Published in final edited form as:

Nano Lett. 2012 December 12; 12(12): 6170–6174. doi:10.1021/nl302991y.

Measuring Uptake Dynamics of Multiple, Identifiable Carbon Nanotube Species via High-Speed Confocal Raman Imaging of Live Cells

Jeon Woong Kang¹, Freddy T. Nguyen^{1,2}, Niyom Lue¹, Ramachandra R. Dasari¹, and Daniel A. Heller³

Daniel A. Heller: hellerd@mskcc.org

¹Laser Biomedical Research Center, G. R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts, 02139

²College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801

³Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York, 10065

Abstract

Carbon nanotube uptake was measured via high-speed confocal Raman imaging in live cells. Spatial and temporal tracking of two cell-intrinsic, and nine nanotube-derived, Raman bands was conducted simultaneously in RAW 264.7 macrophages. Movies resolved single (n,m) species, defects, and aggregation state of nanotubes transiently, as well as cell position, denoted by lipid and protein signals. This work portends the real-time molecular imaging of live cells and tissues using Raman spectroscopy, affording multiplexing and complete photostability.

Keywords

Single-walled carbon nanotube; Raman spectroscopy; Resonance Raman scattering; Raman microscopy; Molecular imaging

Raman scattering has been employed substantially in recent years for biomedical applications. The high signal amplification of surface-enhanced Raman scattering (SERS)¹, has generated excitement for molecular sensing and imaging studies in live cells. Single-walled carbon nanotubes (SWNTs) are a material class with extremely high Raman cross-section due to resonance enhancement, which results in high signals without the need for surface plasmon-generating metals such as silver or gold.² The resonance enhancement of SWNTs stems from the optical absorption bands caused by the Van Hove singularities of the 1D electronic structure of the nanotubes.

Single-walled carbon nanotubes have shown promise in biomedical applications such as drug and nucleic acid delivery,^{3, 4} optical^{5, 6} and electronic⁷ sensors. Carbon nanotube-based probes^{8–11} benefit from the large aspect ratio and surface area, allowing for multivalent binding sites.¹² Raman probe signals are optically stable and quantitative and can be employed for use in the near-infrared “tissue transparent” window. With over 50 different

Correspondence to: Daniel A. Heller, hellerd@mskcc.org.

Supporting Information Available: Movies of nanotube uptake acquired by high-speed confocal Raman imaging, as well as supporting figures, are available free of charge via the internet at <http://pubs.acs.org>.

species, each possessing unique optical and electronic properties, labeling applications could potentially take advantage of the large number of individually addressable probes. The different structures of single-walled carbon nanotubes are denoted by two indices, n and m , which correspond to the two lattice vectors on a sheet of graphene. Each (n,m) designation represents a particular nanotube species with its own unique electronic and optical properties.

Carbon nanotubes produce Raman scattering due to several vibrational modes¹², such as tangential mode of graphene (G-band) at $\sim 1590\text{ cm}^{-1}$ and radial breathing modes (RBM) in the vicinity of $120\text{--}350\text{ cm}^{-1}$ for nanotubes between 0.7 and 2 nm in diameter, as well as intermediate frequency modes^{13, 14} (IFM) which appear between the RBM and G peak regions. The unique Raman shifts of the RBMs and IFMs are highly dependent on the nanotube graphitic species, allowing identification of the nanotube (n,m) species present. The RBM frequencies, caused by the axial vibration of the nanotube's circumference, relate to nanotube diameter with the relation $\omega_{\text{RBM}} = 228/d_t + 16\text{ cm}^{-1}$.¹⁵ The IFMs have not been as extensively studied as the other modes, but certain modes with frequencies between 380 and 650 cm^{-1} have been assigned to nanotube (n,m) species or families of species.¹⁴ Some IFMs exhibit stepwise dispersion, changing in frequency upon excitation at different laser energies, while other modes are non-dispersive.¹³ Other information gained from carbon nanotube samples by Raman spectra includes the presence of metallic nanotubes via the Breit-Wigner-Fano (BWF) lineshape of the tangential mode,² the presence of sp^3 carbon defects via the 1297 cm^{-1} disorder mode (D-peak),² and the presence of van der Waals contact in the RBMs. For instance, at 785 nm excitation, the (10,2) nanotube at 267 cm^{-1} comes into resonance upon such contact, denoting aggregation or "bundling."¹⁶

