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Stimulated Raman scattering of polymer nanoparticles for multiplexed live-cell imaging

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Supporting Information

Experimental Methods

Materials

All materials were purchased from Sigma-Aldrich and used without further purification, except as noted below. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, human recombinant insulin, and transferrin Texas Red conjugate were purchased from Invitrogen. Eagle's Minimum Essential Medium (EMEM) medium was purchased from ATCC. Spectrum Labs dialysis bags were purchased from VWR. Styrene, d_5-styrene, and 4-cyanostyrene were passed over a column of neutral alumina to remove inhibitor prior to polymerization. 4-(trimethylsilylethynyl)styrene and the trisaminocyclopropenium monomer were synthesized according to previously reported procedures[1-2].

Cell culture

HeLa, Cos-7, and MEF cells were grown in DMEM culture medium with 10% *v*/*v* FBS and 1% *v*/*v* penicillin/streptomycin. HT1080 cells were grown in EMEM culture medium with 10% *v*/*v* FBS and 1% *v/v* penicillin/streptomycin. MCF-7 cells were grown in EMEM culture medium with 10% *v*/*v* FBS, 1% *v*/*v* penicillin/streptomycin and 0.01 mg/ml human recombinant insulin. All cultures were incubated in humidified tissue incubators at 37°C and 5% CO_2.

Nanoparticle synthesis and purification

Nanoparticles were synthesized following a general procedure that was scaled accordingly using the indicated weight percentages for each monomer: TAC, styrene, and where applicable, 4-(trimethylsilylethynyl)styrene, 4-cyanostyrene, and d_5-styrene. To the mixture of monomers was added 2,2-azobis(2-methylpropionamidine) dihydrochloride (V-50) and deionized water

such that there was 10 wt% monomers. The mixture was vortexed for 30 s and then added to a two-neck flask fitted with a condenser and stir bar. The solution was sparged with Ar for 15 min and then stirred at 70 °C for 16-24 h. The reaction was then cooled, transferred to a 1k MWCO Spectrum Labs dialysis bag and dialyzed against methanol for 24 h to remove unreacted monomer. The resulting solution was then diluted 1:1 in water to make the nanoparticle stock solution.

Dynamic light scattering

Nanoparticle size and zeta potential were measured on a Malvern Zetasizer Nano ZS (Malvern, United Kingdom). For all measurements, nanoparticles were diluted 1:100 in Milli-Q water at neutral pH. The reported diameters are the average of three measurements, where each measurement comprises at least 10 acquisitions. The zeta potential was calculated according to the Smoluchowski approximation.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on a Zeiss SIGMA VP with an accelerating voltage of 2.0-5.0 keV. Nanoparticles were deposited on clean silicon wafers from solution, dried, and imaged after sputter coating approximately 10 nm of AuPd.

SRS microscopy

A custom-modified integrated laser source (picoEMERALD, Applied Physics & Electronics, Inc.), is used to produce a Stokes beam (1064 nm, 6 ps) with intensity modulated sinusoidally at 8 MHz and a pump beam (tunable from 720 to 990 nm, 5–6 ps) both at 80 MHz repetition rate. Two beams are spatially and temporally overlapped before coupled into an inverted multiphoton laser-scanning microscope (FV1200MPE, Olympus) with optimized near-IR throughput. Lasers are focused onto the cell samples through a 25× water objective (XLPlan N, 1.05 N.A. MP, Olympus) and collected with an oil condenser lens (1.4 N.A., Olympus) after the sample. The Stokes beam is blocked with a high O.D. bandpass filter (890/220 CARS, Chroma Technology) and only the pump beam is collected with a large area Si photodiode (FDS1010, Thorlabs) reverse-biased at a 64 DC voltage. The output photocurrent is electronically filtered (KR 2724, KR electronics), terminated with 50 Ω, and demodulated using a radio frequency lock-in amplifier (SR844, Stanford Research Systems) to extract the stimulated Raman loss signal with near shot-noise-limited sensitivity. The output signal of the lock-in amplifier at each pixel is sent to the analog interface box (FV10-ANALOG, Olympus) of the microscope and images are generated using Fluoview software (Olympus). The imaging experiments are all performed with 40 mW pump beam and 66 mW modulated Stokes beam (measured after the objective) at all frequencies. All images except those in the photo-stability test are acquired

with 30 μs time constant from the lock-in amplifier and 100 μs pixel dwell time with ~27 s per frame (512 × 512 pixels). In photo-stability test with continuous imaging of 100 frames, 3 μs time constant using a fast lock-in amplifier (HF2LI, Zurich instrument) and 8 μs pixel dwell time are used for collecting 100 frames with 2.7 s per frame (512 × 512 pixels).

