Using Stable Isotope Probing and Raman Microspectroscopy to measure growth rates of heterotrophic bacteria

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Running title: SIP-Raman derived bacterial growth rates

Abstract:

The suitability of stable isotope probing (SIP) and Raman microspectroscopy to measure growth rates of heterotrophic bacteria at the single-cell level was evaluated. Label assimilation into E. coli biomass during growth on a complex ¹³C-labeled carbon source was monitored in time course experiments. ¹³C- incorporation into various biomolecules was measured by spectral “red shifts” of Raman-scattered emissions. The ¹³C- and ¹²C-isotopologues of the amino acid phenylalanine (Phe) proved to be a quantitatively accurate reporter molecules of cellular isotopic fractional abundances (f_{cell}). Values of f_{cell} determined by Raman microspectroscopy and independently by isotope-ratio mass spectrometry (IRMS) over a range of isotopic enrichments were statistically
indistinguishable. Progressive labeling of Phe in *E. coli* cells among a range of $^{13}$C/$^{12}$C organic substrate admixtures occurred predictably through time. Relative isotopologue abundances of Phe determined by Raman spectral analysis enabled accurate calculation of bacterial growth rates as confirmed independently by optical density (OD) measurements. Results demonstrate that combining stable isotope probing (SIP) and Raman microspectroscopy can be a powerful tool for studying bacterial growth at the single-cell level when grown on defined or complex organic $^{13}$C-carbon sources even in mixed microbial assemblages.

**Importance:**

Population growth dynamics and individual cell growth rates are the ultimate expressions of a microorganism’s fitness to its environmental conditions, whether natural or engineered. Natural habitats and many industrial settings harbor complex microbial assemblages. Their heterogeneity in growth responses to existing and changing conditions is often difficult to grasp by standard methodologies. In this proof of concept study, we tested whether Raman microspectroscopy can reliably quantify assimilation of isotopically-labeled nutrients into *E. coli* cells and enable determination of individual growth rates among heterotrophic bacteria. Raman-derived growth rate estimates were statistically indistinguishable from those derived by standard optical density measurements of the same cultures. Raman microspectroscopy also can be combined with methods for phylogenetic identification. We report development of Raman-based techniques that enable researchers to directly link genetic identity to functional traits and rate measurements of single cells within mixed microbial assemblages, currently a major technical challenge in microbiological research.
Keywords:
Raman microspectroscopy, stable isotope probing, mass spectrometry, *E. coli*, phenylalanine, cell growth rate, single-cell analysis

Introduction:
Assessing the activity and growth rates of microorganisms is central to our understanding of microbial community dynamics, and contributions of particular taxa to elemental fluxes, and a myriad of biogeochemical processes in the environment. Historically, a variety of techniques, such as cell counts, ATP dynamics, and incorporation of radiolabeled compounds (\(^3\)H-leucine, \(^3\)H-thymidine), has been applied to estimate bulk community production and growth rates (1, 2). However, few studies have examined *in situ* growth rates of specific taxa in mixed microbial assemblages, a necessity for evaluating the impact of individual phylotypes on ecological processes and elemental cycling (3).

Molecular approaches, including analysis of rRNA content or rRNA:rRNA gene ratios have been used to estimate growth rates of individual bacterial and archaeal taxa. These methods are often hampered by cell-to-cell variations in rRNA gene and rRNA copy numbers, thereby yielding loosely-constrained growth estimates (3, 4). Recently developed metagenomic growth estimators, such as codon usage bias and “peak-to-trough ratio” seem to predict growth rates of marine bacterial assemblages with only moderate success (5). Combining microautoradiography with fluorescence *in situ* hybridization (MAR-FISH) enables detection of assimilation of radiolabeled tracers (e.g., \(^3\)H-, \(^14\)C-substates) into individual microbial taxa (6). MAR-FISH has been used extensively to assess abundances of specific taxa that actively assimilate a tracer, but...
this method has rarely been used to estimate microbial assimilation rates (7, 8). Other single-cell
techniques that can be combined with FISH, i.e., nanoscale secondary ion mass spectrometry
(NanoSIMS) and Raman microspectroscopy, enable quantitative tracing of assimilation of stable
isotope labeled substrates into cellular biomass (9–11). Being a non-destructive analysis, Raman
microspectroscopy can also be combined with microfluidics to sort active cells and to construct
genetic libraries from recovered cells (12, 13). Raman-active sorting offers the possibility of higher
phylogenetic resolution than microautoradiography or NanoSIMS methods, in which FISH probe
selection limits taxonomic resolution. Stable isotope probing combined with NanoSIMS (SIP-
NanoSIMS) is increasingly used to measure single-cell assimilation and growth rates (e.g. 14, 15).
The potential for deriving microbial assimilation and growth rates by combining SIP and Raman
microspectroscopy has not yet been fully explored.

