Single-Cell Growth Rates in Photoautotrophic Populations Measured by Stable Isotope Probing and Resonance Raman Microspectrometry

Gordon T. Taylor*, Elizabeth A. Suter, Zhuo Q. Li, Stephanie Chow, Dallyce Stinton, Tatiana Zaliznyak and Steven R. Beaupré

School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY, United States

A new method to measure growth rates of individual photoautotrophic cells by combining stable isotope probing (SIP) and single-cell resonance Raman microspectrometry is introduced. This report explores optimal experimental design and the theoretical underpinnings for quantitative responses of Raman spectra to cellular isotopic composition. Resonance Raman spectra of isogenic cultures of the cyanobacterium, *Synechococcus* sp., grown in $^{13}$C-bicarbonate revealed linear covariance between wavenumber ($\text{cm}^{-1}$) shifts in dominant carotenoid Raman peaks and a broad range of cellular $^{13}$C fractional isotopic abundance. Single-cell growth rates were calculated from spectra-derived isotopic content and empirical relationships. Growth rates among any 25 cells in a sample varied considerably; mean coefficient of variation, CV, was 29 ± 3% ($\sigma/x$), of which only ~2% was propagated analytical error. Instantaneous population growth rates measured independently by *in vivo* fluorescence also varied daily (CV $\approx$ 53%) and were statistically indistinguishable from single-cell growth rates at all but the lowest levels of cell labeling. SCRR censuses of mixtures prepared from *Synechococcus* sp. and *T. pseudonana* (a diatom) populations with varying $^{13}$C-content and growth rates closely approximated predicted spectral responses and fractional labeling of cells added to the sample. This approach enables direct microspectrometric interrogation of isotopically- and phylogenetically-labeled cells and detects as little as 3% changes in cellular fractional labeling. This is the first description of a non-destructive technique to measure single-cell photoautotrophic growth rates based on Raman spectroscopy and well-constrained assumptions, while requiring few ancillary measurements.

Keywords: single-cell analysis, carbon fixation, photosynthesis, productivity, intraspecific trait variability, Raman microspectrometry, carotenoids, stable isotope probing

INTRODUCTION

Ecological theory suggests that predictions about population dynamics in planktonic systems have limited skill if intraspecific trait variation and heterogeneous resource distributions are ignored. Intraspecific trait variation within microalgal and bacterial populations has been shown to non-linearly affect intra- and interspecific interactions and population responses to resource availability,
pathogens, and predators (Hellweger and Kianirad, 2007; Bolnick et al., 2011; Bucci et al., 2012). Such trait variations can arise from any of the following: physiological history of individual cell lines, niche plasticity, natural selection, mutation, genetic drift, or recombinant events (Bolnick et al., 2011). Even clonal populations (isogenic) in apparently homogenous cultures exhibit varying genetics, biochemistry, physiology, and behavior, all of which can produce a range of “growth phenotypes” (Lidstrom and Konopka, 2010; Damodaran et al., 2015; Kopf et al., 2015). This recognition has given rise to agent-based or individual-based models which don’t assume that cell attributes within a population are uniformly or normally distributed around their mean values (e.g., Hellweger and Kianirad, 2007). In addition, aquatic ecologists and biogeochemists now recognize that the planktonic realm has fine-scale structure imposed by heterogeneous distributions of nutrients, oxygen, particles, colonial microbes, and polymeric gels, as well as by metazoan behavior, and symbiotic associations (Azam, 1998; Simon et al., 2002; Wagner et al., 2006; Stocker, 2012; Gemmell et al., 2016). Historically however, the vast majority of measurements have overlooked this microspatial heterogeneity and cryptic material exchanges (e.g., Canfield et al., 2010), and have provided net biogeochemical transformations at best. They also fail to unequivocally link key players to particular processes. These limitations hamper a deeper mechanistic understanding of planktonic dynamics. Single-cell techniques are the only way forward to empirically determine how intraspecific phenotypic/genotypic variations and microspatial architecture (the aquascape) determine individual ecophysiology and translate into collective population responses.

Several single-cell technologies have enabled examination of intra-population variability in genotype, phenotype, activity and cellular growth. Single-cell genomics and transcriptomics provide unique information on genetic variations and are gaining popularity in the aquatic sciences (Stepanauskas, 2012; Wu et al., 2014). The synchrotron X-ray microprobe has provided insights into intraspecific variability in individual cell elemental stoichiometries (Twining et al., 2008). Highly sensitive serial resonant mass sensor arrays have enabled measurement of changes in buoyant masses of bacterial and mammalian cells as they transit through microfluidic channels, thereby tracking somatic growth of individual cells through time (Cermak et al., 2016). Combining fluorescent in situ hybridization (FISH) with microautoradiography (MAR-FISH) (Lee et al., 1999) or with secondary ion mass spectrometry (nano-SIMS-FISH) (Orphan et al., 2002) provides single-cell resolution for linking identity to ecophysiology in complex microbial assemblages. Nano-SIMS-FISH has been combined with SIP to detect nutrient assimilation by individual cells (Musat et al., 2008; Orphan et al., 2009; Foster et al., 2011). Using deuterated water (D2O) and 13NH4 as tracers and SIP-Nano-SIMS analysis enabled Kopf et al. (2015) to demonstrate that intra-population variability in growth rates and ammonium assimilation could be measured in chemostat-grown bacterial cells. However, these techniques generally have low sample throughput, demanding sample preparation requirements, and can be costly in terms of time and/or money invested per cell, all of which can limit the scale of population surveys.

Raman microspectroscopy is amenable to single-cell applications and is complementary to MAR-FISH and nano-SIMS-FISH. Raman microspectroscopy has the advantages of non-destructively yielding intracellular molecular information, of requiring minimal sample preparation, and enabling rapid interrogation of many preserved or live cells. Recent advances in Raman microspectroscopic technology have dramatically broadened its microbiological applications (Brehm-Stecher and Johnson, 2004; Wagner, 2009; Huang et al., 2010; Wang et al., 2016). For example, Raman spectra of single cells have revealed metabolic histories and species identity, through characterization of an organism’s macromolecular composition (e.g., Huang et al., 2004, 2007a; Hermelink et al., 2009; Hall et al., 2011). Huang et al. (2007b) demonstrated that intensity ratios of specific wavenumbers within Raman spectra from individual bacteria varied quantitatively with amount of 13C-glucose available. Furthermore, those cells were phylogenetically identifiable by FISH probing (SIP-Raman-FISH). Li et al. (2012) recently demonstrated that assimilation of 13C-enriched dissolved inorganic carbon (DIC) by individual photoautotrophic cells can be accurately quantified from wavenumber shifts in resonance Raman (SCRR) spectral peaks emanating from carotenoid pigments. Carotenoids are excellent target analytes because all photoautotrophic microbial taxa produce at least one form as accessory light-harvesting pigments or as protection against reactive oxygen species (Garcia-Asua et al., 1998). They are easily resolved by resonance Raman scattering, which increases photon scattering efficiency over spontaneous Raman scattering by at least a 1,000-fold by using laser excitation within the electronic transition frequency band of the analyte (e.g., Taylor et al., 1990; Robert, 2009).

We present a refinement of the SIP-SCRR-FISH approach (Li et al., 2012) that now enables quantitative Raman spectrometric measurement of growth rates in individual photoautotrophic cells. We use this tool to examine growth as the ultimate expression of inter- and intraspecific trait variability. Cells from replicate isogenic Synechococcus sp. cultures provided with varying concentrations of 13C-bicarbonate were interrogated by SCRR through time course experiments to determine their degree of labeling from which single-cell growth rates were calculated and compared to independent measurements of population growth. 13C-labeled populations of Synechococcus sp. and the diatom, Thalassiosira pseudonana, growing at known, but different rates were mixed to demonstrate that SCRR could distinguish among them. Optimal experimental design, underlying assumptions, analytical precision, and intra-population heterogeneity are evaluated. This technique has the advantages of relatively high sample throughput, low analytical costs, and potential application to natural phytoplankton communities in field experiments.
MATERIALS AND METHODS

Media Preparation and Cultivation Conditions

Phytoplankton cultures were grown in f/2 media (Guillard and Ryther, 1962) in which total inorganic carbon (C\textsubscript{0} = CO\textsubscript{2} + H\textsubscript{2}CO\textsubscript{3} + HCO\textsubscript{3}\textsuperscript{-} + CO\textsubscript{3}\textsuperscript{2-}) was either replaced or augmented with varying proportions of 13C-enriched bicarbonate [HCO\textsubscript{3}\textsuperscript{-}; see Supplementary Material (SM) 1]. The 12C- and 13C-bicarbonate solutions (Cambridge Isotope Laboratories, Inc. Andover, MA; 99% 13C, 97% chemical purity) were prepared as 0.4 M working stocks. Nutrient and bicarbonate solutions were aseptically added to autoclaved filtered seawater (<0.22 μm = FSW) in 200-ml sealed septum bottles. C\textsubscript{T} concentrations were then measured using a flow injection analysis system and compared to known C\textsubscript{T} standards (Hall and Aller, 1992).

For the SIP experiment examining isotopic end-members, cultures of the cyanobacterium, Synechococcus sp. (RS9916), were grown at natural 13C abundances and at conditions under which 96% of the C\textsubscript{T} was replaced with 13C-bicarbonate by pH manipulation (see SM 1). For the SIP calibration experiment, complete f/2 media was augmented with sterile bicarbonate solutions with varying 13C abundances (f\textsubscript{media} = 13C\textsubscript{media}/(12C\textsubscript{media} + 13C\textsubscript{media})) and a mean final C\textsubscript{T} of 3.78 ± 0.10 mM C (2 mM added). To minimize variations in growth conditions, C\textsubscript{T} was kept constant while f\textsubscript{media} was manipulated. Nominal (gravimetric) f\textsubscript{media} determinations (0.011, 0.10, 0.20, 0.30, 0.40, and 0.50) were corrected to 0.011, 0.11, 0.22, 0.32, 0.43, and 0.54, respectively, based on actual f\textsubscript{media} measured by isotope ratio mass spectrometry (IRMS) at the UC Davis Stable Isotope Facility. After inoculation, each septum bottle was attached to its own venting system permitting atmospheric exchange of all gases, except CO\textsubscript{2}. Modifications of Li et al.’s (2012) venting system were as follows: Ascarite II\textsuperscript{TM} (Thomas Scientific\textsuperscript{®}) replaced NaOH pellets as our CO\textsubscript{2} trap in open 30 ml syringe barrels, capped with polyester fiber, and connected to 0.2 μm in-line filters that were joined to septum bottles by 1/8” (O.D.) stainless steel swan neck tubes. The swan neck replaced vertical tubes to prevent condensate from the CO\textsubscript{2} trap leaking into septum bottles during extended incubations and contaminating samples. Cultures were subsampled through time while incubating at 20°C on a rotating platform that assured uniform light exposure (48–63 μmol quanta m\textsuperscript{-2} s\textsuperscript{-1}) during a 12:12 h light/dark cycle.

Directly evaluating the SIP-SCRR method’s ability to measure varying f\textsubscript{cell} or growth rate in natural phytoplankton assemblages is difficult, if not impossible, without an independent method to measure non-uniform growth rates among the assemblage members. Therefore, a Synechococcus sp. assemblage was constructed by mixing equal volumes of 12-days cultures from six different f\textsubscript{media} ratios. These cells were ostensibly growing at the same rate, but had different f\textsubscript{cell} signatures due to exposure to different f\textsubscript{media}, essentially mimicking a mixed assemblage with varying growth rates. In a second experiment to compare contemporaneous slow-growing (Synechococcus sp.; mean g = 4.12 days) and fast-growing (T. pseudomana; mean g = 1.44 days) populations, cultures were grown in parallel at natural (0.011 f\textsubscript{media}) and elevated 13C abundances (0.48 f\textsubscript{media}) and subsampled through time. SCRR filters were prepared from mixtures of these cells as a mimic of a heterogeneous environmental sample.