Confocal Raman mapping of biological tissues and single cells has been developed and employed for diagnostic applications.¹⁸ Raman spectroscopy can distinguish macromolecules as well as structural features such as organelles based on their characteristic Raman spectra.^{19, 20} Raman modes visible in single cells include lipids, proteins, and nucleic acids. Recently, the G. R. Harrison Spectroscopy Laboratory developed a high-speed confocal Raman microscopy system using galvano mirrors instead of mechanical stage movement, used in most commercial systems.¹⁷ This high-throughput system has greatly increased the image acquisition speed, allowing the high-resolution mapping of a single cell in under a minute (30×30 pixels over $17\mu\text{m} \times 17\mu\text{m}$ field of view). This development allows for transient imaging of live cells and the tracking of nanomaterials within them.

Herein, we observe the real-time uptake of individual single-walled carbon nanotube species into live macrophages via transient spatial Raman mapping. Signals from DNA-encapsulated carbon nanotubes,^{18, 17, 17} as well as lipid and protein signatures of the cells, are measured concurrently. The uptake of several individual carbon nanotubes is observed via strong Raman scattering of radial breathing mode (RBM) and intermediate frequency mode (IFM) features. The nanotubes are tracked spatially and temporally via rapid, continuous Raman spectral mapping of the cells with a frame-rate approaching one map per minute. The nanotube signals and cell-intrinsic lipid and protein signals are acquired concomitantly, allowing the localization of the nanotubes with respect to the cell position. Early in the experiments, RBM signatures of individual nanotubes account for the majority of the nanotube signal. After 15 minutes, however, the aggregate G-band signal becomes dominant, as multiple nanotube species out of RBM resonance flood the cells. This work demonstrates the real-time tracking of multiple, distinguishable, nanoparticles in cells via Raman mapping for applications in molecular probes, surgical markers, and contrast agents.

A home-built confocal Raman microscopy system (Figure 1a) was described previously.¹⁸ Briefly, a 785nm wavelength Ti:Sapphire laser (3900S, Spectra-Physics) was used for the

excitation. High speed XY scanning was done by galvano mirrors (CT-6210, Cambridge Technology). A 1.2 NA water immersion objective lens (Olympus UPLSAPO60XWIR 60X/1.20) was used to both focus the laser to a cell and to collect the back-scattered light. The back-scattered Raman light from the sample passes through two dichroic mirrors (DM1 = Semrock LPD01-785RU-25, DM2 = Semrock LPD01-785RU-25×36×1.1) and was collected by a multimode fiber (Thorlabs M14L01). Collected signal was delivered to a spectrograph (Holospec f/1.8i, Kaiser Optical Systems) and detected by a TE-cooled, back-illuminated and deep depleted CCD (PIXIS: 100BR_eXcelon, Princeton Instruments). The rejected Rayleigh light from second dichroic mirror (DM2) is collected by a single mode fiber (Thorlabs P1-780AFC-1) and delivered to a photomultiplier tube (PMT, H9656-20, Hamamatsu) which is amplified by a PMT controller (C7169, Hamamatsu). This provides exactly co-registered confocal reflectance image which guides Raman mapping for optimal field of view. Labview 8.6 software (National Instruments) and a data acquisition board (PCI-6251, National Instruments) are used to control the devices.

Single-walled carbon nanotubes synthesized by the HiPCO process¹⁹ (Unidym) were suspended in ss(AT)₁₅ oligonucleotide by sonicating with a probe-tip (Sonics & Materials) at 10 W for 10 minutes in a water bath and centrifuged at 20,000 g for 90 minutes. This produced DNA-encapsulated nanotubes demonstrated to be biocompatible and stable in aqueous solution without the need for a surfactant.^{8, 18} Mouse leukemic monocyte macrophage cells (RAW 264.7) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics. Macrophages were plated onto 60 mm petri dishes modified to incorporate a quartz bottom. The cells were allowed to adhere to the quartz-bottom plates for at least 48 hours before imaging. Immediately prior to imaging, the cell media was replaced with media containing 1 mg/L DNA-encapsulated nanotubes. The near-infrared laser wavelength and excitation power of 60 mW ensured that the cells remain viable during the course of the measurement. The mapping process involved the rapid scanning of a 17μm×17μm area, acquiring 900 (30×30) spectra, each with an integration time of 50 milliseconds. Due to the time delays between each acquisition, effective acquisition time was 75 milliseconds and total acquisition time per image was 68 seconds. With minimal interval between frames, the experiments produced 36,000 (30 × 30 × 40) Raman spectra.