Raman spectroscopy measures inelastically scattered photons emanating from a sample
excited by laser light. The Raman scattering effect varies with the vibrational energy levels of the
excited chemical bonds and thereby provides a molecular fingerprint. The ability to characterize
the molecular composition of complex materials through rapid, label-free and non-destructive
acquisition of spectra at high spatial resolution and with modest sample preparation requirements
are clear advantages of Raman spectroscopy over NanoSIMS (9, 16, 17).

Recently, application of Raman-based approaches in the life sciences has significantly
expanded. Raman spectral peak positions (measured in wavenumbers, cm⁻¹) and intensities are
indicative of an array of biomolecules (e.g., nucleic acids, proteins, lipids, chlorophyll,
carotenoids, polyhydroxybutyrate, starch, polyphosphate) and their abundances. Raman
biomarkers enable molecular imaging of cells for comprehensive examination of physiological
and metabolic processes in cellular and microbiological studies (17–21). For example, molecular
imaging by Raman microspectroscopy enabled tracking compositional changes and subcellular movements of DNA, protein, and lipid pools during cell division of HeLa cells and during viral infection of the cosmopolitan microalga, *Emiliania huxleyi* (18, 22).

Assimilation of stable isotope-labeled substrates elicits useful shifts in a cell’s Raman spectral profile. Raman spectral peaks of isotopically heavy biomolecules shift to lower wavenumbers than light isotopologues (“red-shifted”) because the increased atomic mass slows vibrational frequencies of excited chemical bonds (23). Unlike most other stable isotope probing (SIP) techniques, including those used with NanoSIMS, SIP-Raman non-destructively detects label incorporation into biomolecular pools and does not require extraction or separation of heavy and light fractions of nucleic acids, proteins, or lipids for further analysis. Most SIP-Raman studies use $^2$H-, $^{13}$C-, and $^{15}$N-labeled nutrients or $^2$H in the form of D$_2$O to track these elements’ movement from dissolved pools into individual cellular reporter molecules (9, 24). Huang et al. 2004 (23) were among the first to demonstrate $^{13}$C-glucose assimilation in individual heterotrophic bacteria by means of wavenumber shifts in Raman spectral peaks assignable to carbohydrates, proteins, nucleic acids and individual amino acids. Other $^{13}$C- and $^{15}$N-labeled substrates employed in SIP experiments include galactose, fructose, naphthalene, bicarbonate and ammonium, and their incorporation was detected in multiple macromolecules within bacteria or accessory pigments of photosynthetic organisms (11, 25–28).

Several recent reviews highlight that SIP-Raman and ‘omics’ approaches represent promising paths toward a deeper mechanistic understanding of microbial ecology because these tools can capture genetic identity, function, and process rates of key microbial community members *in situ* (24, 29, 30). Quantitative isotopic labeling of heterotrophic and photoautotrophic cells has been demonstrated in SIP-Raman studies, which usually analyzed cells grown in varying
ratios of $^{13}$C/$^{12}$C- or $^{15}$N/$^{14}$N-labeled substrates (e.g., glucose, naphthalene, bicarbonate, ammonium). Cellular isotopic content was shown to correspond with either spectral peak ratios of Raman biomarkers (e.g., $^{13}$C/$^{12}$C-phenylalanine peak intensities) in heterotrophic cells (23, 26, 28, 31) or with progressive shifts in wavenumber position of carotenoids in photoautotrophic cells (11). Building on the aforementioned, Taylor et al. 2017 (25) used resonance Raman microspectroscopy of carotenoid pigments to derive single-cell growth rates of photoautotrophic organisms from quantifying time-dependent $^{13}$C-assimilation. To date, there have been no SIP-Raman studies of heterotrophic bacterial growth dynamics relying on quantitative tracing of $^{13}$C-assimilation into cellular biomass. To address this knowledge gap, we performed time course SIP experiments with *E. coli* grown on complex organic substrates with a range of $^{13}$C-fractional abundances. Then progressive change in isotopic fractional abundances of cells was measured by Raman microspectroscopy to derive single-cell bacterial growth rates.