Population Growth in SIP Experiments

Daily change in in vivo chlorophyll a fluorescence was used to measure population growth in all treatments. After gentle agitation, triplicate 200-μl subsamples were assayed in a Turner Designs\textsuperscript{®} Aquafluor\textsuperscript{TM} fluorometer, calibrated according to manufacturer’s instructions. When required, subsamples were diluted with sterile f/2 to remain below 80% detector saturation. Population growth rates (μ\textsubscript{pop}) were calculated from arbitrary fluorescence units (AFU) either as the regression slope of ln AFU vs. time for the entire exponential growth phase (mean μ\textsubscript{pop}) or as the difference between neighboring ln AFUs within the time course (instantaneous μ\textsubscript{pop,inst} = [ln AFU\textsubscript{t+1}−ln AFU\textsubscript{t}]/(1+(t−1))]. Direct microscopic cell counts in the control sample confirmed that AFU values were highly correlated with cell concentrations (r\textsuperscript{2} = 0.97, p < 0.001) during exponential growth phase, signifying that AFU/Δt is a reliable proxy for population growth under our experimental conditions.

SCRR Sample Preparation from SIP Experiments

For SCRR microspectrometry sampling, volumes removed (0.25–5.00 ml) were adjusted to obtain cell densities of 20–50 cells per microscope field and replaced with N\textsubscript{2} gas to prevent a partial vacuum within the incubation bottles. After vortexing subsamples, cells were collected on 25 mm 0.2 μm polycarbonate membranes (Millipore\textsuperscript{®} GTP\textsuperscript{TM}) and rinsed with phosphate-buffered saline and filtered to dryness. Membranes were then air-dried in Millipore\textsuperscript{®} PetriSlides\textsuperscript{TM} and stored at −20°C.

In early experiments, potential interference of Cy3 fluorescent oligonucleotide probes with SCRR data acquisition was evaluated on membrane subsamples subjected to FISH. Membrane wedges were hybridized with a general bacterial probe (EUBMIX: Amann et al., 1990; Daims et al., 1999) in 35% formamide for 2 h and washed with buffer for 30 min and stored at −20°C until analysis (Pernthaler et al., 2001).

Although polycarbonate membranes are widely used for FISH and other fluorescence microscopies, these and most other types of filters and membranes produce contaminating Raman emissions (Raman-active). Likewise, borosilicate glass slides and cover slips and most mounting fluids (Citifluor\textsuperscript{®}, Vectashield\textsuperscript{TM}, Cargille\textsuperscript{®} Type A\textsuperscript{TM} immersion oil, and glycerol) are Raman-active, water being the exception. Therefore, we developed a technique to transfer cells from polycarbonate membranes to mirror-finished 304 stainless steel slides (1 × 3 × 0.0235”) supplied by Stainless Supply\textsuperscript{®} (Monroe, NC USA). Prior to use, slides were sequentially washed in an ultrasonic bath using acetone, isopropanol, and methanol, followed by MilliQ\textsuperscript{TM} water rinsing at each step, and then air-dried.

To prepare both probe-hybridized (FISH) and non-hybridized cells for SCRR, samples were transferred to cleaned metal slides using a modified filter-transfer-freeze (FTF) technique (Hewes...
and Holm-Hansen, 1983). Briefly, each membrane wedge was placed sample-side down on a small droplet of sterile, particle-free MilliQ™ water (2–5 μL depending on wedge size) and the slide was placed on an aluminum cooling (−80°C) block. The membrane was peeled from the steel surface immediately after freezing, leaving most cells frozen to the slide, which was then air-dried in the dark (Suter, 2017). Transfer efficiency of Synechococcus cells was determined microscopically on filter wedge replicates; one set analyzed prior to freeze transfer and the other afterwards.

**Single-Cell Resonance Raman Microspectrometry**

SCRR measurements were performed using a Renishaw® inVia™ confocal Raman microspectrometer configured with a modified upright Leica® DM2700™ fluorescence microscope, a computer-controlled motorized XYZ stage (0.1 μm step size), 514 nm Ar⁺ ion laser, and 1,040 × 256 CCD Peltier-cooled detector. After cells were manually targeted by mouse clicks on a Cy3-fluorescent or autofluorescent digital image, the automated stage centered each cell under the laser beam for Raman data acquisition. For each sample, 25–40 cells (0.5–1.8 μm equivalent spherical diameters) were individually interrogated at 10% laser power (92 ± 7 μW at sample) through a Leica® dry 100× (NA = 0.90) objective lens which produced a spot diameter of 0.7 μm. Spectra were obtained using a 65 μm slit centered at 1,752 μm, a 1,800 line/mm diffraction grating, aligned to wavenumber region between ∼400 and 2,000 cm⁻¹ (1,350 cm⁻¹ center) and acquisition of two 1 s CCD detector exposures.

**Spectral Analysis**

Spectra were processed using Renishaw’s® Wire 4.1™ software by first subtracting baselines using the software’s standard best fit polynomial algorithm, and its intensity normalization function to standardize all spectra to a maximum peak value of 1, because cell-to-cell Raman scattering intensities are variable. Effects of 13C enrichment on behavior of dominant resonance Raman (RR) peaks were evaluated by two methods; peak-picking and curve-fitting. For peak-picking, center positions and widths of dominant spectral peaks were simply tabulated by the software and recognition thresholds were adjusted manually when required.

For curve-fitting analyses, two of the dominant RR peaks, usually positioned at ∼1,521 and 1,157 cm⁻¹, were deconvolved using Wire 4.1’s function with our own empirical scripts. Peak shape and position are controlled by relative contributions of each isotopologue bond (e.g., 12C12C, 12C13C, 13C12C) to total peak area (see SM 2). Center positions (⟨Δν⟩ = wavenumber, cm⁻¹) for pure 12C12C and 13C13C isotopologues were estimated from the regression intercept and slopes of calibration curves [e.g., ν (C=C) vs. fcell], respectively. Center positions for the intermediate isotopologue peaks were estimated as the midpoint between the end-members. After local baseline correction, contribution of each isotopologue to triplet peak area was determined by allowing peak positions to float ± 1 cm⁻¹ and peak widths to float ± 10 cm⁻¹ and by running a full Voigt fit routine (floating Lorentzian and Gaussian contributions to peak form) for 5,000 iterations or a < 0.00001 tolerance (difference between reduced chi-square values of two successive iterations), whichever came first. The curve fit function continuously compared the sum of the isotopologues’ areas (synthetic peak) to observed data and reported the reduced Chi square values (goodness of fit).

**Determination of Cell Labeling and Single Cell Growth Rates**

Since CO₂ in the septum bottles is not allowed to exchange with the atmosphere, the isotopic signature of autotrophic cells (fcell) must predictably approach that of the media’s DIC (fmedia) scaled for isotopic fractionation, α with every cell division. Fractional labeling of populations was thus computed as follows.

To estimate α, an average δ13C value of −23‰ for marine plankton and a mean δ13C value of +1.5‰ for the ocean’s mixed layer C T pool were selected from global summaries (Goericke and Fry, 1994; Hoefs, 2009). Cellular 13C fractionation, αcell–DIC, was calculated from the isotope ratios (R = 13C/12C) of phytoplankton and seawater DIC using Equation (1).

\[
α_{cell–DIC} = \frac{R_{cell}}{R_{DIC}} = \frac{δ^{13}C_{cell} + 1000}{δ^{13}C_{DIC} + 1000} = \frac{-23 + 1000}{+1.5 + 1000} = 0.976
\]

(1)

Values for fcell in specific samples were calculated via Equation (2), with uncertainties propagated from individual terms (see SM 3 and 4 for derivations of equations).

\[
f_{cell} = \frac{αf_{media}}{1 + (α - 1)f_{media}} + \left(\frac{αf_o}{1 + (α - 1)f_o} - \frac{αf_{media}}{1 + (α - 1)f_{media}}\right) e^{-nln(2)}
\]

(2)

where f_o = ancestral fractional isotopic signature of the media (0.011 13C) and n = number of generations completed in the presence of enriched DI13C, which was calculated by Equation (3) using the population’s specific growth (μpop, day⁻¹) measured by in vivo fluorescence.

\[
n = \frac{t}{g} = t\frac{μ_{pop}}{ln (2)}
\]

(3)

where t = time elapsed (days) and g = generation time (days).

Single-cell growth (μsc) can be calculated independently of population growth data if the fcell of individual cells can be measured. SCRR enables such determinations if a spectral feature, such as mean peak wavenumber (⟨Δν⟩) for the ν(C=C) vs ν(C=C) bonds in carotenoids varies predictively with fcell. This relationship can be utilized according to Equation (4).

\[
⟨Δν⟩ \approx b_0 + b_1 f_{cell}
\]

(4)

After determining the values of b_0 and b_1 experimentally, the number of generations (n) completed after spiking samples with DI13C can be calculated from measurements of ⟨Δν⟩ (Raman), fmedia, and f_o with Equation (5). Once n is derived, g or μsc is calculated from Equation (3).

\[
n \approx \frac{1}{ln(2)} ln \left(\frac{f_{media} - f_o}{f_{media} - (1 + (α - 1)f_{media}) (⟨Δν⟩ - b_0) / αb_1}\right)
\]

(5)
**RESULTS**

**SIP Experimental Design**

For SIP experimental design, it was initially critical to establish whether DIC replacement or augmentation is preferable, to determine how much $^{13}$C-bicarbonate tracer is required, and then to accurately establish the value of $f_{\text{media}}$. In principle, a known amount of the $C_T$ pool must be removed before replacing with sufficient $^{13}$C-bicarbonate to return media to the original $C_T$ pool size. Among published DIC replacement strategies, such as microwaving (Li et al., 2012), $N_2$ purging, and pH manipulation, only pH manipulation adequately constrained the $C_T$ and $f_{\text{media}}$ terms (see SM 1).

DIC replacement may be suitable for experiments with cultivated populations in synthetic media, but is clearly inappropriate for experiments with natural field assemblages. DIC augmentation requires less manipulation and therefore potentially introduces fewer artifacts. More than doubling DIC augmentation requires less manipulation and therefore is absolutely essential for accurate growth calculations.

Accordingly, experiments reported here used media augmented with the same $C_T$ but varying $f_{\text{media}}$, and pH was titrated back to 8.0 prior to inoculation with 0.1N NaOH (SM1), unless otherwise noted.

**SIP-Raman-FISH Spectra**

While the identities of our cultures are known, phylogenetic identification of target cells by FISH enables application of SIP-SCRR to field samples. Cells hybridized against the Cy3-EUBMIX probe fluoresce brightly (Figure 1A) and can also be observed under bright-field illumination (Figure 1B). Cy3 fluorescence did not interfere with SCRR spectral acquisition when exciting within the carotenoid spectral absorption band (370–530 nm) at 514 nm (Figure 1C). Spectral responses to $^{13}$C enrichment of cellular biomass are evident in spectra i–v (Figure 1C). Spectrum $i$ obtained in 2 s from a single cell grown in control media ($f_{\text{media}} = 0.011$) was dominated by three major peaks attributed to carotenoids. The 1,521 cm$^{-1}$ peak has previously been assigned to $\nu$ (C–C) in-phase stretching, the 1,156 cm$^{-1}$ to $\nu$ (C–C) in-phase stretching, and 1,004 cm$^{-1}$ to a combination of $\delta$ (C=CH$_3$) methyl deformation and $\delta$ (C–H) out-of-plane bending modes in $\beta$-carotene (Marshall and Marshall, 2010). Evolution of redshifted peaks is evident as cells are grown in 54% DI$^{13}$C for 3, 6, 12, and 24 days (spectra ii–v). Thus, cells actively growing photoautotrophically in DI$^{13}$C are easily recognized by redshifted positions of any of these three SCRR peaks.