Spontaneous Raman spectroscopy

The spontaneous Raman spectra are collected using the LabSpec 6 software on a confocal Raman microscope (Xplora, Horiba Jobin Yvon) at room temperature. A 27 mW (after the objective), 532-nm diode laser was used to excite the sample through a 50 × air objective (MPlan N, 0.75 N.A., Olympus). The acquisition time for nanoparticle solution samples was 10 s and for live-cell samples were 40 or 80 s.

Live-cell SRS imaging

For all SRS imaging experiments, cells are first seeded on glass coverslips in 24-well plates in ~0.5 mL culture medium for 2 days at 37°C and 5% CO_2 before experiments.

For multicolor experiments, HeLa cells are incubated with ~1 nM alkyne, nitrile or deuterium labeled polymer dots (500-1500× dilution from stock solution) for 2 h before imaging.

For endosome co-localization experiment, HeLa cells are incubated with ~1 nM alkyne labeled polymer nanoparticles and 25 μg/ml transferrin Texas Red conjugate for 1 h before imaging.

For low-temperature cellular entry experiment, HeLa cells are incubated with ~1 nM alkyne labeled polymer dots at 4°C for 2 h before imaging.

For time-dependent cellular entry experiments, HeLa cells are separately incubated with ~1 nM alkyne, nitrile and deuterium labeled polymer dots for 1, 2 and 4 h before imaging.

For photo-stability experiment, HeLa cells are incubated with ~1 nM alkyne labeled polymer dots for 2 h before continuous imaging of 100 frames.

For imaging experiments in multiple cells lines, all cells (COS-7, MEF, MCF-7 and HT1080) are incubated with ~1 nM alkyne labeled polymer dots for 2 h before imaging.

For multiplexed cell-type sorting experiments, COS-7, MEF and HeLa cells are specifically incubated with ~1 nM alkyne, nitrile and deuterium labeled polymer dots for 2.5 h. Then cells are trypsinized, mixed and seeded on coverslips in co-culture for 4 h and 24 h before imaging.

After all incubations, cells are washed with phosphate-buffered saline (PBS) solution for three times and the glass coverslips are taken out to assemble into imaging chambers filled with PBS for spontaneous Raman measurements or live-cell SRS imaging.

Cell viability

All cell lines are incubated with ~1nM alkyne, nitrile, and deuterium labeled polymer dots for 4 h. This media was then removed and replaced with fresh media and the cells were allowed to grow in the presence of internalized nanoparticles for two days. Trypan blue dye exclusion cell counting was then performed in triplicate with an automated cell counter (ViCell, Beckman-Coulter). Cell viability under experimental conditions is the viable cell count reported as a percentage relative to untreated cells.

Imaging processing

All images are acquired with FluoView scanning software, assigned color and analyzed by ImageJ.

Supplementary Tables and Figures

Table S1. Raman-active nanoparticles synthesis and characterization

Raman-active nanoparticle	TAC wt[a]%	Styrene wt%	Raman-labeled styrene wt%	D_H (nm)	ζ potential (mV)
Alkyne	5	47.5	47.5	55 ± 9	35 ± 5
Nitrile	5	70	25	60 ± 10	20 ± 10
Carbon-Deuterium	5	-	95	50 ± 7	35 ± 10