**Materials and Methods**

**Bacterial growth in isotopically enriched media**

Two *E. coli* strains, K and Bs-1, were chosen for isotopic labeling experiments. The ultraviolet-sensitive mutant strain *E. coli* Bs-1, acquired from the American Type Culture Collection (ATCC® 23224™), was selected for grazing experiments (to be presented elsewhere), because UV-radiation induces irreparable DNA lesions and results in cessation of growth. Both strains were routinely grown in Bacto® nutrient broth medium (Difco Laboratories USA). In isotopic labeling experiments, *E. coli* strains were grown in replicate flasks of a complex Bioexpress® medium (Cambridge Isotope Laboratories, chemical purity $\geq$ 98%) which contained
nearly equivalent total carbon concentrations, but varying proportions of labeled and unlabeled substrates. Bioexpress® media are derived from hydrolysates of cyanobacterial cultures grown in either $^{12}$C- or $^{13}$C-bicarbonate and their exact compositions are proprietary. The media’s fractional isotopic abundances ($f_{\text{media}} = ^{13}\text{C}/(^{12}\text{C} + ^{13}\text{C})$) were established by mixing varying proportions of $^{13}$C-Bioexpress® medium (98.5% $^{13}$C-enrichment in all carbon substrates) with $^{12}$C-Bioexpress® medium (98.9% $^{12}$C). Total carbon concentrations of both labeled and unlabeled media were directly determined using a Thermo EA1112 CNS elemental analyzer (32). To accomplish this, quartered pre-combusted (450°C for 4 h) GF/F filters were placed in 10 x 12 mm tin capsules (CE Elantech, Inc.), soaked with 100 µl of the respective Bioexpress® medium (10x working stock solution) and dried over night at 65°C. Blanks were prepared in the same manner without adding medium. All measurements were performed in duplicate. Nominal volumetric ($vol$) $f_{\text{media}}$ values of media admixtures were corrected using measured total carbon concentrations ($[C_{\text{T enr}}]$, $[C_T]$) of both media, their respective $^{13}$C-fractional abundances (enriched $f_{\text{enr}} = 0.985$ and natural $f_0 = 0.011$), and equation 1.

$$f_{\text{media}} = \frac{vol [C_{\text{T enr}}] f_{\text{enr}} + vol [C_T] f_0}{vol [C_{\text{T enr}}] + vol [C_T]}$$

Eqn 1

If not stated otherwise (e.g., determined by IRMS), all $f_{\text{media}}$ values of media preparations presented in this study are corrected based on carbon analyzer values for total organic carbon.

**Validation of Raman microspectroscopy for determining cellular $^{13}$C fractional abundance**

To test the accuracy of Raman-derived estimates of cellular fractional $^{13}$C abundances ($f_{\text{cell}} = ^{13}\text{C}/(^{13}\text{C} + ^{12}\text{C})$), *E. coli* Bs-1 cells were grown in media with a range of $^{13}$C-fractional abundances and sampled during early and late stationary phase as cells approached isotopic equilibrium with their medium. Specifically, 50 µl of an overnight *E. coli* Bs-1 culture grown in
unlabeled Bioexpress® medium was inoculated into 5 ml of 11 different $^{13}$C-labeled Bioexpress® medium admixtures ranging from natural $^{13}$C abundance to 98.5% ($f_{\text{media}} = 0.011, 0.09, 0.17, 0.26, 0.35, 0.44, 0.54, 0.65, 0.75, 0.87, 0.985$). Suspensions were incubated in 30 ml tissue culture flasks with vented caps at 37°C in the dark on an orbital shaker at 100 rpm. Subsamples (900 µl) from each bacterial suspension were fixed with borate-buffered, filtered formaldehyde (2% final conc.) after incubating 4.5 and 18 h. Fixed cells were collected on 0.2 µm polycarbonate filters (25 mm, Millipore™ GTTP) and stored at -20°C for Raman interrogations.

As an independent validation of Raman-derived $f_{\text{cell}}$ estimates, $^{13}$C-fractional abundances of E. coli Bs-1 cells and their media were directly determined by isotope-ratio mass spectrometry (IRMS). For the media’s isotopic ratio measurements, quartered pre-combusted GF/F filter were placed in 10 x 12 mm tin capsules (CE Elantech, Inc.), soaked with 80µl of three $^{13}$C/$^{12}$C Bioexpress® growth media admixtures ($f_{\text{media}} = 0.011, 0.216, 0.444$) and dried over night at 65°C. For blanks, pre-combusted GF/F filter sections were treated the same way but omitting the medium. Wrapped tin foil cups were stored at room temperature until IRMS analysis.

For IRMS measurements of $f_{\text{cell}}$, 15 ml of the same medium preparations were seeded with 150 µl of an overnight E. coli Bs-1 culture (grown in unlabeled Bioexpress® medium) and incubated for 4.5 h, as described above. After incubation, E. coli Bs-1 cells were immediately inactivated by UV-irradiation to avoid further growth during subsequent handling and to obviate the need for a fixative, such as formaldehyde that would dilute the $^{13}$C signature. Cell suspensions in tissue culture flasks were exposed to UV radiation for 60 sec under constant shaking to a total intensity of 21,000 ergs mm$^{-2}$ on a UVP™ Benchtop UV-Transilluminator M-26XV. Cells from 12 ml of each culture were captured on pre-combusted GF/F filters and rinsed with filter-sterilized (0.2 µm) distilled water to remove excess media. For blanks, GF/F filters were rinsed with the
same volume of filter-sterilized distilled water. Filters were dried overnight at 65°C and cut into equal quarters before wrapping in tin foil cups for IRMS analysis. Replicate aliquots (900 µl) of each bacterial suspension were also fixed with formaldehyde (2% final conc.) and stored for Raman interrogations.

IRMS measurements of media, cell samples and blanks were performed in triplicate by UC Davis Stable Isotope Facility (https://stableisotopefacility.ucdavis.edu). Technical limitations of the IRMS system prevent reliable carbon isotopic quantifications exceeding 50 atom% and therefore media and bacterial preparations with higher labeling were not submitted for analysis.