**Quantitative Application of Single-Cell Resonance Raman Microspectrometry**

To evaluate whether SCRR microspectrometry can accurately quantify fractional labeling of individual photoautotrophic cells ($f_{\text{cell}}$), parallel time course incubations of *Synechococcus* sp. were conducted under varying $f_{\text{media}}$. Populations maintained exponential growth for the first 18 days at a mean rate of $\mu_{\text{pop}} = 0.220 \pm 0.002 \text{ day}^{-1}$ ($g = 3.20 \pm 0.03 \text{ days}$) for all treatments as measured by in vivo fluorescence (Figure S1) and thus were unaffected by $f_{\text{media}}$ values between 0.011 and 0.54 ($p > 0.98$; Kruskal-Wallis one-way ANOVA). Thus, the range of $f_{\text{media}}$ used did not appear to introduce artifacts to the SIP-SCRR experiments and theory dictates that all populations would incorporate $^{13}$C into intracellular pools in proportion to their particular $f_{\text{media}}$.

SCRR spectra were subjected to Renishaw® Wire 4.1™ curve-fitting routine to illustrate how resonances from $^{12}$C$^{12}$C, $^{12}$C$^{13}$C, and $^{13}$C$^{13}$C bonds (isotopologues) contribute to the total
area, position, and shape of peaks as cells become $^{13}$C-enriched. As an example, cells grown in $f_{\text{media}} = 0.011$ (Figure 2A) and 0.54 for 3–9 days illustrate the effects of $^{13}$C enrichment on the 1,521 cm$^{-1}$ ($\nu$ (C=C)) peak (Figures 2B–D). During early stages of labeling (Figures 2B,C), the triplet peak widens with a distinct shoulder that disappears as $^{12}$C$=^{13}$C replaces $^{12}$C$=^{12}$C (Figure 2D). This transition causes red-shifting of the triplet peak’s mean resonance ($\langle \Delta \nu \rangle$) and eventually leads to bandwidth narrowing (Figures 1, 2D). Curve-fitting skill is illustrated by the coherence between fit curve solutions (gray lines; Figure 2) and observed data (green lines; Figure 3). The mean reduced Chi Square was 1.00 ± 0.25 (S.D.) for the entire 0.54 $f_{\text{media}}$ time course ($N = 172$ cells).

Curve-fitting analysis of spectra from the entire $f_{\text{media}} = 0.32$ time course illustrate that the proportion of underlying peak areas attributed to $^{12}$C$=^{12}$C bonds decreased asymptotically to ~50%, while those of $^{12}$C$=^{13}$C and $^{13}$C$=^{13}$C bonds increased asymptotically to ~44 and ~6%, respectively, as $^{13}$C was incorporated into cellular biomass (non-linear least-squares regression, solid lines, Figure 3). Observed changes are consistent with our model for $^{13}$C-incorporation at random positions in a representative carotenoid molecule ($\beta$-carotene) (dashed lines, Figure 3; see SM 2 and SM 3 for derivation). In the present example, fractionation would theoretically limit $f_{\text{cell}}$ to a maximum value of 0.31, at which the expected proportions of $^{13}$C$=^{13}$C, $^{12}$C$=^{13}$C, and $^{13}$C$=^{13}$C bonds and their associated peak areas would approach 48, 43, and 10%, respectively (Equations S11, S20). However, unlike $^{12}$C$=^{12}$C and $^{13}$C$=^{13}$C bonds, the observed and predicted proportions of $^{13}$C$=^{13}$C bonds were significantly different after 24 days (Figure 3C). We attribute this discrepancy to lower curve-fitting skill caused by the baseline correction method employed, which could undoubtedly be improved upon. The same time/growth-dependent trends in isotopologue contributions
to the \( \nu (C=C) \) peak area were observed at all levels of DI\(^{13}\)C augmentation (not presented). Curve-fitting skills for the isotopologues contributing to the 1,156 cm\(^{-1} \) \( (\nu (C-C)) \) and 1,004 cm\(^{-1} \) \( (\delta (C=CH)) \) peak areas were less satisfactory due to baseline uncertainties and overlapping spectral features from other molecular bonds, particularly near 1,004 cm\(^{-1} \) (not presented).

As an alternative to curve-fitting peak areas as a function of time (e.g., Figure 3), we simply compared software-determined center positions \( (\Delta \nu) \) of major peaks in randomly selected cells \( (N = 25 \text{ cells sample}^{-1}) \) for all DI\(^{13}\)C-augmented cultures to the control \( (0.011 f_{\text{media}}) \). Figure 4 illustrates typical asymptotic responses of the three major peak wavenumbers as functions of growth in the \( f_{\text{media}} = 0.32 \) treatment. Declines in peak wavenumber (red shifts) are steep over the first 2 generations, slowing as they approach an asymptote in about 5–7 generations. Clearly, growth-dependent changes in mean Raman peak wavenumbers \( (\Delta \nu) \) of the three carotenoid signature peaks observed in single-cells (boxes and whiskers) agree well with theoretical predictions (dashed lines) of red-shifting resonances for a population isotopically equilibrating with its media (see SM 2 for derivation of prediction).

The most useful, generalized trend is obtained by constructing calibration curves directly comparing \( (\Delta \nu) \) with \( f_{\text{cell}} \). Knowing \( f_{\text{media}} \) and population growth \((\mu_{\text{pop}} \text{ or } g)\) from independent chemical and fluorescence measurements (Figure S1) and assuming an isotopic fractionation factor \((\alpha)\) of 0.976 Equation 1), \( f_{\text{cell}} \) can be calculated from Equation 2 and Equation 3. Mean SCRR wavenumbers \((\Delta \nu)\) of the three major carotenoid peaks declined linearly across all values of \( f_{\text{cell}} \) examined; from natural \(^{13}\)C abundances \( f_{\text{cell}} = 0.0107 \) to an \( f_{\text{cell}} \) of \( \sim 0.53 \) (Figure 5). The regressions' coefficients of determination \( (r^2 = 0.88–0.97) \), the slopes' standard errors \( (s.e. = 0.18–0.26) \) and 99% confidence intervals demonstrate well-constrained relationships between \( f_{\text{cell}} \) and \( (\Delta \nu) \). Collectively, the robust linear regressions and their agreement with theory suggest that \( f_{\text{cell}} \) can be reliably approximated by simply measuring \( (\Delta \nu) \).

Analogous examination of the curve-fitting approach across the entire \( f_{\text{cell}} \) range yielded results consistent with peak position analysis, i.e., the contributions of individual isotopologues to triplet peak area varied predictably with cell \(^{13}\)C-content (not presented). However, scatter evident in isotopologue peak contributions, largely due to variable curve-fitting skill, renders isotopologue peak area analysis less suitable than the highly predictable relationship between \( (\Delta \nu) \) and \( f_{\text{cell}} \) for quantitative applications. Nevertheless, empirical curve-fitting results conform to theory and illustrate the mechanics behind red-shifting peak wavenumbers.

**Variability and Sensitivity of SCRR**

Scatter in SCRR microspectrometric data emanate from both natural cell-to-cell variability in \(^{13}\)C-content inherent to different growth phenotypes and from analytical uncertainty. Our sample size \((25–40 \text{ cells})\) was adequate to distinguish between time points and treatments and to represent intra-population variability (e.g., Figures 3–5). If required, uncertainty could be reduced somewhat with larger sample sizes. Potential sources of analytical error in Raman microspectrometry include: improper spectrometer calibration, beam misalignment, varying laser intensity, poor beam focusing, variable target geometry, and contaminating materials associated with target cells. However, current instrumentation and best practices eliminate or minimize most of these issues. Concerns about small variations in focus and target geometry are irrelevant because they primarily affect emission intensity which is not critical to our analyses.

*Synechococcus* sp. cells perpetually grown on natural \(^{13}\)C/\(^{12}\)C abundances serve as the best candidate for an authentic standard to assess analytical error. Table 1 summarizes spectral data obtained from all control cultures. Assuming \(^{13}\)C and \(^{12}\)C...
atomic abundances (g = 3.3 days) exhibited considerable day-to-day oscillations in growth rates from time course (e.g., Figure 4) or brief end-point incubations. To illustrate, 25 single-cell growth rates ($\mu_{ac}$) throughout exponential growth phase ($r = 0.32$ and $0.43 f_{med}$ incubations are compared to proximal daily population growth rates ($\mu_{pop, inst}$) determined at $t = 1$, $t = 5$, and $t = t + 1$ (open boxes and whiskers Figures 6A,B). While mean $\mu_{pop}$ from 0 to 18 days was $0.21$ day$^{-1}$ for both populations (broken horizontal lines), these relatively slow-growing phototrophs will necessarily approach the isotopic signature of their photosynthetic carbon source. This is also true for $\delta$ (C=CH) peak positions in cells growing at natural $^{13}$C abundances spanning 10.2 and 8.1 cm$^{-1}$, respectively, while the $\nu$ (C-C) peak spanned just 2.4 cm$^{-1}$ (Table 2). Furthermore, $\nu$ (C-C) peak wavenumbers observed in photoautotrophic protists were statistically indistinguishable from those of cyanobacteria (ANOVA p-value = 0.905), whereas the $\nu$ (C-C) and $\delta$ (C=CH) peak positions were more phylogenetically distinctive (Table 2). Therefore, even though the $\nu$ (C-C) peak had the broadest dynamic range and highest precision within our Synechococcus cultures, we chose the more phylogenetically conserved $\nu$ (C-C) peak for further quantitative analyses.

Theoretical sensitivity or limit of detection (LOD) of the mean $\nu$ and $\delta$ wavenumbers was calculated using the formula $\text{LOD} = 3 \sigma / b$, where $\sigma$ is the pooled standard deviation (±0.305 cm$^{-1}$) of $\nu$ and $\delta$ observed at each value of $f_{cell}$ and $b = 0.305 \times f_{cell}$ (Winefordner and Long, 1983). This calculation yielded a LOD of 0.03 leading us to conclude that $\Delta \nu$ of $\sim$3% is detectable by our technique.

### Single-Cell Growth Rates Determined by SCRR

Autotrophs will necessarily approach the isotopic signature of $^{13}$C-augmented media ($f_{med}$) as a function of time, growth rate, and isotopic fractionation (Equation 2 and Equation 3). Therefore, the empirical linear relationship between $\Delta \nu$ and $f_{cell}$ (Figure 5) can be used to estimate single-cell growth rates from time course (e.g., Figure 4) or brief end-point incubations. To illustrate, 25 single-cell growth rates ($\mu_{ac}$) throughout exponential growth phase ($r = 0.32$ and $0.43 f_{med}$ incubations are compared to proximal daily population growth rates ($\mu_{pop, inst}$) determined at $t = 1$, $t = 5$, and $t = t + 1$ (open boxes and whiskers Figures 6A,B). While mean $\mu_{pop}$ from 0 to 18 days was $0.21$ day$^{-1}$ for both populations (broken horizontal lines), these relatively slow-growing phototrophs exhibited considerable day-to-day oscillations in $\mu_{pop, inst}$ (diamonds). We speculate that these oscillations result from intrinsic cell cycle periodicity and/or extrinsic variations in incubation conditions. Nonetheless, median growth rates computed over the entire exponential phase of $f_{med}$ 0.32 and
TABLE 1 | Analytical precision of single-cell resonance Raman spectral features assessed from 150 Synechococcus sp. (RS9916) continuously cells grown at natural $^{13}$C abundances ($f_{\text{media}} = 0.011$).

<table>
<thead>
<tr>
<th>Source</th>
<th>$\nu$ (C=CH) ($\sigma_{\nu}$)</th>
<th>$\nu$ (C=C) ($\sigma_{\nu}$)</th>
<th>$\delta$ (C=CH)/$\delta$ (C-H) ($\sigma_{\nu}$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROKARYOTES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechocystis sp. (PCC 6803)</td>
<td>1.517 1.158 1.004</td>
<td>Li et al. (2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechocystis elongatus (PCC 7942)</td>
<td>1.522 1.158 1.006</td>
<td>Li et al. (2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechococcus sp. (RS9916)</td>
<td>1.521 1.158 1.006</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prokaryote Mean</td>
<td>1519.9 1156.5 1005.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>2.8 1.1 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>0.18 0.09 0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUKARYOTES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arctic &quot;AMA&quot; microalgae</td>
<td>1.524 1.157 1.003</td>
<td>Li et al. (2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalassiosira weissflogii (Bacillariophyta)</td>
<td>1.526 1.157 1.010</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalassiosira pseudonana (Bacillariophyta)</td>
<td>1.526 1.157 1.010</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterocapsa triquetra (Dinoflagellata)</td>
<td>1.525 1.156 1.011</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aureocoluma lagunensis (Pelagophyceae)</td>
<td>1.527 1.158 1.009</td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eukaryote Mean</td>
<td>1525.6 1156.6 1008.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>1.2 0.9 3.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%CV</td>
<td>0.08 0.08 0.32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA p-value</td>
<td>0.006 0.905 0.250</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*$^a$%CV = coefficient of variation = standard deviation $\times$ 100/mean.