[a]All weight percentages determined by monomer feed

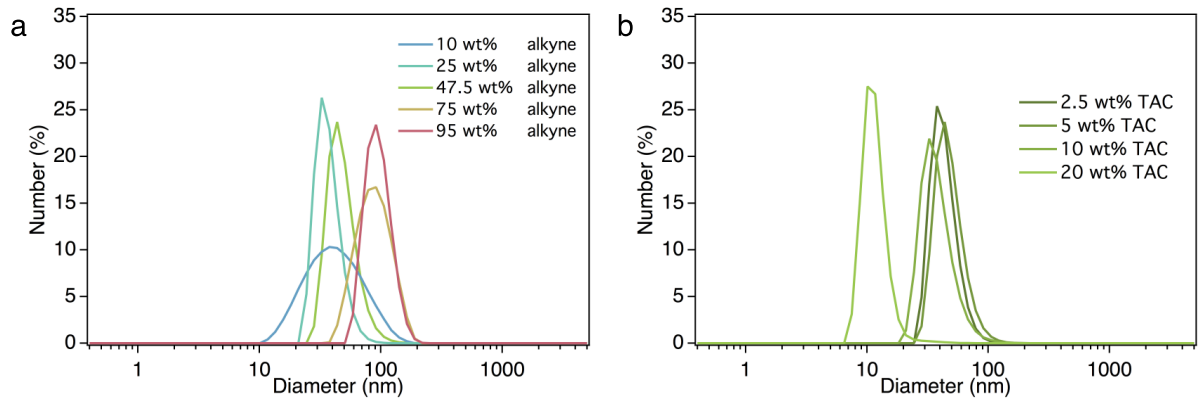

Figure S1. DLS data of alkyne labeled nanoparticles with varying loadings of alkyne monomer (a) and trisaminocyclopropenium monomer (b).

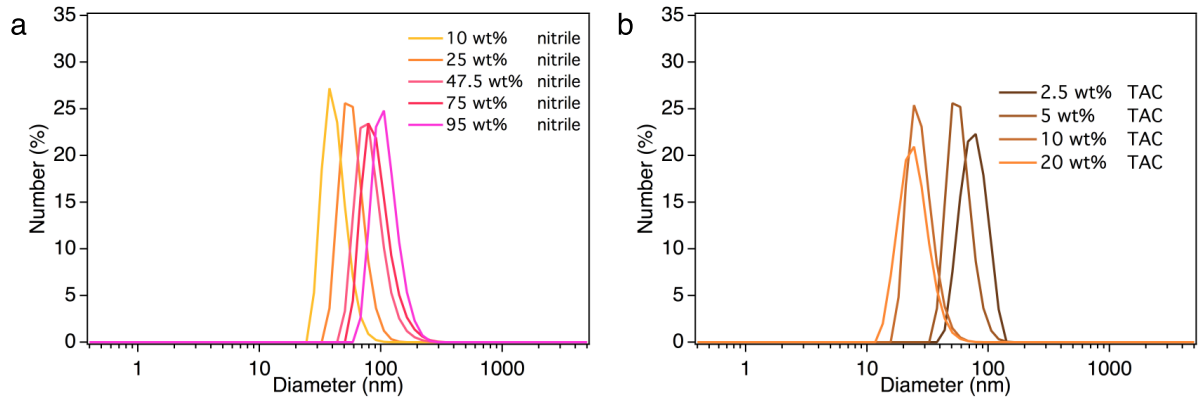

Figure S2. DLS data of nitrile labeled nanoparticles with varying loadings of nitrile monomer (a) and trisaminocyclopropenium monomer (b).

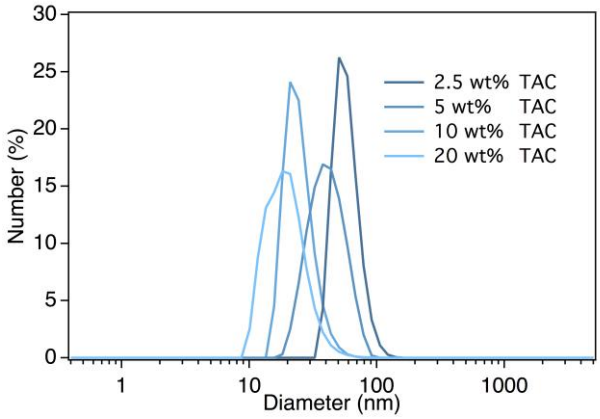

Figure S3. DLS data of deuterium labeled nanoparticles with varying loadings of trisaminocyclopropenium monomer.