Initial Raman measurements of the cell exhibited only cell-intrinsic Raman features, including lipids (1450 cm⁻¹) and proteins (1004 cm⁻¹) (Figure 1b). Five minutes after injection of the nanotubes, they began to enter the cell. While typical signal strengths of cellular lipids and proteins were 7~10 times that of the noise, nanotube RBM peaks reached 150 times and G-band reached 500 times. In the initial minutes of the experiment, carbon nanotube signals often appeared to favor RBMs, often exhibiting a higher signal than the G-band mode in some spectra. Notably, a sole RBM or IFM peak was often recorded in a single spectrum, denoting the presence of only one visible nanotube species in a particular location (Figure 1c). The disorder mode and BWF lineshape were present in the cell, but not in all spectra containing nanotube species, suggesting that metallic nanotubes and those with defects were spatially discerned within the cells. An aggregate spectrum of the entire region at the same timepoint shows few of the individual features seen in the spatially-resolved spectra (Figure 1d), instead resembling a spectrum of the original DNA-nanotube stock solution (Figure 1e).

All Raman spectra were fit with a linear combination of normalized basis Lorentzian spectra with non-negative restriction. Basis spectra were generated with known peak centers from the literature. To avoid data over-fitting, the process was carefully monitored with restricting the fitting coefficient as non-negative. As a result, we acquired 36,000 coefficients for each basis spectrum. These coefficients were used to construct images based on their spatial

(30×30) and temporal (40 temporal points) coordinates. The data analysis software was written by the authors in MATLAB. The experiments detected RBM peaks at 203, 215, 225, 233, and 267 cm^{-1} , corresponding to the (13,3), (9,7), (10,5), (11,3), and (10,2) species/bundling, respectively. The IFM modes at 401 and 844 cm^{-1} , disorder mode at 1297 cm^{-1} and G-band at 1590 cm^{-1} were also detected within cells and included in the fitting process (Figure 2a). The fitting coefficient of each basis spectrum represents the relative strength of each component and was used to reconstruct images. Figure 2b shows the spatially-mapped intensity of each fitted nanotube-based mode, as well as the 1450 cm^{-1} lipid and 1004 cm^{-1} protein peaks at 32.4 minutes during the experiment. The cellular lipid signal was used to define the cell location and delineate cell boundaries. This peak was used to determine that a particular feature, present in the upper-right corner of the G-band, 203 cm^{-1} , 267 cm^{-1} , 401 cm^{-1} , and 1297 cm^{-1} mode images, was in fact not within the cell membrane and likely adsorbed to the quartz substrate. It is reasoned that the spot denotes a carbon nanotube aggregate (bundle) due to the presence of a large 267 cm^{-1} (bundling) peak.

The dynamics of macrophage uptake of nanotubes were measured by quantifying cell-localized nanotube Raman signals over the course of the experiment. A plot of the average intensity of each Raman feature over time within the confines of the cell shows the relative accumulation of each nanotube species within the macrophage (Figure 3a, S1). While the G-band signal exhibited an increasing trend through much of the course of the experiment, the RBM and IFM features did not increase at the same rate. The relative G-band signal in the cell, normalized to the total nanotube-derived signal, is plotted versus time in Figure 3b. In the first few minutes of nanotube incubation, the G-band signal accounted for a small proportion of the total Raman signal in the macrophage. After approximately 14 minutes, the G-band signal intensity equaled the aggregate RBM signal. The inverse effect is apparent by plotting the normalized RBM signal versus time. A control experiment performed in the absence of cells showed no such relative increase in the G band (Figure S2).

We attribute the apparent increase in the G-band to two photophysical factors: the RBMs do not have the same resonances as the G-band, and the bandwidths of the resonance windows differ.²⁰ Early in the accumulation process, the nanotubes are spatially separated within the cell so that each spectrum is acquired while the laser is focused on one or few nanotubes. The spectra at these timepoints often contain a single RBM peak. Individual RBM modes excited on resonance appear relatively intense compared to the G-band. Convolution of the spectrum increases as multiple nanotube species begin to accumulate at the same relative location, resulting in spectra containing species whose RBMs are weak or absent but whose tangential modes still contribute to the aggregate G-band signal due to its relatively large resonance window.