*$^b$99% confidence intervals.

0.43 cultures were $0.22 \pm 0.07$ and $0.20 \pm 0.07$ day$^{-1}$ for single cells (Raman-derived) and $0.22 \pm 0.07$ and $0.22 \pm 0.07$ day$^{-1}$ for populations (fluorescence-derived), respectively (Figures 6A,B). In fact, $\mu_{sc}$ computed from SCRR measurements between 0 and 18 d for the 0.11, 0.32, 0.43, and 0.54 $f_{\text{media}}$ treatments were statistically indistinguishable from $\mu_{\text{pop,inst}}$ measured over the same period ($p > 0.05$; ANOVA). Inexplicably, $\mu_{sc}$ rates derived from the 0.22 $f_{\text{media}}$ treatment were lower than those from $\mu_{\text{pop,inst}}$ measurements in all but the 3 day sample ($p < 0.05$ ANOVA; not presented).

Both in vivo fluorescence and SIP-SCRR based measurements of specific growth rate are subject to errors that may contribute differing amounts to the observed variability. For comparison, the mean %CV for ($\Delta \nu$) values obtained by SCRR ($\pm 0.05$%; $N = 861$) within individual samples was two orders of magnitude smaller than that obtained by daily triplicate in vivo fluorescence measurements ($\pm 3.7$%; $N = 144$; S.E. $= 0.23$). The mean relative error for $f_{\text{cell}}$ was $\pm 2.0$% (S.E. $= 0.25$), which is controlled by uncertainties in $b_0$ and $b_1$ in Figure 5 and measurements of $f_{\text{media}}$. CVs for $\mu_{sc}$ tend to be higher at lower $f_{\text{media}}$ and shorter incubations times (see SM 4 for discussion of uncertainties).

Of the observed variance for $\mu_{sc}$ through time (CV $= 29\% \pm 2.4$ S.E.) illustrated in Figure 6, more than 93% appears to be due to cell-to-cell heterogeneity in physiological state within these isogenic populations. Likewise, most variance observed in $\mu_{\text{pop,inst}}$ through time (CV $= 64\% \pm 24$ S.E.) is likely due to actual variations in cell division, rather than analytical imprecision. We note that in vivo fluorescence measurements were made at approximately the same time every day (±1.5 h). Consequently, samples were undoubtedly withdrawn at different points within the 3.3 days cell cycle of these populations.

An approach routinely used in biomedical studies for evaluating agreement between two independent methods of measuring a single variable is to visualize the distributions of the differences between the two measurements plotted against their means (Bland and Altman, 1986). The Bland-Altman plot is more appropriate than regression analysis when those independent measurements have small dynamic ranges that are surpassed by the measurements’ observed variability. Distributions of observed $\mu_{sc} - \mu_{\text{pop,inst}}$ in the $f_{\text{media}} = 0.32$ and 0.43 at each time point are compared to $(\mu_{sc} + \mu_{\text{pop,inst}})/2$ in Figure 6C, where the $\mu_{\text{pop,inst}}$ term for each $\mu_{sc}$ was the mean determined at $t_{x-1}$, $t_x$, and $t_{x+1}$. If the two measurement methods consistently produced identical results, all points would fall on the horizontal zero line.
In the current case, the average difference is +0.003, meaning that on average the $\mu_{sc}$ method returns a 0.3% higher value than the $\mu_{pop,inst}$ method among all observations. The +1.96 and −1.96 boundaries (broken horizontal lines) represent the 95% confidence intervals, and 16 of the 17 outliers are positive, again illustrating that the $\mu_{sc}$ method tends to return slightly higher values than the $\mu_{pop,inst}$ method. Absolute accuracy of our $\mu_{sc}$ method cannot be determined from this analysis largely due to cell-to-cell variability and uncertainties over which of the daily $\mu_{pop,inst}$ measurements are most representative of a particular $\mu_{sc}$ time point. Nonetheless, the data distribution in Figure 6C illustrates that the vast majority of SIP-SCRR-derived growth rates are within the 95% confidence intervals of independently-measured population growth rates. Furthermore, the preceding error analysis suggests that most observed variability is attributable to real differences in growth activity and not to analytical error.

**SIP-SCRR-Derived Growth Rates in Mixed Assemblages**

Our final objective was to demonstrate that SIP-SCRR can measure growth rates of individual cells in a mixed photoautotrophic assemblage whose members are assimilating $^{13}$C-bicarbonate at contrasting rates. In the absence of an independent method to measure single-cell growth in natural communities, we constructed an artificial assemblage by dispensing equal volumes of *Synechococcus* cell suspensions from the six $f_{media}$ treatments (Figure 5) at a single time point. Figure 7A presents the SCRR spectra obtained from 13 individual cells in the mixed assemblage (Figure 7B). After interrogating multiple fields, the statistical distribution of *Synechococcus* sp. cells with six distinctive $^{13}$C signatures added to the mixture were indistinguishable (ANOVA; $p = 1.00$) from those recognized by SIP-SCRR (Figure 7C). Therefore, morphologically identical cells with distinctive $^{13}$C signatures can be recognized and reliably quantified within an isotopically mixed assemblage by this method.

To further evaluate application of this technique to phylogenetically diverse field assemblages, subsamples of parallel cultures of relatively slow-growing cyanobacteria and fast-growing diatoms were mixed at several time points. Grown in identical media ($f_{media} = 0.011$ and 0.48), the diatom, *T. pseudonana*, and cyanobacterium, *Synechococcus* sp., maintained exponential growth for 6 days, attaining population growth rates of 0.483 and 0.161 day$^{-1}$, respectively. In Figure 8, the distribution of $f_{cell}$ values computed by plugging SCRR observations of $\langle \Delta \nu_{C-C} \rangle$ into Equation (4) are compared to $f_{cell}$ values predicted from $\alpha$, $f_{media}$, population growth measured by *in vivo* fluorescence of the cultures and Equation (2). The trend line computed from all cells ($N = 253$) spanning $f_{cell}$ values of 0.0107 to 0.449 is not significantly different from the 1:1 line. These results demonstrate that within experimental error and intra-population variability, fractional $^{13}$C-labeling of single diatom and cyanobacterial cells computed from SIP-SCRR measurements closely agrees with fractional labeling of entire populations grown at natural and enhanced $^{13}$C-DIC abundances. Results presented in Figures 7, 8 support the proposition that single-cell growth rates within mixed natural photoautotrophic assemblages can be computed from SIP-SCRR measurements.

**DISCUSSION**

**Single-Cell Productivity Techniques**

Individual or agent-based ecological models suggest that intra-population variability in genetics, cell line history and microspatial geochemical heterogeneity can influence population responses to environmental forcing along multiple pathways and lead to divergent outcomes (e.g., Hellweger and Kianirad, 2007; Bolnick et al., 2011; Bucci et al., 2012; Fredrick et al., 2013). Empirical testing of such models is challenging because the requisite single-cell productivity measurements have been limited historically to a few laboratory techniques with cultures. For example, division of individual cells encapsulated in mineral
oil has been followed through time microscopically (Dewan et al., 2012; Damodaran et al., 2015) and increasing mass of individual cells has been measured through time in serial microfluidic mass sensor arrays (Cermak et al., 2016). MAR-FISH provides single-cell activity measurements, but does not yield growth rates per se. Recent evidence suggests that two complementary techniques, SIP-nano-SIMS-FISH and SIP-Raman-FISH, hold the most promise for providing single-cell growth rates in both the lab and field experiments.

SIP-nano-SIMS-FISH has been used to estimate growth of anaerobic phototrophic bacteria as well as for cells in syntrophic and symbiotic partnerships (Musat et al., 2008; Orphan et al., 2009; Foster et al., 2011). However, the growth computations in these studies depended upon several variables with unspecified uncertainties, such as substrate pools’ isotopic composition estimates, assumed assimilative/dissimilative relationships, and microscopic biovolume measurements used to derive elemental content based on allometry. Recognizing this limitation, Kopf et al. (2015) recently demonstrated that accurate single-cell growth rates could be derived by applying appropriate mathematical models to data obtained from bacterial chemostat cultures labeled with $^2$H$_2$O under well-constrained conditions and analyzed by SIP-nano-SIMS.

Previous studies applying SIP-Raman to aquatic microbiology measured cell activity levels by altered Raman spectral features and then cataloged them either morphometrically or by combining with FISH (e.g., Huang et al., 2007b; Li et al., 2012; Berry et al., 2014). Huang et al. (2007b) were the first to demonstrate that red shift ratios in single-cell Raman spectra of cultivated bacteria varied predictably with $^{13}$C-content of the media. Li et al. (2012) recently demonstrated that SIP-SCRR red shifts in the three major carotenoid peaks ($\Delta \nu$) vary quantitatively with $f_{cell}$ of *Synechocystis* sp. and *Synechococcus elongatus*, grown in $^{13}$C-bicarbonate enriched media. In fact, when their data from these two cyanobacteria are combined, we obtain functional responses that closely agree with those presented in Figure 5; e.g., $\langle \Delta \nu_{C-C} \rangle = 1156.5 \pm 31.0 \pm 1.4 f_{cell}$; $r^2 = 0.99$ (computed from Figure 3 in Li et al., 2012). The $f_{cell}$ values derived from mass spectrometric analysis of extracted cellular protein (Li et al., 2012) yielded essentially the same functional responses as carotenoid $\langle \Delta \nu \rangle$. This is consistent with the postulates that turnover of major intracellular molecular
pools are tightly coupled under optimal culture conditions and that the isotopic signatures are equivalent, i.e., \( \alpha_{\text{protein}} \approx \alpha_{\text{carotenoids}} \), which supports the validity of our approach. Furthermore, it corroborates the proposition that as long as \( \alpha \) and \( \mu_{\text{pop}} \) are known and \( f_{\text{media}} \) is well-constrained, mean \( f_{\text{cell}} \) of an autotrophic culture can be reliably computed and precludes the need for mass spectrometry. To our knowledge, the present study is the first to extend SIP-SCRR data interpretation beyond fractional labeling and relative activity determinations to quantify absolute single-cell growth rates from photoautotrophic populations.

**Single-Cell Growth Rates by SIP-SCRR-FISH**

Our approach is well-suited to examine cell to cell heterogeneity in photoautotrophic growth in both cultures and field samples. Almost all carbon assimilated by aquatic photoautotrophs is drawn from the C\(_T\) pool. Therefore, dissolved \(^{13}\)C-bicarbonate, which equilibrates with C\(_T\) species, is an unsurpassed tracer of autotrophic production. Surface ocean values of C\(_T\) only vary between \( \sim 1.95 \) and \( 2.25 \) mM C globally (Key et al., 2004), and can be empirically measured in samples by several methods (e.g., Hall and Aller, 1992; Key et al., 2004; Kaltin et al., 2005). Therefore, the experimentalist can add precisely measured masses of \(^{13}\)C\(_{\text{CO}}^-\) to samples and accurately calculate \( f_{\text{media}} \), which is ultimately needed to derive \( \mu_{\text{sc}} \). In contrast, DIC replacement manipulations lead to uncertainties in C\(_T\) and calculation of \( f_{\text{media}} \) (Table S1), necessarily alter water chemistry, and are impractical for field experiments. Therefore, we conclude that spiking unaltered samples with a sterile \(^{13}\)C\(_{\text{CO}}^-\) concentrate to achieve the desired \( f_{\text{media}} \) is preferable to any DIC replacement protocol (e.g., Li et al., 2012). In principle, our experiments demonstrated that \(^{13}\)C\(_{\text{CO}}^-\) amendments as small as 10% are adequate to exceed our LOD (3% \( \Delta f_{\text{cell}} \)) within 0.5 generations (Figure 5) and enable recognition of active cells. However, if accurate and precise determination of \( \mu_{\text{sc}} \) is the objective, then \(^{13}\)C\(_{\text{CO}}^-\) amendments yielding 0.3–0.5 \( f_{\text{media}} \) and incubation durations of one generation are recommended to constrain the optimal propagated relative uncertainties (\( \pm \sigma_{\mu}/\mu \)) to between 6.6 and 11.0% (see Figures S2–S4). These amendments do not appear to significantly alter photoautotrophic growth in seawater (Figure S1).

