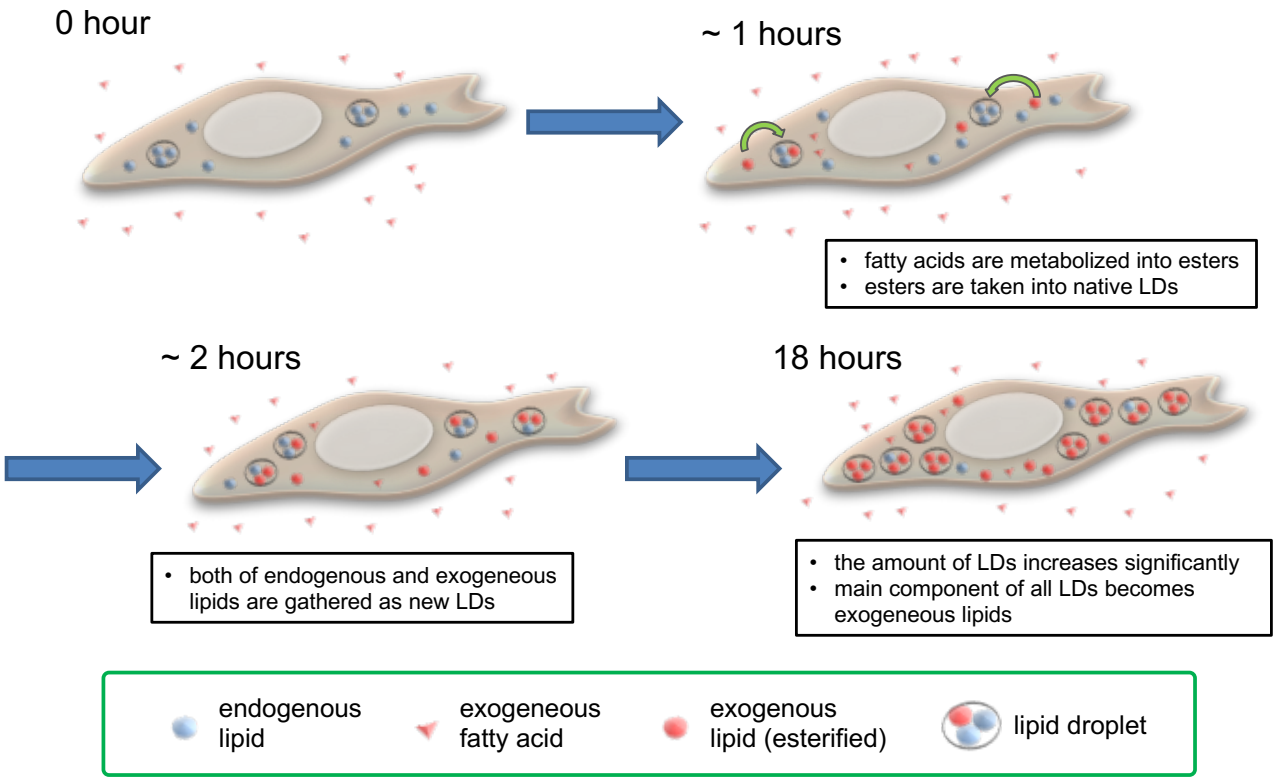

Fig. S7 Model of the formation process of LDs by the introduction of exogenous fatty acids into a cell.

4. References

1 M. Takeuchi, S. Kajimoto and T. Nakabayashi, *J. Phys. Chem. Lett.*, 2017, **8**, 5241–5245.

5. Author Contributions

S.K. and T.N. conceived and conducted the research. H.T. and A.Y. carried out the experiments. H.T. analysed the data. S.K. and T.N. checked the data. H.T. prepared the figures and ESI. S.K. and T.N. wrote the manuscript. H.T., A.Y., S.K and T.N. checked the manuscript and ESI.