$^{13}$C-label assimilation dependence on growth rates

Inocula (250 µl) of cultures grown overnight in unlabeled Bioexpress® medium were introduced into 25 ml of labeled medium admixtures ($f_{media} = 0.011, 0.348, 0.985$ for *E. coli* Bs-1 and $f_{media} = 0.011, 0.985$ for *E. coli* K). Cells were incubated in 60 ml vented tissue culture flasks at 37°C on an orbital shaker at 100 rpm for 4.5 h in the dark. Immediately after inoculation and every 20–30 min thereafter, *E. coli* cell abundance was indirectly monitored by triplicate optical density (OD) measurements at 400 and 600 nm on a Perkin-Elmer Lambda 2 UV-Vis spectrophotometer using sub-micro cuvettes. Time course OD measurements provided an independent means to calculate population doubling times ($g$) and first-order growth rates ($\mu$). Replicate subsamples were also formaldehyde-fixed at all time points and filtered onto 0.2 µm polycarbonate filters for Raman interrogations.

Raman interrogations of bacteria

Polycarbonate membranes typically used to concentrate microorganisms are Raman-active and severely interfere with analysis. Therefore, preserved cells from all experiments were freeze-transferred from membrane filters to mirror-finished stainless steel slides (1x3 in) as detailed in...
and stored at -20°C until performing Raman interrogations. This technique semi-quantitatively transfers cells onto a “Raman-silent” surface and enables acquisition of high quality single-cell Raman spectra free from background interference.

Raman measurements were performed at the NAno-Raman Molecular Imaging Laboratory (NARMIL) at Stony Brook University. Analyses were performed using a Renishaw® inVia™ confocal Raman microspectrophotometer configured with a modified upright Leica® DM2700™ epifluorescence microscope, a computer-controlled motorized XYZ stage (0.1 μm minimum step size), 633 nm He/Ne laser, and a 1,040 × 256 CCD Peltier-cooled detector. Single *E. coli* cells (30-40 individuals) from each sample were targeted from reflected bright-field inVia camera micrographs (magnification 1000x) and individually interrogated at 100% laser power (~4 mW at sample) through a Leica® dry 100x (NA = 0.9) objective lens which produced a laser spot diameter of 0.86 μm. Spectra were obtained using a 1200 line/mm diffraction grating, aligned to wavenumber region between ~220 and 2060 cm\(^{-1}\) (1200 cm\(^{-1}\) center), yielding spectral resolution of 1.2 cm\(^{-1}\). CCD detector exposures were 2 s each and 20 exposures were acquired for each spectrum. Additionally, cell clusters were identified as regions of interest (ROI) and their location and size were determined from micrographs (Fig. S1). Raman spectra for ROI were acquired automatically in StreamHR mapping mode at grid nodes (1 μm step size) using the Renishaw WiRE™ Raman software (Version 5.2). The optical configuration and spectral acquisition in the mapping mode followed the same specifications as described above, except for the exposure time which was set to 18 s per grid node. For each sample in the validation experiment and for each time point in the growth experiment (2-4 and usually 3) Raman maps were produced from approximately 1000 to 5000 individual spectra (average ≈ 3000), generating spectra from approximately 300 to 800 (average ≈ 500) individual cells (Fig. S1). Processed data from both
single cell and mapped spectral data delivered comparable average results, but the latter had narrower confidence intervals (lower relative standard deviations) due to larger sample size. Furthermore, Raman mapping reduces observer bias because all cells lying on grid nodes are automatically interrogated, thereby randomizing cell selection. Thus, all results presented in this study are based on Raman maps, except for the illustration of “red shifts” in Raman spectral profiles over time in Figure 1, which are based on single-cell spectra from 30-40 individuals.

**Raman spectral analysis**

Each mapped sample contained spectra from approximately 500 cells. All spectra were saved as separate text files using the Renishaw WiRE™ Raman software (Version 5.2). Further data processing was performed using a custom Python script that performed the following tasks. From an individual map, all spectra not exceeding a filtering threshold of 40 counts above baseline were excluded as these originate from barren nodes within the map’s grid. The map’s averaged spectrum was then truncated into two regions, the Phe diagnostic region from 950 to 1050 cm\(^{-1}\), and the signal-free region from 1800 to 2000 cm\(^{-1}\) to calculate the analytical noise level. A linear baseline correction was applied to the “noise” region to determine the background signal variance (±1 SD). The mean spectrum within the diagnostic region was normalized to a value of 1 using the highest peak, and analytical noise among the ~500 spectra was scaled accordingly. A quadratic polynomial was applied for baseline correction of the diagnostic region. This spectral region was then subjected to a non-linear least-squares peak-fitting routine that iteratively fit five Voigt probability distribution profiles positioned at 966, 990, 1002, 1007/1009, and 1029 cm\(^{-1}\) to the original curve. Positions and widths of 966 and 1002 cm\(^{-1}\) peaks were allowed to vary simultaneously during fitting iterations, but the distance between 100% \(^{13}\)C and \(^{12}\)C isotopologue peaks (966 and 1002 cm\(^{-1}\)) was fixed at 36.5 cm\(^{-1}\) and sigma and gamma Voigt parameters were...
limited to 15. The 1029 cm\(^{-1}\) peak is a secondary mode of the Phe ring and the unassigned peaks
at 990 and 1007/1009 cm\(^{-1}\) were introduced to optimize convergence of the total fit. The peak
position typically occurring between 1006 and 1009 cm\(^{-1}\) was allowed to vary between 1004 and
1012 cm\(^{-1}\), and positions of the 1029 and 990 cm\(^{-1}\) peaks were allowed to vary ±10 cm\(^{-1}\). Widths
of unassigned and secondary peaks were allowed to vary between 0.1 and 15 cm\(^{-1}\). The statistical
goodness-of-fit was assessed on the fly by reduced Chi-square comparison of the total fit with
original data. The fitting routine ceased when the reduced Chi-square tolerance level (1.0e\(^{-7}\)) and
minimum divergence were attained.