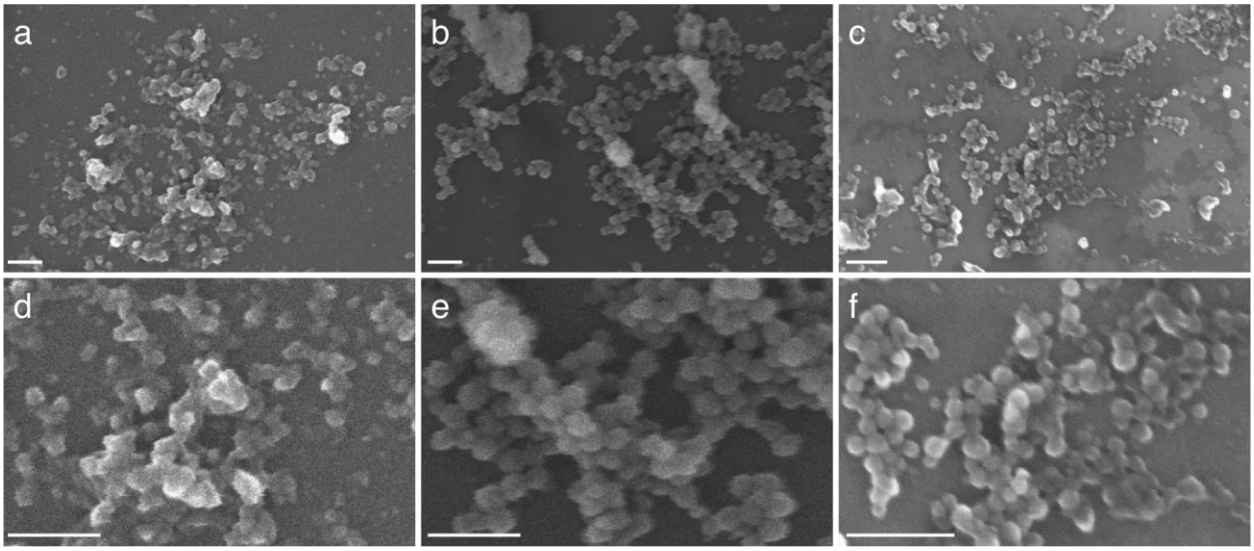

Figure S4. Scanning electron micrographs of CC-dots (a,d), CN-dots (b,e), and CD-dots (c,f). All scale bars are 200 nm.

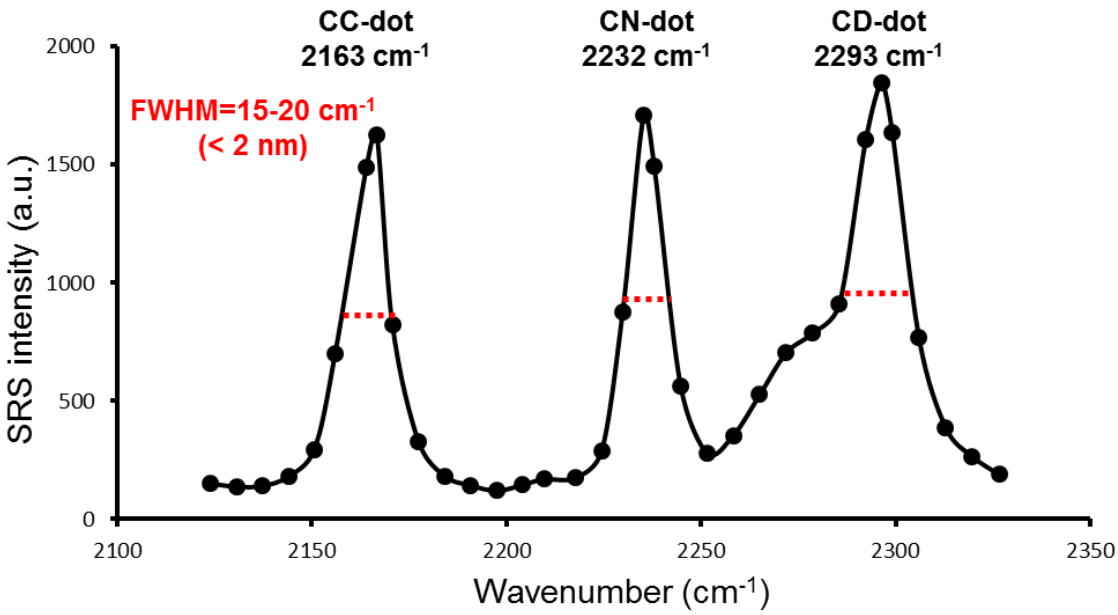

Figure S5. SRS spectrum of Raman-active polymer dots mixture. Three Raman-active polymer dots (CC-dots, CN-dots and CD-dots) show spectrally orthogonal Raman peaks with narrow bandwidth (FWHM=15-20 cm^{-1}, <2 nm) under SRS microscopy. SRS sweeping step size is 0.5 nm.