Movies (Supplementary Information) of endogenous and exogenous features were constructed from nanotube-derived and cell-intrinsic Raman modes (Figure 3c-g). A movie showing the carbon nanotube G-band and the cellular lipid peak (Movie M1) together demonstrates the transient encroachment of the nanotubes into the cellular space over a time-frame of several minutes (Still image at 32.4 minutes shown in Figure 3c). The transient uptake of three Raman features, including two RBMs and the G-band, are shown in Movie M2 (Corresponding still image at 32.4 minutes shown in Figure 3d and time series in Figure 3g). Note that the progression shows individual RBM features present in the cell early after its exposure to nanotubes. The RBM signals outweigh the G-band signals, especially at the beginning of cell uptake. Later in the movie, the G-band becomes more prominent. Bundled nanotubes, denoted by the 267 cm^{-1} peak (Movie M3, Figure 3e), were detected with a low rate of incidence, corresponding to the overall low intensity of the 267 cm^{-1} mode in the original nanotube sample (Figure 1). The 844 cm^{-1} IFM (Movie M4, Figure 3f), was also easily tracked with respect to the G-band, showing few species present.

We have demonstrated the first transient cellular uptake imaging of nanomaterials and the first transient imaging of carbon nanotubes with the ability to distinguish multiple pristine carbon nanotube species. Using our unique instrumentation, we acquired movies of both nanotube-based and cell-intrinsic Raman signals. This data allowed the cellular transient tracking of four nanotube (n,m) species by RBMs, as well as the presence and location of bundles, two IFM bands, the presence of defects, and the graphene tangential mode, in concert with endogenous lipids and proteins from the cell. This work demonstrates the possibility of transient confocal molecular imaging of live cells and tissues using Raman spectroscopy, affording unique photostability and much greater multiplexing ability than conventional imaging methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Prof. Peter So for helpful discussions. This work was performed at the MIT Laser Biomedical Research Center and was supported by the NIH National Institute of Biomedical Imaging and Bioengineering, grant P41EB015871-26A1. DAH thanks the Damon Runyon Cancer Research Foundation (DFS-#2050-10) and the Frank A. Howard Fellows Program at Memorial Sloan-Kettering Cancer Center.

References

1. Haes AJ, Van Duyne RP. *J. Am. Chem. Soc.* 2002; 124:10596–10604. [PubMed: 12197762]
2. Dresselhaus MS, Dresselhaus G, Saito R, Jorio A. *Phys. Rep.* 2005; 409:47–99.
3. Kam NWS, O'Connell MJ, Wisdom JA, Dai H. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:11600–11605. [PubMed: 16087878]
4. Herrero MA, Toma FM, Al-Jamal KT, Kostarelos K, Bianco A, Da Ros T, Bano F, Casalis L, Scoles G, Prato M. *J. Am. Chem. Soc.* 2010; 132:1731–1731.
5. Heller DA, Jin H, Martinez BM, Patel D, Miller BM, Yeung TK, Jena PV, Hobartner C, Ha T, Silverman SK, Strano MS. *Nat. Nanotechnol.* 2009; 4:114–120. [PubMed: 19197314]
6. Barone PW, Baik S, Heller DA, Strano MS. *Nat. Mater.* 2005; 4:86–92. [PubMed: 15592477]
7. Allen BL, Kichambare PD, Star A. *Adv. Mater.* 2007; 19:1439–1451.
8. Heller DA, Baik S, Eurell TE, Strano MS. *Adv. Mater.* 2005; 17:2793–2799.
9. Liu ZA, Li XL, Tabakman SM, Jiang KL, Fan SS, Dai HJ. *J. Am. Chem. Soc.* 2008; 130:13540–13541. [PubMed: 18803379]
10. Keren S, Zavaleta C, Cheng Z, de la Zerda A, Gheysens O, Gambhir SS. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:5844–5849. [PubMed: 18378895]
11. (a) Jin H, Heller DA, Strano MS. *Nano Lett.* 2008; 8:1577–1585. [PubMed: 18491944] (b) Lacerda L, Pastorin G, Gathercole D, Buddle J, Prato M, Bianco A, Kostarelos K. *Adv. Mater.* 2007; 19:1480–1484.
12. Kalbacova M, Broz A, Kromka A, Babchenko O, Kalbac M. *Carbon.* 2011; 49:2926–2934.
13. Jorio A, Saito R, Hafner JH, Lieber CM, Hunter M, McClure T, Dresselhaus G, Dresselhaus MS. *Phys. Rev. Lett.* 2001; 86:1118–1121. [PubMed: 11178024]
14. Fantini C, Jorio A, Souza M, Saito R, Samsonidze GG, Dresselhaus MS, Pimenta MA. *Phys. Rev. B.* 2005; 72:085446.
15. Luo ZT, Papadimitrakopoulos F, Doorn SK. *Phys. Rev. B.* 2007; 75:205438.
16. Fantini C, Jorio A, Souza M, Strano MS, Dresselhaus MS, Pimenta MA. *Phys. Rev. Lett.* 2004; 93:147406. [PubMed: 15524844]
17. Heller DA, Barone PW, Swanson JP, Mayrhofer RM, Strano MS. *J. Phys. Chem. B.* 2004; 108:6905–6909.