As the 13C-fractional abundance of Phe increases within cellular protein, the 966 cm\(^{-1}\) peak
intensifies as the 1002 cm\(^{-1}\) peak intensity weakens. At higher fractional abundances, the low
intensity 1002 cm\(^{-1}\) of 12C-Phe was observed to be masked by the neighboring peak at 1006 cm\(^{-1}\).
However, our curve-fitting routine allowed us to deconvolve the two peaks and obtain the intensity
of 12C-Phe ring breathing vibration at lower abundances. Fractional abundances of 13C-Phe and
standard deviations were calculated from the curve-fitted peak heights (intensity ratios as
I\(_{966}/I_{966+1002}\)) and used as a proxy for total cellular 13C isotopic content (f\(_{cell}\)).

**Labeling kinetics and Raman-derived growth rates**

Considering that bacterial growth parameters can vary through time course experiments
(e.g., lag, exponential, and stationary phases), growth rate constants (\(\mu_i\)) and generation times (\(g_i\))
were calculated between all consecutive time points (e.g., \(t_0\), \(t_1\), \(t_2\), ..., \(t_n\)) using the OD
measurements (e.g., \(A_0\), \(A_1\), \(A_2\), ..., \(A_n\)) at 400 and 600 nm and equations 2 and 3, respectively.

\[
\mu_1 = \frac{(\ln A_1 - \ln A_0)}{(t_1 - t_0)}
\]

Eqn 2
The cumulative number of generations \( n \) completed while growing in the \(^{13}\text{C}\)-labeled medium was calculated by summing \( n_i \) for each time interval (e.g., from \( t_0 \) to \( t_i \)) of consecutive time points and their respective generation time \( g_i \) using equation 4.

\[
n = \sum_{t=1}^{t=x} \left( \frac{(t_1-t_0)}{g_1} + \ldots + \frac{(t_x-t_{x-1})}{g_x} \right)
\]

Eqn 4

To calculate Raman-derived growth rates \( \mu, \text{h}^{-1} \) for each time interval within a growth curve, \( n \) must first be calculated from \( f_{\text{cell}}, f_0, f_{\text{media}}, \) and \( \alpha \) values using equation 5 which was derived from equation 6 (25). The \(^{13}\text{C}\)-fractional abundance of the ancestral growth medium \( (f_0) \) is assumed to be constant at natural abundance of 0.011 and media \(^{13}\text{C}\)-enrichment \( (f_{\text{media}}) \) is either titrated from known admixtures of enriched and light media or measured directly. The isotopic fractionation factor \( (\alpha) \) in proteins for heterotrophic bacteria is assumed to be 0.9989 (34).

\[
n = -\frac{1}{\ln(2)} \ln \left( \frac{f_{\text{cell}} - \alpha f_{\text{media}}}{\alpha (f_0 - f_{\text{media}})} \right)
\]

Eqn 5

\[
f_{\text{cell}} = \alpha f_{\text{media}} + \alpha (f_0 - f_{\text{media}}) e^{-n \ln(2)}
\]

Eqn 6

Then to obtain \( \mu \) from \( n \) at each time interval \( (t) \), equation 7 was applied.

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

Eqn 7

Results:

Diagnostic Raman peak selection
Raman spectra of *E. coli* cells were acquired between 550 and 2000 cm\(^{-1}\), a region that contains peaks from many biomolecules such as nucleic acids, proteins and lipids. Observed Raman peak positions were assigned to biomolecules and chemical moieties based on those reported in the literature (35–38) (Fig. 1, Tab. 1).

Spectral features of *E. coli* cultivated at natural isotopic abundances did not change significantly through the growth phases (not presented), except for peaks emanating from nucleic acids. *E. coli* cells displayed distinct peaks assigned to vibrational modes of purine and pyrimidine bases (e.g., ring breathing modes of adenine at 723 cm\(^{-1}\) and cytosine and thymine at 779 cm\(^{-1}\)) in the exponential and the stationary growth phase. These peaks were either absent or their intensity was significantly reduced in lag phase cells (0-30min) (compare spectral profiles of panels a and b in Fig. 1A and B at peak numbers 2, 3 and 6; detailed assignment in Table 1).