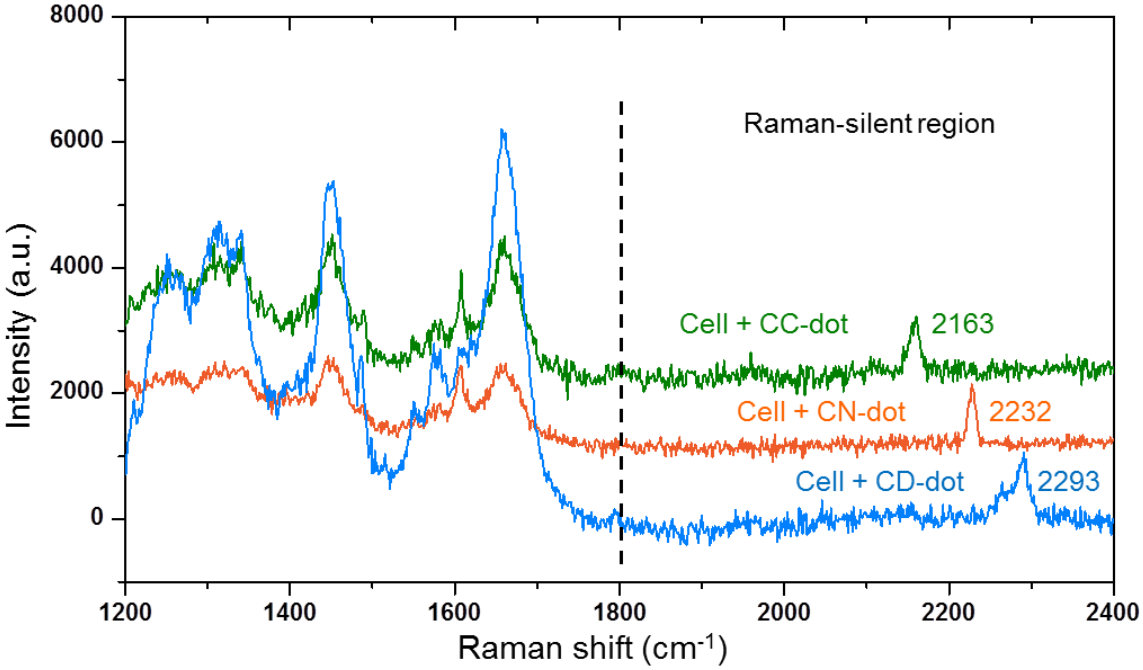

Figure S6. Spontaneous Raman spectra of three Raman-active polymer dots in live cells. HeLa cells are separately incubated with CC-dots (green), CN-dots (orange) and CD-dots (cyan) for 4 hours. PBS background is subtracted from all spectra. The intensity is scaled based on the peaks in the silent region.