Sample processing requirements for SCRR or SCRR-FISH are relatively simple. Samples can be captured on membranes and frozen or preserved with formaldehyde or paraformaldehyde, but not glutaraldehyde (due to fluorescence interference). If FISH is required, samples are processed by standard membrane-based protocols (e.g., Pernthaler et al., 2001; Stoecker et al., 2010). In contrast, previous SIP-Raman-FISH studies required performing hybridizations on cell suspensions (liquid-FISH) concentrated by centrifugation or resuspension from a membrane, then spotting suspensions onto quartz or Al-coated glass slides, followed by drying, rinsing and DAPI counter-staining (Huang et al., 2007b; Berry et al., 2014). Membrane-based approaches
are advantageous because cell recovery exceeds that obtained
by centrifugation or by resuspension from membranes.
Furthermore, the risk of cell loss from the microscope slide
during rinsing or counter-staining in the liquid-FISH technique
is eliminated.
Hybridization of fluorophore-labeled oligonucleotide FISH
probes into cells may introduce two challenges to Raman
interrogation; unwanted fluorescence and isotope dilution.
Persistent fluorescence from a sample illuminated by a laser
within its electronic transition band can prevent acquisition of
Raman spectra altogether. As demonstrated by Huang et al.
(2007b), bacterial cells hybridized with FISH probes labeled
with Cy3, Cy5, or Fluorescein (Fluos) were all amenable to
single-cell Raman microspectroscopy using a 532 nm laser,
but cells labeled with Cy3 required brief photobleaching
prior to acquisition of spectra. We only tested a Cy3-labeled
probe using our 514 nm laser and could obtain high quality
SCRR spectra of carotenoids without significant fluorescent
interference. Nonetheless, Cy5, Fluos and perhaps other yet-
to-be tested fluorophores are probably preferable to Cy3 for
most applications, but practitioners should test fluorophores with
desired laser line.
Isotope dilution created by incorporation of a FISH probe
is a concern with SIP-nano-SIMS because the instrument is
measuring secondary ions generated from individual atoms
ablated from a specimen, irrespective of source molecules. Thus,
ionized carbon from isotopically-enriched biomass can be diluted
with ionized light carbon from a FISH probe. In the SIR-Raman-
FISH approach, however, isotopic content determinations arise
from wavenumber shifts in signature molecular bond vibrations,
in the present case from v(C-C) in carotenoids. Therefore,
aliasing $f_{cell}$ determinations from diagnostic SCRR peaks by
isotopically-light FISH probes is improbable in the present
study and can be avoided in other Raman assays. For example,
Huang et al. (2007b) determined $^{13}$C-content in FISH-hybridized
cells from red shift ratios in diagnostic peaks emanating from
the essential amino acid, phenylalanine, were indistinguishable
from unhybridized cells. Cells hybridized by CARD-FISH
(catalyzed reporter deposition) may not be amenable to Raman
interrogation because required reagents may be Raman-active
and contaminate the spectra, but this requires future evaluation.
While membrane-based cell collection, hybridization, and
staining techniques present significant advantages for working
with microbes in natural waters, all tested membranes and
filters are Raman-contaminating and consequently can’t
be used directly in the instrument. Therefore, we were
motivated to develop a rapid, simple method to efficiently
transfer concentrated and stained cells from low sorption
membranes (polycarbonate) to a Raman-neutral substrate
for interrogation. With minimal optimization efforts, the
filter-transfer-freeze (FTF) technique met those goals and
achieved transfer efficiencies of 51–77% for *Synecococcus*
to mirror-finished stainless steel slides. For most anticipated
applications, complete cell transfer is not required to adequately
subsample populations captured on membranes. However,
evaluation of other membrane materials may be warranted to
maximize transfer efficiencies and minimize selective retention
on membranes. The steel slides have several advantages. Firstly,
they are completely reusable after solvent cleaning. Secondly,
they cost 1–3% that of the quartz and Al-coated slides used
in previous Raman studies (e.g., Huang et al., 2007b; Li et al.,
2012). The third advantage is that potentially higher quantum
efficiencies improve the method’s sensitivity. This is explained
as follows: Raman-shifted photons tend to scatter more or less
in a 360° sphere (isotropic), depending on sample morphology,
opacity, volume, and substrata properties. However, reflective
opaque metal slides or Al-coated glass substrata limit scattering
geometry to 180°, reflecting photons upward and thus potentially
doubling photon capture efficiency compared to transparent
substrata. Lastly, the highly reflective surface enables recognition
of cells as small as bacteria under bright-field illumination
without using any staining or FISH procedures.

**Intra-Population Growth Rate Heterogeneity**

In this study, computed growth rates within a given isogenic
*Synechococcus* sp. population varied from cell to cell by ~27%
(CV) around the mean at any particular time point after
removal of the ~2% propagated analytical error. Similar intra-
population variations have been observed in isogenic populations
of photoautotrophic protists. For example, microscopic cell
count measurements of $\mu_{sc}$ for *Chlorella vulgaris* varied between
0.55 and 1.52 day$^{-1}$ ($\bar{X} = 1.16 \pm 0.37$ SD) among mineral oil-
capsulated droplets of media within a microfluidic device,
yielding a CV of 32%, while $\mu_{pep}$ in bulk media was 1.12
day$^{-1}$ (Dewan et al., 2012). Similarly, subpopulations in a
synchronized isogenic *Chlamydomonas reinhardtii* culture grew at
significantly different rates among oil-encapsulated droplets
within a millifluidic sampler (Damodaran et al., 2015). Using SIP-
nano-SIMS, Kopf et al. (2015) reported CVs of 19–51% for $\mu_{sc}$
of chemostat cultures of the bacterium, *Staphylococcus aureus*.
Interestingly, the heterogeneity in single cell growth rate (CV)
varied inversely with chemostat dilution rate, i.e., behavior of
individual cells in slow growing *S. aureus* populations was less
uniform than that of fast growers. We observed a similar trend
with fast-growing *T. pseudonana* (CV = 8–9%) and slower-
growing *Synecococcus* sp. (CV = 23–34%) (Figure 8).

We were unaware of any comparable measurements of $\mu_{sc}$
in cyanobacteria. However, significant variations in elemental
stoichiometry within *Synecococcus* field populations have been
observed using synchrotron X-ray fluorescence microscopy.
For example, an average CV of 120% in Si/P ratios was
apparent among cells within discrete tropical and subtropical
water samples (Baines et al., 2012). Similarly, quotas for
P and Fe varied by 68 and 97% CV, respectively, among
individual *Synechococcus* cells in the Sargasso Sea (Twining
et al., 2010). Even individual *Synechococcus*, *Prochlorococcus*,
and *Thalassiosira* cultures exhibit significant intra-population
variability in intracellular P content (CV = 17–86%), which
can affect the collective population growth rate (Fredrick
et al., 2013). Thus, considerable cell-to-cell variation in
elemental composition, activity, and growth of photoautotrophic
populations can be expected whether they are isogenic and
exposed to ostensibly homogenous conditions or are anisogenic and living in dynamic heterogeneous environments. Such findings and agent-based ecological models underscore the need for reliable single-cell measurements to better understand processes driving plankton population dynamics as well as biological cycling of key elements (Bolnick et al., 2011; Bucci et al., 2012; Fredrick et al., 2013).

CONCLUSIONS

We conclude that, compared to other technologies, our approach to single-cell growth measurement has some clear advantages. SCRR has minimal sample preparation requirements, provides relatively high sample throughput, and at low cost per analysis (excluding capital expenses). Specimens can be interrogated in vivo, in vitro, frozen, preserved, or dried, and don’t require special analytical conditions, such as electrical conductivity, embedding, or high vacuum (as in nano-SIMS). Organisms can be recognized by bright-field, autofluorescence, fluorescent stains, or FISH illumination. All targeted cells in a field can then be immediately analyzed by Raman microspectrometry by altering the optical path with a few computer keystrokes and allowing the automated stage to center each target under the laser spot. Raman microspectrometry offers a unique and powerful set of capabilities to chemically interrogate individual cells and investigate biogeochemical processes at spatial scales relevant to microorganisms. Combining SIP with FISH and Raman microspectrometry allows identification of key players in cycling of a particular element or compound and thereby enables linking function with phylogeography. Furthermore, rates of material cycling can be determined from features within single-cell Raman spectra. If the stable isotope traces the sole source of that element into cells and $f_{\text{media}}$ of that element can be measured, then single-cell growth rates can be determined.

As demonstrated in this study, a $^{13}$C-bicarbonate tracer and SCRR of carotenoids appear to be particularly well-suited to investigate intra- and inter-population variability in growth of cultured photoautotrophs. As documented by Goericke and Welschmeyer (1993), carotenoid labeling rates closely match population growth rates during balanced growth which occurs during exponential phase in cultures. However, they also observed that nutrient and light stresses may alter this relationship, conditions which may be encountered in field samples. We recommend conducting SIP experiments over an entire diel cycle (24 h) to account for variability in light field responses and intrinsic biosynthetic periodicities and to produce sufficient signal. Resolution of the impact of light and nutrient limitation on broad application of the SIP-SCRR approach to examining intra- and inter-population growth variability in the field requires further systematic evaluation under a range of conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

Authors are grateful to J. Collier for *Synechococcus* cultures, S. Zegers for *T. pseudonana* cultures, C. Heilbrun for DIC analyses and M. Pachiadaki for insightful comments on an earlier draft. IRMS analyses of DI$^{13}$C were conducted by the UC Davis Stable Isotope Facility. Raman data were acquired in SoMAS’ NANO-Raman Molecular Imaging Laboratory (NARMIL), a community resource dedicated to environmental science applications and founded with NSF-MRI grant OCE-1336724. This research was also partially supported by NSF grants OCE-1335436 and OCE-1259110 and Gordon and Betty Moore Foundation Grant #5064. School of Marine and Atmospheric Sciences contribution number 1436.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01449/full#supplementary-material

REFERENCES


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Supplementary Material

Single-Cell Growth Rates in Photoautotrophic Populations Measured by Stable Isotope Probing and Resonance Raman Microspectrometry

Gordon T. Taylor*, Elizabeth Suter¹, Zhuo Qun Li¹, Stephanie Chow¹, Dallyce Stinton¹, Tatiana Zaliznyak¹ and Steven R. Beaupré¹

1. School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY, USA

*Correspondence: Gordon T. Taylor: gordon.taylor@stonybrook.edu

SM1: Evaluation of ¹³C-enriched media preparation methods

The first critical steps in performing quantitative SIP experiments with cultures is to establish whether DIC replacement or augmentation is preferable, to determine how much ¹³C-bicarbonate tracer can be added without significantly altering seawater chemistry, and then to accurately determine the fractional contribution ($f_{media} = \frac{¹³C_{media}}{¹³C_{media} + ¹²C_{media}}$) of the heavy isotope to the total inorganic carbon pool ($C_T = CO_2 + H_2CO_3 + HCO_3^- + CO_3^{2-}$).