Red shifts were detected in a multitude of Raman peaks that are inherent to nucleobases, amino acids, amides and lipids. However, precise quantification of the relative contribution of \(^{12}\)C and \(^{13}\)C-isotopologues was often hampered by several factors. For instance, red-shifted peaks of nucleobases (adenine, guanine, cytosine and thymine), amino acids (tyrosine, glutamate, aspartate) and amides (I, III) were evident, but precise quantification of their respective \(^{12}\)C-parent peaks was prevented by overlapping vibrational modes emanating from other moieties (e.g., peak numbers 1, 2, 3, 6, 7 and 10 in Table 1). Conversely, red shifts to wavenumbers already occupied by other functionalities impedes quantitation of some heavy isotopologues. This was observed for the tyrosine peak that shifted to a Phe position during labeling (peak number 1 in Table 1). Peaks of several nucleobase ring-breathing modes moved to peak positions indicative of proteins, lipids or unsaturated fatty acids (peak number 7 and 8 in Table 1). Further, the CH\(_2\) and CH\(_3\) vibrational modes of proteins and lipids experience small, poorly-resolved red shifts due to relative differences.
in atomic masses of C and H (peak number 8 in Table 1). Low signal intensities of lipid and carbohydrate vibrational modes caused by C-C and C-O stretching did not permit reliable quantification of their respective isotopologues (peak number 5 in Table 1).

Unlike the aforementioned, the ring-breathing mode of Phe isotopologues with 6 \(^{12}\text{C}\) atoms or 6 \(^{13}\text{C}\) atoms in the phenyl ring display two well-defined peaks at 1002 and 966 cm\(^{-1}\), respectively. Their positions and intensities enable reliable calculation of \(^{13}\text{C}\)-Phe peak intensity fractions to use as a proxy for \(f_{\text{cell}}\). In order to achieve utmost precision in quantifying peak intensity at 966 cm\(^{-1}\) and 1002 cm\(^{-1}\) we developed a robust curve-fitting procedure that allowed precise deconvolution of partially overlapping peaks. Otherwise, a “contaminating” neighboring peak (1006/1009 cm\(^{-1}\)) would have prevented proper intensity measurements of the unlabeled Phe ring-breathing mode at 1002 cm\(^{-1}\) (Fig. S2).

Validation of \(^{13}\text{C}\)-Phe as a proxy for cellular \(^{13}\text{C}\)-fractional abundance (\(f_{\text{cell}}\))

Among the diagnostic Raman peaks detected in this study, the essential amino acid phenylalanine (Phe), present in all cells, is the most promising reporter molecule for Raman-derived \(f_{\text{cell}}\) estimates. In order to validate \(^{13}\text{C}\)-Phe as an accurate proxy of cellular \(^{13}\text{C}\)-fractional abundance, we compared direct Raman-based measurements with two alternative determinations of \(f_{\text{cell}}\). Firstly, separate \textit{E. coli} Bs-1 cultures were grown to early and late stationary phase in media with 11 different admixtures of the same \(^{13}\text{C}\) and \(^{12}\text{C}\) carbon substrates. Contributions of \(^{13}\text{C}\) and \(^{12}\text{C}\) additions to total carbon (\(f_{\text{media}}\)) in each medium was calibrated by total organic carbon analysis. Cells grown thusly are well-suited for testing the validity of Raman signature-derived \(f_{\text{cell}}\) estimates under varying isotopic enrichments, because they should have been very close to isotopic equilibrium with their medium (\(f_{\text{media}} \cong f_{\text{cell}}\)) and intra-population variability should be minimal.
compared to earlier growth stages. Raman-derived $f_{\text{cell}}$ estimates after 4.5 and 18 hours of incubation are statistically indistinguishable over the entire range of labeled media ($f_{\text{media}} = 0.011$ to 0.985), illustrating that the shorter incubation time was sufficient for $E. \text{coli}$ Bs-1 populations to reach the maximum labeling in their respective media (Fig. 2). Hence, a maximum duration of 4.5 h was chosen for all subsequent labeling experiments. Raman-based $f_{\text{cell}}$ measurements were highly correlated with $f_{\text{media}}$, yielding regression slopes of 0.91 to 0.93 and very high coefficients of determination ($R^2 = 0.99$) (Fig. 2). The fact that the slope of $f_{\text{cell}}$ over $f_{\text{media}}$ is $<1$ suggests that either $f_{\text{cell}}$ was slightly underestimated by our Raman approach, or $f_{\text{media}}$ which was not measured directly in this experiment was slightly overestimated, or that cells did not reach isotopic equilibrium due to isotopic discrimination.