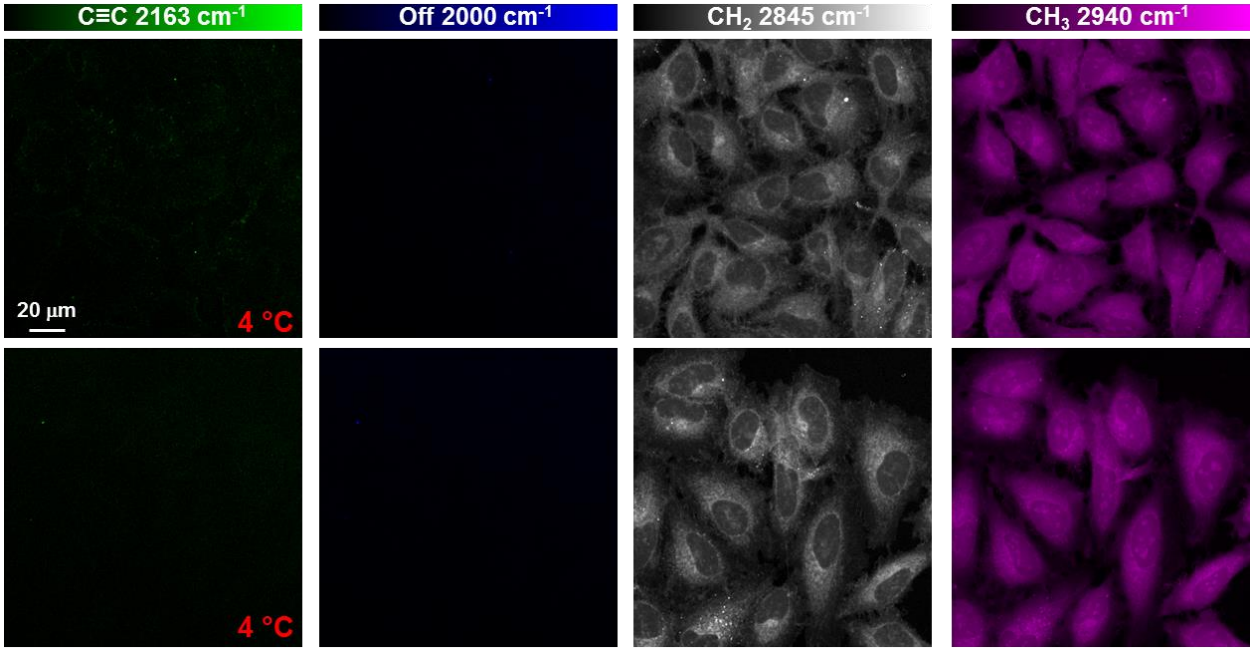

Figure S7. Low-temperature entry inhibition of Raman-active polymer nanoparticles in live HeLa cells. HeLa cells are incubated with alkyne labeled polymer nanoparticles for 2 hours at 4°C with suppressed endocytosis and nanoparticle entry.