Three DIC replacement methods were evaluated for how effectively DI¹²C was removed prior to media preparation and ¹³C-bicarbonate amendment. The first method was boiling filtered seawater (FSW) for 1 min in a microwave oven according to Li et al. (2012). The second was to actively sparge FSW with N₂ for 10 min. The third approach was to acidify FSW to pH 3.5 with 1N HCl, then seal in a gas-tight vessel and autoclave. Once cool, pH was returned to 8.0 with 0.1N NaOH. Standard f/2 nutrients and sodium bicarbonate were aseptically transferred to 200-ml septum bottles to yield a final $C_T$ of ~2 mM in nominal $f_{media}$ ratios of 0.011 (natural abundance), 0.25, 0.50, 0.75, and 1.00. However, media prepared in this way usually became viscous and turbid due to mineral precipitation and *Synechococcus* sp. cultures did not grow well under these conditions.

DI¹³C augmentation to the culture media was evaluated for pH and growth effects. At the extreme, 2.3 mM DI¹³C additions (final $C_T$ ~4.1 mM or 130% enrichment) only depressed pH by 0.23 units (Table S1). At $C_T$ enrichments of <50%, pH excursions were <0.13, suggesting that amendments at these levels will not significantly affect photoautotrophic growth.

Total dissolved inorganic carbon ($C_T$) concentrations were determined using a flow injection analysis (FIA) system (Hall and Aller, 1992). This instrument permits transfer of CO₂ from an acidified ($\Sigma CO_2$) reagent stream across a gas-permeable membrane into a receiving carrier stream which flows over a conductivity detector. $C_T$ levels of unknown samples were compared to conductivity measurements of known standards. pH measurements were made on a Thermo
Scientific® Orion 2-Star™ Benchtop pH meter, which was calibrated immediately prior to all measurements.

In principle, for accurate DI$^{13}$C replacement, all or a known amount of the C$_T$ pool must be removed before replacing with sufficient $^{13}$C-bicarbonate to return media to the original C$_T$ pool size. Microwave heating as employed by Li et al. (2012) or N$_2$ sparging only removed 30 or 60% of the original C$_T$ pool, respectively, while acidification effectively removed 96% of the C$_T$ pool as determined by FIA-conductivity measurements of ΣCO$_2$ (Table S2). Microwave heating had the additional effect of raising the pH above 9.0. Adding 1.8 mmol L$^{-1}$ bicarbonate to previously acidified and neutralized FSW returned DIC to the original pool size and an acceptable pH. Doing the same to the microwaved or sparged samples in the present case would have increased C$_T$ by 71 and 40% and without independent verification, the actual $f_{\text{media}}$ would be poorly constrained. Therefore, pH manipulation is clearly the most reliable approach for DIC replacement.

Partial or complete DIC replacement may be a suitable approach for media preparation and culture experiments, but clearly is inappropriate for field experiments with natural assemblages. DIC augmentation requires far less manipulation and therefore introduces fewer potential artifacts. Alteration of pH is foremost among potential artifacts for DIC augmentation and was therefore evaluated. We note that pH of the aged seawater (Table S1) was higher than that of average seawater (pH = 7.5-8.4), which we attribute to prolonged photoautotrophic growth within the storage vessel. Increasing C$_T$ by 11% to as much as 130% depressed the pH of FSW from 8.79 to 8.56 (Table S1). Consequently, our experimental data were derived from media augmented with a constant amount of total DIC, but varying $^{13}$C-bicarbonate content, and then returned to a pH of 8.0. Reported $f_{\text{media}}$ are based on IRMS and FIA-conductivity results, which were within ~6% of nominal ratios (gravimetrically formulated from C$_T$ concentration in FSW) (Table S2).
## Table S1. Summary of effects of dissolved inorganic carbon manipulations on cultivation medium.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;&lt;sup&gt;f&lt;/sup&gt; (mean mM ± SD)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered coastal seawater (FSW)</td>
<td>1.78 ± 0.10</td>
<td>8.76</td>
</tr>
<tr>
<td>Microwaved to boiling FSW&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.06</td>
<td>9.04</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;-purged FSW&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.08</td>
<td>8.30</td>
</tr>
<tr>
<td>Acidified FSW&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.07 ± 0.07</td>
<td>3.50</td>
</tr>
<tr>
<td>Previously acidified FSW, pH adjusted &amp; bicarbonate added&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.86 ± 0.06</td>
<td>8.00</td>
</tr>
<tr>
<td>FSW – 11% DIC enriched&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.98</td>
<td>8.79</td>
</tr>
<tr>
<td>FSW – 22% DIC enriched&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.16</td>
<td>8.76</td>
</tr>
<tr>
<td>FSW – 51% DIC enriched&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.69</td>
<td>8.66</td>
</tr>
<tr>
<td>FSW – 77% DIC enriched&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.16</td>
<td>8.57</td>
</tr>
<tr>
<td>FSW – 130% DIC enriched&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.09</td>
<td>8.56</td>
</tr>
</tbody>
</table>

<sup>a</sup> – FSW brought to a boil in microwave and held for 1 min according to Li et al. (2012).

<sup>b</sup> – FSW sparged for 10 min with N<sub>2</sub> for 10 ml FSW – sparged for 30 min for 200 ml.

<sup>c</sup> – FSW acidified with 1.0 N HCl, shaken in a closed vial, and opened to release CO<sub>2</sub> gas.

<sup>d</sup> – pH of FSW from c was adjusted with 0.1 N NaOH.

<sup>e</sup> – Unmanipulated FSW was amended with increasing volumes of a 10 mM NaHCO<sub>3</sub> working stock. pH was returned to 8.0 prior to inoculation with Synechococcus cells.

<sup>f</sup> – C<sub>T</sub> = CO<sub>2</sub> + CO<sub>3</sub><sup>-</sup> + HCO<sub>3</sub><sup>-</sup> and determined as ΣCO<sub>2</sub> by flow injection and conductivity analysis of acidified sample (Hall and Aller, 1992).
**Table S2.** Comparison of $C_T$ values derived from three methods for SIP experiments using 2 mM DIC amendments with varying $f_{\text{media}}$.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (mM)</th>
<th>S.D.</th>
<th>N</th>
<th>Significantly Different $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric (G)</td>
<td>3.78</td>
<td>na$^b$</td>
<td>1</td>
<td>from IRMS</td>
</tr>
<tr>
<td>Flow Injection Analysis (FIA)</td>
<td>3.78</td>
<td>0.10</td>
<td>8</td>
<td>from IRMS</td>
</tr>
<tr>
<td>Isotope Ratio Mass Spectrometry (IRMS)</td>
<td>3.56</td>
<td>0.05</td>
<td>12</td>
<td>from G and FIA</td>
</tr>
</tbody>
</table>

$^a$ – $p < 0.05$; **Dunn’s Method pairwise ANOVA**

$^b$ – not applicable because concentration based on a single measurement
Figure S1. Growth curves determined by *in vivo* fluorescence (460 nm excitation / >665 nm emission) for *Synechococcus* sp. (RS9916) grown in normal f/2 media ($f_{\text{media}} = 0.011$, control) and f/2 media augmented with 2 mM $^{13}$C-bicarbonate to yield $f_{\text{media}} = 0.12, 0.22, 0.32, 0.43,$ and 0.54. Mean growth rates ($\mu_{\text{pop}}$) determined by slopes over first 18 days were not significantly different among treatments (ANOVA $p > 0.90$). Aggregate curve and regression include data from all six cultures. Diamonds indicate dates on which samples were collected for Raman microspectrometry.
SM2: Expected relationships between mean SIP-SCRR wavenumbers ($\langle \Delta \nu \rangle$) and fractional isotopic abundance ($f_{\text{euc}}$)

Slight differences in vibrational frequencies upon substituting $^{13}$C for $^{12}$C in a carbon-carbon bond are manifested as unique peaks in the Raman spectrum. Since these isotopologue peaks tend to overlap, their relative areas are imprecise predictors of the fractional isotopic abundance of $^{13}$C ($f_{13}$). However, a strong relationship exists between the mean Raman wavenumber of overlapping isotopologue peaks and fractional isotopic abundance, which can be derived as follows.

First, we assume that all isotopologue bonds have symmetric Raman peaks with characteristic central wavenumbers ($\Delta \nu_i$, where the subscript $i = 0, 1, 2$ is the number of $^{13}$C atoms in $^{12}$C$^{12}$C, $^{12}$C$^{13}$C, or $^{13}$C$^{13}$C bonds, respectively). Therefore, the mean wavenumber ($\langle \Delta \nu \rangle$) for overlapping isotopologue bond peaks is the average of their characteristic wavenumbers weighted by their peak areas ($A_i$).

\[
\langle \Delta \nu \rangle = \frac{A_0 \Delta \nu_0 + A_1 \Delta \nu_1 + A_2 \Delta \nu_2}{A_0 + A_1 + A_2}
\]

The areas are obtained by integrating each peak over all values of $\Delta \nu$. We assume that the peak heights are equal to the products of their Raman efficiencies ($\varepsilon_i(\Delta \nu)$), the effective wavenumber-dependent cross-section with units of intensity $\cdot$ effective cell area $\cdot$ bond$^{-1}$) and the number of isotopologue bonds of interest in the optical path. The latter is equal to the product of the average cell thickness ($l$), concentration of the molecule of interest in the cell ($[m]$), stoichiometric number of bonds in that molecule expressing the desired resonance (e.g., $b = 9$ conjugated double bonds in the chain per $\beta$-carotene), and expected proportions of isotopologues among those bonds ($\langle \phi_i \rangle$). These terms are constants on the timescale of acquiring a Raman spectrum can be factored out of the peak integral. Therefore, the peak area (intensity $\cdot$ cm$^{-1}$) is the product of these constants and a bulk efficiency ($\varepsilon'_i$, with units of intensity $\cdot$ effective cell area $\cdot$ bond$^{-1}$ $\cdot$ cm$^{-1}$) that is equal to the integral of $\varepsilon_i(\Delta \nu)$ over all values of $\Delta \nu$ (Eq. S2).

\[
A_i = \varepsilon'_i[l[m]b\langle \phi_i \rangle]
\]

The expected proportions of $^{12}$C$^{13}$C, $^{12}$C$^{13}$C, and $^{13}$C$^{13}$C bonds can be estimated by assuming a stochastic distribution of $^{13}$C atoms throughout the principle resonance structure of the molecule (i.e., assuming negligible isotopic clumping). Hence, the proportions (probability = $P(k|n,f_{13})$) of each set of isotopomers with $k$ $^{13}$C atoms in a molecule containing $n$ carbon atoms will be given by the individual terms of the binomial distribution (Eq. S3), where the bulk binomial coefficient (“n over k” term) represents the number of possible unique isotopomers in that set.

\[
P(k|n,f_{13}) = \binom{n}{k} f_{13}^k (1 - f_{13})^{n-k}
\]
As such, any molecule with \( n \) carbon atoms for which \( k = 0 \) to \( n \) \(^{13}\)C atoms are randomly substituted could exist as \( 2^n \) possible isotopic species. For example, \( \beta \)-carotene (\( \text{C}_{40}\text{H}_{56} \)) has \( 2^{40} \approx 1.1 \text{ trillion} \) possible isotopologues with respect to stable carbon isotopes.

\[
\sum_{k=0}^{n} \binom{n}{k} = 2^n
\]

Of this total, the sum of all combinations of unique isotopologue bonds (\( \eta_{i,k} \)) *in each set of isotopomers defined by k* must be equal to the product of the number of all possible isotopologues and the number of bonds of interest in that molecule.

\[
\sum_{i=0}^{2} \eta_{i,k} = b \binom{n}{k}
\]

Thus, expressions for the number of possible \(^{12}\text{C}^{12}\text{C}^{13}\text{C}^{12}\text{C}\), \(^{12}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\), and \(^{13}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\) bonds *in each set of isotopomers defined by k* can be obtained by factoring combinations out of the bulk binomial coefficient that are consistent with the physical limitations of isotopic substitution (Eq. S6). Specifically, \( n \) is decreased by the number of atoms in a bond (i.e., 2) and \( k \) is decreased by \( i \) \(^{13}\)C atoms because, for example, there cannot be any \(^{13}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\) bonds in a molecule with just \( k = 0 \) or 1 \(^{13}\)C atoms, nor can there be any \(^{12}\text{C}^{12}\text{C}^{13}\text{C}^{12}\text{C}\) bonds in a molecule with at least \( n-1 \) \(^{13}\)C atoms. Accordingly, each combination is only meaningful for values of \( k \) that range from \( i \) to \( n-2+i \).