To assess whether the disparity between Raman $f_{\text{cell}}$ and $f_{\text{media}}$ resulted from inaccuracies in either $^{13}$C-media preparation (e.g., pipetting errors) or Raman measurements of $f_{\text{cell}}$, fractional isotopic abundances were independently measured by isotope-ratio mass spectrometry (IRMS). Our carbon analyzer-corrected determinations of $f_{\text{media}}$ were almost identical to the two $^{13}$C-enriched admixtures (IRMS vs. carbon analyzer, 0.217±0.0001 vs. 0.216 and 0.436±0.003 vs. 0.444). Both Raman and IRMS-determined $f_{\text{cell}}$ values varied linearly with IRMS-determined $f_{\text{media}}$ ($R^2 = 0.99$) and similar to Fig. 2 slopes were less than 1; 0.897 and 0.896, respectively (Fig. 3a). Reassuringly, accuracy of Raman $f_{\text{cell}}$ determinations is strongly supported by direct comparison with IRMS-determined $f_{\text{cell}}$ values, which are essentially identical to within analytical uncertainty (Fig. 3b). We interpret the departure from the 1:1 line in Figure 3a as emanating entirely from isotopic discrimination ($\alpha$, fractionation). Alternatively, we are left with the unlikely possibility that both Raman and IRMS underestimate $f_{\text{cell}}$ by exactly the same amount.

**Derivation of bacterial growth parameters from SIP-Raman experiments**
In order to follow $^{13}$C-labeling of *E. coli* populations through all growth phases, cells were incubated in equivalent carbon concentrations of Bioexpress® with varying $^{13}$C:$^{12}$C admixtures ($f_{\text{media}} = 0.011, 0.348, 0.985$ for *E. coli* Bs-1 and $f_{\text{media}} = 0.011, 0.985$ for *E. coli* K). OD measurements on cell suspensions of both *E. coli* strains confirmed that growth curves were nearly identical among populations grown in the different media admixtures (Fig. S3). Thus, population growth among treatments can be considered replicated measurements of the same variable. Furthermore, previous studies with *E. coli* revealed that monitoring growth by OD at different wavelengths can yield significantly different calculated growth rates (39). Thus, OD measurements were performed at 400 and 600nm, wavelengths commonly used to measure bacterial cell density (40). In either case, both *E. coli* strains produced very similar, typical sigmoidal population growth curves.

Theoretical $^{13}$C-labeling kinetics were calculated using OD$_{400}$ and OD$_{600}$ based growth curves to compute number of generations ($n$) during each sampling interval using equation 6 and then compared to Raman-derived $f_{\text{cell}}$ estimates. As expected, isotopic signatures of cells incubated in natural $^{13}$C abundance media did not change over the time course (data not shown). Raman $f_{\text{cell}}$ measurements agree remarkably well with predicted $f_{\text{cell}}$ estimates in their general sigmoidal trajectory through time among all $^{13}$C-enriched treatments as exemplified by *E. coli* Bs-1 (Fig. 4a,b). Within individual labeling treatments, Raman-based $f_{\text{cell}}$ values strongly covaried ($R^2 = 0.96 - 0.98$) with those predicted from independent OD data, yielding regression slopes that are nearly 1; 1.11 and 0.99 for $f_{\text{media}} = 0.348$ and 0.985 treatments, respectively (Fig. 4a,b). Results from all time course treatments for *E. coli* Bs-1 and K were tested for their overall agreement between $f_{\text{cell}}$ values derived from Raman measurements and predicted from OD-based growth curves, by means of a Bland-Altman difference plot (41) (Fig. 5). Mean differences of Raman-based $f_{\text{cell}}$ from OD-
predicted \( f_{\text{cell}} \) were -0.04 and -0.023 for OD\(_{600}\) and OD\(_{400}\), respectively (Fig. 5a,b). The mean difference of OD\(_{600}\) derived \( f_{\text{cell}} \) determinations from those of OD\(_{400}\) was +0.017 (Fig. 5c). This comparison illustrates that while OD\(_{600}\) and OD\(_{400}\) coherently track growth trends, subtle differences are evident among time intervals. In all cases, no more than two observations of the difference values (Raman measured and predicted \( f_{\text{cell}} \)) lie outside the 95% confidence limits. Distribution patterns indicate that agreement of both methods is consistent across the entire range of measurements, and not biased towards higher or lower \( f_{\text{cell}} \) values (Fig. 5a,b).

Finally, growth rates derived independently from Raman and OD spectroscopic techniques were compared during exponential phase for both *E. coli* strains grown in the three \(^{13}\)C-enriched media. Exponential phase was defined from ln-normal plots of OD data. SIP-Raman-based single-cell growth rates were computed using Eqn 5 and Eqn 7. OD-based population growth rates were calculated with Eqn 2. Growth rates (\( \mu \)) of both *E. coli* strains derived from the SIP-Raman measurements agreed remarkably well with those computed from both OD\(_{600}\) and OD\(_{400}\) measurements and were not significantly different from each other (\( p > 0.05 \); one way ANOVA) (Fig. 6a,b).