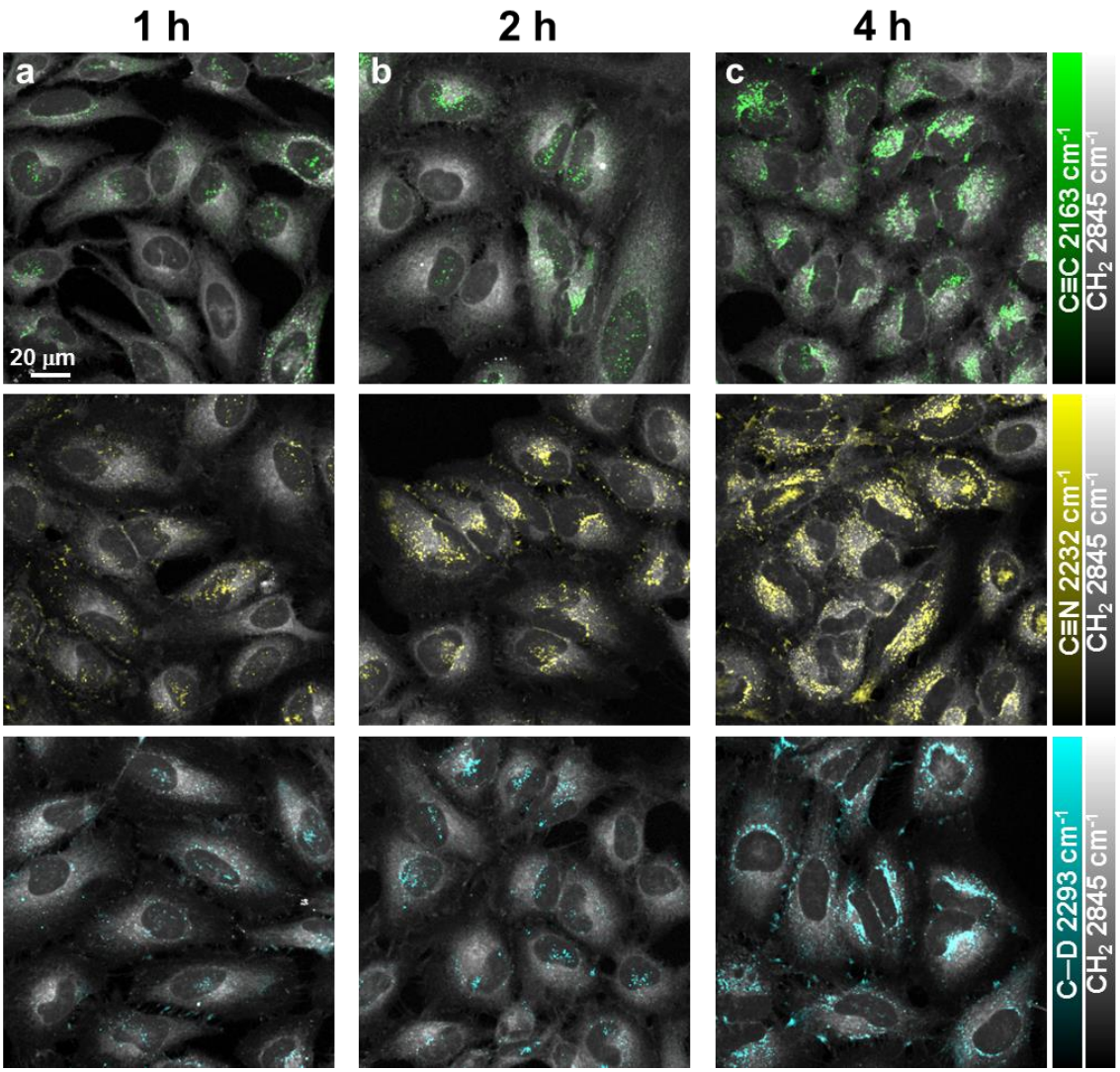

Figure S8. Time-dependent entry kinetics of Raman-active polymer nanoparticles in live HeLa cells. Cells are incubated with alkyne, nitrile and deuterium labeled polymer nanoparticles for various time of 1 h (a), 2 h (b) and 4 h (c). Rapid cellular entry is observed in 1 h for all three polymer nanoparticles.