The additional binomial coefficient ("2 over \( i \)"") accounts for two possible sites of \(^{13}\)C substitution in a \(^{12}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\) bond (\(^{12}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\) vs. \(^{13}\text{C}^{13}\text{C}^{12}\text{C}\)), while the coefficient \( b \) scales the result to account for all possible bonds of interest in the set.

\[
\eta_{i,k} = \begin{cases} 
b \binom{2}{i} \binom{n-2}{k-i}, & \text{for } i \leq k \leq n-2+i \\ 0, & \text{otherwise} \end{cases}
\]

Therefore, the proportions (\( \phi_{i,k} \)) of the numbers of possible \(^{12}\text{C}^{12}\text{C}^{13}\text{C}^{12}\text{C}\), \(^{12}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\), and \(^{13}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\) bonds *in each set of isotopologues defined by k* are found by dividing Eq. S6 by Eq. S5.

\[
\phi_{i,k} = \begin{cases} 
\binom{n}{k}^{-1} \binom{2}{i} \binom{n-2}{k-i}, & \text{for } i \leq k \leq n-2+i \\ 0, & \text{otherwise} \end{cases}
\]

The expected proportions of \(^{12}\text{C}^{12}\text{C}^{13}\text{C}^{12}\text{C}\), \(^{12}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\), and \(^{13}\text{C}^{13}\text{C}^{13}\text{C}^{12}\text{C}\) bonds in the entire suite of \( 2^n \) isotopic species are equal to the averages of their proportions in each set defined by \( k \).
(S8) \[ \langle \phi_i \rangle = \sum_{k=i}^{n-2+i} P(k|n, f_{13}) \phi_{i,k} \]

Substituting Eq. S3 and S7 into S8 gives an expression for the expected proportions of \(^{12}\text{C}^{12}\text{C}, ^{12}\text{C}^{13}\text{C}, \) and \(^{13}\text{C}^{13}\text{C}\) bonds as a function of \(f_{13}, n, \) and \(k.\)

(S9) \[ \langle \phi_i \rangle = \sum_{k=i}^{n-2+i} \binom{2}{i} \binom{n-2}{k-i} f_{13}^k (1-f_{13})^{n-k} \]

These expected proportions can be greatly simplified by first implementing a change of variables and factoring (let \(n' = n - 2, \) and \(k' = k - i).\)

(S10) \[ \langle \phi_i \rangle = \binom{2}{i} f_{13}^i (1-f_{13})^{2-i} \sum_{k'=0}^{n'} \binom{n'}{k'} f_{13}^{k'} (1-f_{13})^{n'-k'} \]

The summation in Eq. S10 represents a complete binomial distribution and is therefore equal to one. Thus, the expected stochastic proportions of \(^{12}\text{C}^{12}\text{C}, ^{12}\text{C}^{13}\text{C}, \) and \(^{13}\text{C}^{13}\text{C}\) bonds are independent of the number of atoms or bonds of interest in a molecule (where \(n \geq 2).\) Instead, they are equivalent to the proportions expected for a diatomic molecule via binomial expansion.

(S11) \[ \langle \phi_i \rangle = \binom{2}{i} f_{13}^i (1-f_{13})^{2-i} \]

or, after expanding the terms:

\[ \langle \phi_0 \rangle = (1-f_{13})^2 \]
\[ \langle \phi_1 \rangle = 2f_{13}(1-f_{13}) \]
\[ \langle \phi_2 \rangle = f_{13}^2 \]

Finally, substituting Eq. S2 and S11 into Eq. S1 and simplifying yields the expected general relationship between mean SCRR shift and fractional \(^{13}\text{C}\) abundance.

(S12) \[ \langle \Delta \nu \rangle = \frac{\epsilon_0' \Delta \nu_0 + 2(\epsilon_1' \Delta \nu_1 - \epsilon_0' \Delta \nu_0) f_{13} + (\epsilon_0' \Delta \nu_0 - 2 \epsilon_1' \Delta \nu_1 + \epsilon_0' \Delta \nu_0) f_{13}^2}{\epsilon_0' + 2(\epsilon_1' - \epsilon_0') f_{13} + (\epsilon_0' - 2 \epsilon_1' + \epsilon_0') f_{13}^2} \]

This expression is consistent with our intuition that the mean Raman shift \(\langle \Delta \nu \rangle\) is equal to the characteristic wavenumber of a \(^{12}\text{C}^{12}\text{C}\) resonance \(\langle \Delta \nu_0 \rangle\) when \(f_{13} = 0,\) or a \(^{13}\text{C}^{13}\text{C}\) resonance \(\langle \Delta \nu_2 \rangle\)
when \( f_{13} = 1 \), and it is symmetrically weighted around the resonance of a \(^{12}\text{C}^{13}\text{C}\) bond (\( \Delta \nu_i \)) when \( f_{13} = 0.5 \). This function is linear when assuming approximately equal efficiencies (\( \varepsilon'_0 \approx \varepsilon'_1 \approx \varepsilon'_2 \)) and spacing between peaks (i.e., \( \Delta \nu_0 - \Delta \nu_i \approx \Delta \nu_i - \Delta \nu_2 \)). If we define \( f_{\text{cell}} \) as the \( f_{13} \) value of a specific compound in a cell, then \( \langle \Delta \nu \rangle \) can be expressed as a linear function of \( f_{\text{cell}} \) with an intercept and a slope predicted from theory (\( \Delta \nu_0 \) and \( 2(\Delta \nu_1 - \Delta \nu_0) \); Eq. S13) or obtained by experiment (\( b_0 \) and \( b_1 \); Eq. S14).

\[
\langle \Delta \nu \rangle \approx \Delta \nu_0 + 2(\Delta \nu_1 - \Delta \nu_0) f_{\text{cell}}
\]

or

\[
\langle \Delta \nu \rangle \approx b_0 + b_1 f_{\text{cell}}
\]

This line of reasoning predicts that the observed intercept (\( b_0 \)) is equal to the \(^{12}\text{C}^{12}\text{C}\) resonance (\( \Delta \nu_0 \)), and that the slope (\( b_1 \)) is both negative (because \( \Delta \nu_2 < \Delta \nu_1 < \Delta \nu_0 \)) and approximately equal in magnitude to twice the spacing between adjacent peaks. This model is supported by the observed linearity between \( \langle \Delta \nu \rangle \) and \( f_{\text{cell}} \) (\( r^2 = 0.96 \)), and by agreement between the values of \( \Delta \nu_1 \) and \( \Delta \nu_2 \) that are observed (1498 cm\(^{-1}\), 1474 cm\(^{-1}\)) and expected (\( \Delta \nu_{1,\text{expected}} = b_0 + b_1/2 = 1498.7 \pm 0.3 \) cm\(^{-1}\), \( \Delta \nu_{2,\text{expected}} = b_0 + b_1 = 1475.4 \pm 0.3 \) cm\(^{-1}\)) from regression parameters (\( b_1 = -44.9 \pm 0.30 \), and \( b_0 = 1521.8 \pm 0.08 \) cm\(^{-1}\)) for \( \beta \)-carotene’s \( v(C=C) \) stretch in \( \text{Synechococcus} \) cells. However, the model should be applicable to any resonance that meets the stated assumptions.
SM3: Expected relationship between fractional isotopic abundance \( (f_{\text{cell}}) \), generation number \( (n) \), and mean SIP-SCRR wavenumbers \( (\Delta \tilde{\nu}) \)

The relationship between \( f_{\text{cell}} \) and the number of generations \( (j, \text{where} \ 0 \leq j \leq n) \) can be estimated assuming that (1) shifts in fractional isotopic abundances due to Rayleigh distillations are negligible on the timescale of a typical incubation, (2) variations in the isotopic fractionation factor \( (\alpha) \) associated with biosynthesis of carotenoids are within error of \( \alpha \) for bulk cellular production, (3) all cells in the population have approximately the same concentration of carbon atoms \( \text{moles/cell} \), (4) all cellular constituents turn over on approximately the same timescale, and (5) that ancestral and newly assimilated biomass are isotopically homogenized prior to cell fission. Under these assumptions, the number of cells \( (N) \) in the original inoculum \( (\text{subscript} \ 0) \) doubles with each new generation \( (j) \).

\[
N_j = N_0 e^{j \ln(2)}
\]

According to isotopic mass balance, the fractional isotopic abundances of daughter cells \( (f_{\text{cell},j+1}) \) will be the simple average of the isotopic abundances of their parent cells \( (f_{\text{cell},j}) \) and of the \( ^{13}\text{C} \)-enriched biomass that was assimilated prior to fission \( (f_{\text{new}}) \).

\[
f_{\text{cell},j+1} = (f_{\text{cell},j} + f_{\text{new}}) \frac{N_j}{N_{j+1}}
\]

Recursive application of Eq. S16 and simplification permits calculation of the fractional isotopic abundance of the \( j = n^{\text{th}} \) generation of cells from the number \( (N_n) \) and fractional isotopic abundance \( (f_{\text{cell},0}) \) of the original generation of cells \( (j = 0) \).

\[
f_{\text{cell},n} = f_{\text{cell},0} \frac{N_0}{N_n} + f_{\text{new}} \frac{1}{N_n} \sum_{j=0}^{n-1} N_j
\]

Substituting Eq. S15 into Eq. S17 reduces the summation to \( N_n - N_0 \) and the ensuing \( N_n/N_0 \) terms to \( e^{-n \ln(2)} \), producing a general isotopic mass balance expression for \( f_{\text{cell}} \) after \( n \) generations.

\[
f_{\text{cell},n} = f_{\text{new}} + (f_{\text{cell},0} - f_{\text{new}}) e^{-n \ln(2)}
\]

When constructing a calibration curve \( ((\Delta \tilde{\nu}) \text{vs.} \ f_{\text{cell}}) \) it is easier to directly measure the fractional isotopic abundances of growth media \( (f_{\text{media}}) \) than of discrete cellular constituents. Therefore, the definitions for fractionation factors \( (\alpha_{\text{biomass-media}} = R_{\text{biomass}}/R_{\text{media}}) \), isotope ratios \( (R = ^{13}\text{C}/^{12}\text{C}) \), and fractional isotopic abundances \( (f_{13} = ^{13}\text{C}/(^{12}\text{C}+^{13}\text{C})) \) can be used to predict the isotopic composition of cellular biomass from the isotopic composition of growth media.
Single-Cell Growth Rates by Raman Spectrometry
GT Taylor et al

(S19) \[ f_{\text{biomass}} = \frac{\alpha f_{\text{media}}}{1 + (\alpha - 1) f_{\text{media}}} \]

Substituting Eq. S19 for \( f_{\text{new}} \) and \( f_{\text{cell,o}} \) in Eq. S18 gives the following expressions for calculating \( f_{\text{cell}} \) and its uncertainty (\( \sigma_{f_{\text{cell}}} \)), where \( f_o \) and \( f_{\text{media}} \) are the fractional isotopic abundances of the original and \( ^{13}\text{C} \)-enriched growth media, respectively.