18. Puppels GJ, de Mul FF, Otto C, Greve J, Robert-Nicoud M, Arndt-Jovin DJ, Jovin TM. *Nature*. 1990; 347:301–303. [PubMed: 2205805]
19. Kang JW, Lue N, Kong CR, Barman I, Dingari NC, Goldfless SJ, Niles JC, Dasari RR, Feld MS. *Biomed. Opt. Exp.* 2011; 2:2484–2492.
20. Uzunbajakava N, Lenferink A, Kraan Y, Volokhina E, Vrensen G, Greve J, Otto C. *Biophys. J.* 2003; 84:3968–3981. [PubMed: 12770902]
21. Zheng M, Jagota A, Semke ED, Diner BA, Mclean RS, Lustig SR, Richardson RE, Tassi NG. *Nat. Mater.* 2003; 2:338–342. [PubMed: 12692536]
22. Bronikowski MJ, Willis PA, Colbert DT, Smith KA, Smalley RE. *J. Vac. Sci. Technol. A*. 2001; 19:1800–1805.
23. Jiang J, Saito R, Gruneis A, Chou SG, Samsonidze GG, Jorio A, Dresselhaus G, Dresselhaus MS. *Phys. Rev. B*. 2005; 71:205420.

Figure 1.

a.) Schematic of high-speed confocal Raman imaging microscope employing galvano mirrors for raster-scanning the laser beam across the specimen. b.) Overlaid Raman spectra of one full raster scan of a cell just after introducing carbon nanotube-containing media ($T=0$) showing cellular lipid (1450 cm^{-1}) and protein (1004 cm^{-1}) Raman features but no carbon nanotube features. c.) Overlaid Raman spectra of one full raster scan of the same cell at 32.4 minutes after the introduction of nanotube-containing media showing strong nanotube RBM, tangential, IFMs, and disorder modes. Cellular signal is present but dwarfed by the nanotube modes. d.) Aggregate Raman spectrum of nanotubes in the cell showing convoluted Raman peaks. e.) Spectrum of the ss(AT)₁₅ oligonucleotide-suspended carbon nanotube sample used in this study.

Figure 2.

a.) Example Raman spectrum of multiple clearly differentiable nanotube RBMs with intensities approaching that of the G-band. The spectrum was spectrally deconvoluted to quantify the RBM, IFM, disorder, and G-band. Inset: Magnified RBM region showing deconvoluted peaks. b.) Raman intensity maps of a single RAW 264.7 macrophage at 32.4 minutes after introducing nanotubes. Each map was compiled using the intensity of a single Raman mode from an endogenous cell-based peak (lipids and proteins), or a nanotube-derived peak (RBMs, IFMs, degradation, and G-band). The cell shape was derived from the lipid peak.

Figure 3.

a.) Uptake of several carbon nanotube Raman modes measured within a live macrophage. b.) Relative G-band signal, normalized to the total nanotube-derived Raman signal in the cell, plotted over time. The G-band represents an increasing proportion of total nanotube signal in the cell. The inverse is seen by plotting the RBM signal versus total signal. c-f.) Single frames taken at 32.4 minutes from movies (in Supporting Information) showing cell-intrinsic features and nanotube-based features within the confines of the cell. g.) Time series of movie frames from Movie M2 showing uptake of two nanotube species, as defined by their RBMs, and the G-band. Earlier images show few G-band features with respect to the total Raman signal, while later images exhibit the opposite.