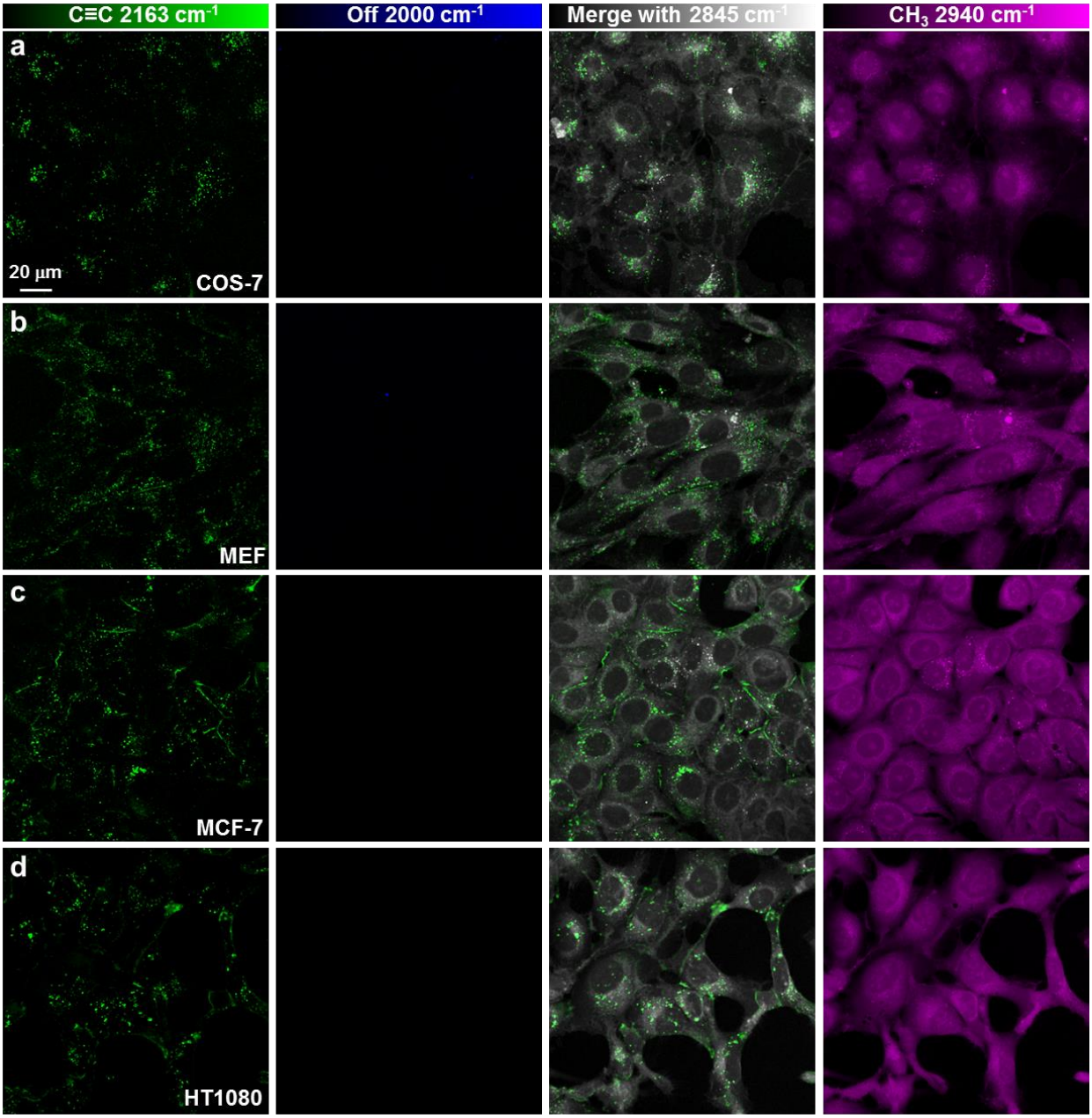

Figure S9. SRS imaging of Raman-active polymer dots in multiple cell lines. COS-7 (a), MEF (b), MCF-7 (c) and HT1080 (d) cells are incubated with alkyne labeled polymer dots for 2 hours. Both non-cancerous and cancerous cell lines are efficiently labeled by Raman-active nanoparticles.

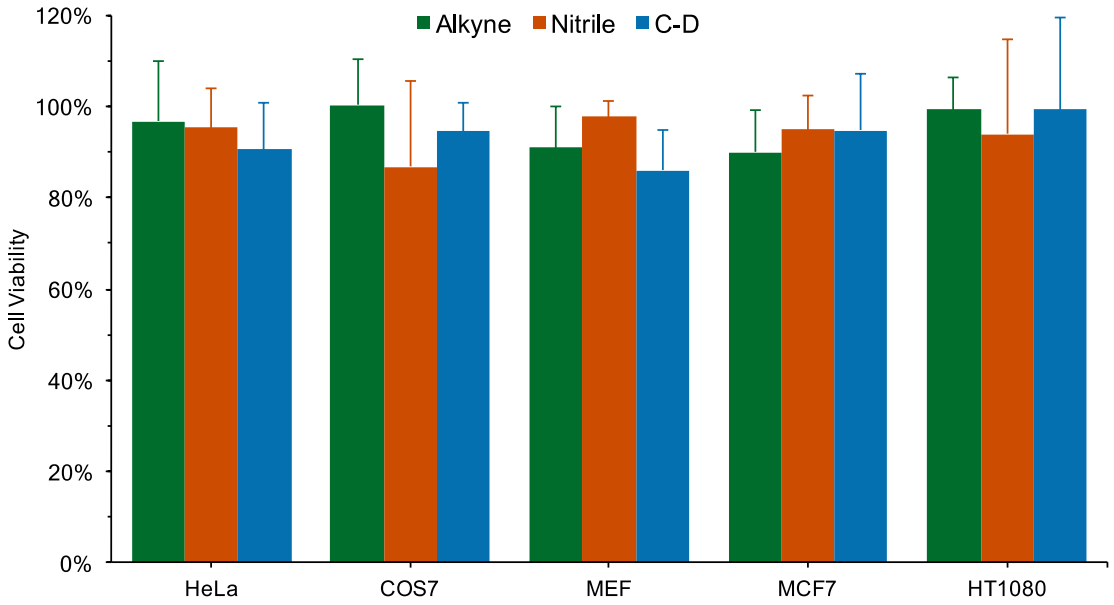

Figure S10. Viability assay of multiple cell lines incubated with alkyne (green), nitrile (orange), and deuterium (cyan) labeled polymer nanoparticles. All cells are incubated with nanoparticles for four hours before exchanging with fresh media and allowing the cells to grow for two days. All five tested cell lines shows greater than 85% viability after 48 h with the three Raman-active polymer nanoparticles. Cell viability is calculated as a percentage relative to control with error bars representing standard deviation of triplicate measurements.

Supplementary references:

1. Jiang, Y.; Freyer, J. L.; Cotanda, P.; Brucks, S. D.; Killops, K. L.; Bandar, J. S.; Torsitano, C.; Balsara, N. P.; Lambert, T. H.; Campos, L. M., The evolution of cyclopropenium ions into functional polyelectrolytes. *Nat. Commun.* **2015,** *6*, 5950.

2. Fleischmann, S.; Komber, H.; Voit, B., Diblock Copolymers as Scaffolds for Efficient Functionalization via Click Chemistry. *Macromolecules* **2008,** *41* (14), 5255-5264.