(S20) \[ f_{\text{cell}} = \frac{\alpha f_{\text{media}}}{1 + (\alpha - 1) f_{\text{media}}} + \left( \frac{\alpha f_o}{1 + (\alpha - 1) f_o} - \frac{\alpha f_{\text{media}}}{1 + (\alpha - 1) f_{\text{media}}} \right) e^{-n ln(2)} \]

(S21) \[ \sigma_{f_{\text{cell}}} = \sqrt{\left( \frac{\partial f_{\text{cell}}}{\partial f_o} \right)^2 \sigma_o^2 + \left( \frac{\partial f_{\text{cell}}}{\partial f_{\text{media}}} \right)^2 \sigma_{f_{\text{media}}}^2 + \left( \frac{\partial f_{\text{cell}}}{\partial n} \right)^2 \sigma_n^2 + \left( \frac{\partial f_{\text{cell}}}{\partial \alpha} \right)^2 \sigma_{\alpha}^2} \]

where

\[ \left( \frac{\partial f_{\text{cell}}}{\partial f_o} \right) = \frac{\alpha}{(1 + (\alpha - 1) f_o)^2} e^{-n ln(2)} \]

\[ \left( \frac{\partial f_{\text{cell}}}{\partial f_{\text{media}}} \right) = \frac{\alpha}{(1 + (\alpha - 1) f_{\text{media}})^2} (1 - e^{-n ln(2)}) \]

\[ \left( \frac{\partial f_{\text{cell}}}{\partial n} \right) = \frac{\alpha (f_{\text{media}} - f_o) ln(2)}{(1 + (\alpha - 1) f_{\text{media}})(1 + (\alpha - 1) f_o)} e^{-n ln(2)} \]

\[ \left( \frac{\partial f_{\text{cell}}}{\partial \alpha} \right) = \frac{(1 - f_{\text{media}}) f_{\text{media}}}{(1 + (\alpha - 1) f_{\text{media}})^2} (1 - e^{-n ln(2)}) + \frac{(1 - f_o) f_o}{(1 + (\alpha - 1) f_o)^2} e^{-n ln(2)} \]

Substituting Eq. S20 for \( f_{\text{cell}} \) in Eq. S14 yields the expected relationship between \( \langle \Delta \nu \rangle \) and \( n \) where, again, \( b_0 \) and \( b_1 \) are experimentally determined coefficients that are theoretically equal to \( \Delta \nu_0 \) and \( 2(\Delta \nu_1 - \Delta \nu_0) \), respectively.

(S22) \[ \langle \Delta \nu \rangle \approx b_0 + b_1 \left( \frac{\alpha f_{\text{media}}}{1 + (\alpha - 1) f_{\text{media}}} + \left( \frac{\alpha f_o}{1 + (\alpha - 1) f_o} - \frac{\alpha f_{\text{media}}}{1 + (\alpha - 1) f_{\text{media}}} \right) e^{-n ln(2)} \right) \]
SM4: Calculating the number of generations (n) from SCRR $\langle \Delta \nu \rangle$ measurements, associated uncertainties, and experimental design

The general equation for calculating the number of generations (n) from $f_{\text{media}}$, $f_o$, $\alpha$, $b_0$, and $b_1$ is obtained by rearranging Eq. S22.

\[
(S23) \quad n \approx \frac{1}{\ln(2)} \ln \left( \frac{f_{\text{media}} - f_o}{(1 + (\alpha - 1)f_o) \left( f_{\text{media}} - (1 + (\alpha - 1)f_{\text{media}}) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1} \right)} \right)
\]

Ignoring the $(1+(\alpha-1)f_o)$ term in the denominator of the logarithm will introduce a constant but negligible offset of +0.0004 generations for $\alpha = 0.976$ and $f_o = 0.011$. Therefore, $n$ can be calculated more simply via Eq. S24.

\[
(S24) \quad n \approx \frac{1}{\ln(2)} \ln \left( \frac{f_{\text{media}} - f_o}{f_{\text{media}} - (1 + (\alpha - 1)f_{\text{media}}) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1}} \right)
\]

The anticipated uncertainty on $n$ and the experimental conditions needed to minimize this uncertainty, can be evaluated by propagating the uncertainties (single standard deviations, $\sigma_i$) on all terms in Eq. S24.

\[
(S25) \quad \sigma_n = \sqrt{\left( \frac{\partial n}{\partial f_o} \right)^2 \sigma_f^2 + \left( \frac{\partial n}{\partial f_{\text{media}}} \right)^2 \sigma_{f_{\text{media}}}^2 + \left( \frac{\partial n}{\partial b_1} \right)^2 \sigma_{b_1}^2 + \left( \frac{\partial n}{\partial b_0} \right)^2 \sigma_{b_0}^2 + \left( \frac{\partial n}{\partial \langle \Delta \nu \rangle} \right)^2 \sigma_{\langle \Delta \nu \rangle}^2 + \left( \frac{\partial n}{\partial \alpha} \right)^2 \sigma_{\alpha}^2}
\]

where

\[
\left( \frac{\partial n}{\partial f_o} \right) = \frac{-1}{(f_{\text{media}} - f_o) \ln(2)}
\]

\[
\left( \frac{\partial n}{\partial f_{\text{media}}} \right) = \frac{1}{(f_{\text{media}} - f_o) \ln(2)} \left( \frac{f_o - (1 + (\alpha - 1)f_o) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1}}{f_{\text{media}} - (1 + (\alpha - 1)f_{\text{media}}) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1}} \right)
\]

\[
\left( \frac{\partial n}{\partial b_1} \right) = \frac{-1}{b_1 \ln(2)} \left( \frac{(1 + (\alpha - 1)f_{\text{media}}) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1}}{f_{\text{media}} - (1 + (\alpha - 1)f_{\text{media}}) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1}} \right)
\]

\[
\left( \frac{\partial n}{\partial b_0} \right) = \frac{-1}{\alpha b_1 \ln(2)} \left( \frac{1 + (\alpha - 1)f_{\text{media}}}{f_{\text{media}} - (1 + (\alpha - 1)f_{\text{media}}) \frac{\langle \Delta \nu \rangle - b_0}{\alpha b_1}} \right)
\]
Finally, the relative uncertainty of SIP-SCRR generation times \((g = t/n)\) and specific growth rates \((\mu_c = \ln(2) \cdot n/t)\) can be estimated through propagation of uncertainties and the reasonable assumption that \(\sigma_g/t \ll \sigma_n/n\).

\[
\frac{\sigma_{\mu_c}}{\mu_c} = \frac{\sigma_g}{g} = \frac{\sigma_n}{n}
\]

The values of most parameters in Eq. S24 and S25 are beyond the analyst’s control \((\alpha, b_1, b_0, \langle \Delta \varphi \rangle, f_o)\). However, an experiment can be optimized by careful selection of the \(^{13}\text{C}\)-enriched growth medium \((f_{\text{media}})\) and the duration of an incubation (i.e., \(t\) or \(n\)). First, the most precise measurements of \(n\) and the growth rate must happen after shifts in \(f_{\text{cell}}\) become detectable \((n > 0)\) but before \(f_{\text{cell}}\) reaches its asymptote (i.e., \(f_{\text{cell}} = \alpha f_{\text{media}}/(1 + (\alpha - 1)f_{\text{media}})\), as per Eq. S20) (Figs. S2 and S3). This optimum condition was readily found for the \(\nu(C-C)\) stretch by numerically locating the minimum value of the relative uncertainty \((\pm \sigma_n/n)\) as a function of \(\langle \Delta \varphi \rangle\) for various values of \(f_{\text{media}}\) (ranging from 0 to 1, \(\pm 0.002\)). This required assuming reasonable values and uncertainties for \(\alpha (0.976 \pm 0.003), f_o (0.0110 \pm 0.0002),\) and \(\langle \Delta \varphi \rangle\) (ranging from 1120 to 1160 cm\(^{-1}\), with \(\sigma_{\langle \Delta \varphi \rangle} = \pm 0.34\) based on known measurements. The two remaining quantities, \(b_1\) (-30.34 \(\pm 0.18\) cm\(^{-1}\)) and \(b_0\) (1157.04 \(\pm 0.05\) cm\(^{-1}\)), were obtained from a least squares linear regression of 961 individual measurements \((r^2 = 0.97; \text{Fig. S2})\).

This analysis suggests that the most precise determinations of \(n\) will be obtained by measuring \(\langle \Delta \varphi \rangle\) after \(~1.5\) cell divisions. More importantly, the minimum relative uncertainties \((\pm 1\sigma_n/n)\) can be significantly reduced from 0.356 \((f_{\text{media}} = 0.10)\) to a theoretically lowest achievable value of 0.033 (i.e., in the limit of \(f_{\text{media}} = 1.0\)) by using more isotopically-enriched growth media (Fig. S2). In addition, cells grown in highly enriched media exhibit both broader and flatter relative uncertainty minima (Fig. S2), making the highest precision measurements much less sensitive to the number of generations or the precise timing of measurements. As a practical compromise between measurement performance, incubation artifacts, and costs, we advocate amending routine samples to \(f_{\text{media}}\) values between 0.3 and 0.5, where the optimum value of \(n\) has a minimum theoretical relative uncertainty between 0.11 and 0.066, respectively (Fig. S3).

Further analysis of Eq. S25 suggests that the greatest contribution of uncertainty (i.e., the individual \(\langle \partial n/\partial x \rangle^2 \sigma_{\alpha}^2 n^2\) terms under the radical, where \(x = \alpha, f_o, f_{\text{media}}, b_0, b_1, \) or \(\langle \Delta \varphi \rangle\)) to SCRR determinations of \(n\) comes from measurements of \(\langle \Delta \varphi \rangle\) (Fig S4). Under optimal measurement conditions, the uncertainty contributions from measurements of \(\alpha\) and \(f_o\) are at least 3 to 5 orders of magnitude smaller than that of \(\langle \Delta \varphi \rangle\), respectively, and therefore can be ignored. The remaining
measurements ($f_{\text{media}}$, $b_0$, and $b_1$) make approximately equal contributions to the total uncertainty and suggest avenues for future improvements in performance. Therefore, the accuracy and precision of SIP-SCRR growth rate determinations are presently limited by individual $\langle \Delta \tilde{v} \rangle$ measurements and the calibration curve ($b_0$ and $b_1$), rather than uncertainty or variations in the isotopic composition of original growth medium ($f_0$) or fractionation factors for carotenoid biosynthesis ($\alpha$).
Figure S2. Modeled number of generations (n, thin black line), their uncertainties (± 1σ_n, gray shaded area), and their relative uncertainties (σ_n/n, heavy black line) as a function of measured mean SCRR ν(C-C) shift for f_media values of (a) 0.10, (b) 0.20, (c) 0.50, and (d) 0.90, assuming α = 0.976 ± 0.003, b_1 = -30.34 ± 0.18 cm\(^{-1}\), b_0 = 1157.04 ± 0.05 cm\(^{-1}\), f_o = 0.0110 ± 0.0002, and σ_ν(C-C) = 0.34 cm\(^{-1}\). Minimum values of ±σ_n/n and corresponding (optimal) values of n are indicated by the filled and open circles, respectively.
Figure S3. Estimated minimum relative uncertainties (±σ_n/n, filled circles, heavy line) and corresponding number of cell generations (n, open circles, thin line) as a function of f_media assuming α = 0.976 ± 0.003, b_1 = -30.34 ± 0.18 cm^{-1}, b_0 = 1157.04 ± 0.05 cm^{-1}, f_o = 0.0110 ± 0.0002, and σ_v(C-C) = 0.34 cm^{-1}. 
Figure S4. Modeled contributions of each measurement’s uncertainty \((f_o = \text{blue}, \alpha = \text{green}, f_{\text{media}} = \text{orange}, b_1 = \text{purple}, b_0 = \text{red}, \text{and } \langle \Delta \tilde{\nu} \rangle = \text{brown})\) toward the total uncertainty \((\pm \sigma_n; \text{heavy black line})\) of the number of generations \((n)\) determined via SCRR for \(f_{\text{media}} = (a) 0.3 \text{ and (b) } 0.5\), as a function of mean wavenumber. The uncertainty \((\pm \sigma_n)\) for the optimum conditions (minimum \(\pm \sigma_n/n\)) to measure \(n\) are indicated by the filled circles. The contributions to the total uncertainty were calculated as the individual terms for each measurement under the radical of Eq. S25; i.e., as \((\partial n/\partial x)^2 \sigma_x^2\), where \(x = \alpha, f_o, f_{\text{media}}, b_0, b_1, \text{or } \langle \Delta \tilde{\nu} \rangle\).