**Discussion:**

**Challenges for accurate quantification of \(^{12}\)C and \(^{13}\)C Raman peaks**

The consistent positions of Raman spectral peaks in *E. coli* cells grown at natural \(^{13}\)C abundances illustrate this technique’s analytical reproducibility. Thus, peak shifts to lower wavenumbers (red shifts) observed in cells grown in \(^{13}\)C-enriched media can be attributed almost exclusively to
assimilation of heavy isotopes. However, among the various red-shifted biomolecules and chemical moieties, precise quantification of spectral peaks from isotopologues is often complicated by overlapping peaks with other origins (Fig. 1, Table 1). Such occurrences have impeded peak quantification in prokaryotic and eukaryotic cells and limit Raman spectral features suitable for estimates of cellular $^{13}$C-enrichment (20, 42, 43). Similar to Xie et al. 2005 (44), we found some peaks emanating from DNA have very low intensities in lag and early exponential phases of population growth, which impede proper quantification of DNA isotopologues (Fig. 1A). Raman spectral profiles are known to vary among cells in different growth stages or between cells assimilating different substrates (23, 44, 45). In our time course experiments, the effect of different substrates was largely avoided by inoculating pre-conditioned cells grown on the same media (Bioexpress® medium). Using pre-conditioned inocula that were more or less synchronized to be in exponential phase likely also reduced observed variations in Raman spectra in the earliest time points.

We chose Raman peaks that emanate from Phe isotopologues as a proxy for cellular isotopic abundance. Phenylalanine is an essential amino acid present in all organisms, making it a universal biomarker for all cells (43). As reported in numerous SIP-Raman studies focused on detecting cellular $^{13}$C-incorporation of prokaryotes and eukaryotes (20, 21, 23, 24, 26, 28, 42, 46–49), Phe produces distinct and easily quantifiable peaks in Raman spectral profiles of E. coli cells of this study (Fig. 1c). Intensities of the phenyl ring breathing mode at 966 and 1002 cm$^{-1}$ representing 100% $^{13}$C and 100% $^{12}$C in the ring, respectively, were used to compute the $^{13}$C-fractional abundance of Phe. This ratio was then extrapolated to $^{13}$C-isotopic abundance of cells ($f_{cell}$) on the assumption that all cellular carbon pools are equally isotopically enriched. However, two more Raman peaks (989 and 978 cm$^{-1}$) can occur that are attributed to partially labeled Phe-
isotopologues in cells fed with $^{13}$C-glucose (42, 50). In our study, these peaks could not be confidently detected above baseline and analytical noise. The rich Bioexpress® labeling medium used here consists of hydrolyzed $^{13}$C-labeled cyanobacterial biomass and thus represents a complex amalgam of organic substrates, presumably including peptides and free amino acids.

Assimilation of $^{13}$C-labeled amino acid mixtures (51) or $^{13}$C-labeled phenylalanine (52) results in the appearance of the red-shifted 967 cm$^{-1}$ Phe peak as well as the ancestral 1002/3 cm$^{-1}$ peak, but no intermediate peaks indicating 2 or 4 $^{13}$C substitutions in the phenyl ring. Thus, we interpret our spectral data as indicating that cells are not synthesizing Phe de novo when presented with pre-formed $^{12}$C-Phe and $^{13}$C-Phe in the medium. Under our experimental conditions, cells appear to be opting for energy-saving salvage pathways to meet their Phe requirement.

**Phenylalanine as a quantitative reporter for cellular isotopic abundance**

Use of Phe as a proxy for $f_{cell}$ was validated by two independent methods. Analogous to the approach of Huang et al. 2007 (28), we calibrated $E. coli$ $f_{cell}$ estimates against isotopic signatures of the media using a broad range of isotopic enrichments, spanning natural abundance to 98.5% $^{13}$C (Fig. 2). This approach assumes that cells reach isotopic equilibrium with their medium ($f_{cell}$ = $f_{media}$) after populations are well into stationary phase (in our case 4.5 and 18h). We also compared Raman-based values to direct $f_{cell}$ determinations by IRMS of cells with three different isotopic contents ($f_{media}$ = 0.011, 0.216 and 0.444) (Fig. 3). In both cases, Raman-based $f_{cell}$ values were highly reproducible and covaried predictably with $f_{media}$. In the pseudo-calibration curve, Raman $f_{cell}$ values were on average 11% isotopically lighter than the media in which they grew. Comparing IRMS data for $f_{media}$, Raman and IRMS $f_{cell}$ values were both on average 6% lighter than their growth media. Are these disparities indicative of isotopic disequilibrium due to insufficient time, or fractionation? In Bioexpress® media, both $E. coli$ strains entered stationary phase within 3 h of
inoculation, so when these cells were harvested (4.5 and 18 h) they should have been as isotopically equilibrated as possible. However, the observed 6-11% depletions result in isotopic fractionation factors ($\alpha$) of 0.89-0.94, which to our knowledge represent significantly more fractionation than previously reported. Independent IRMS measurements of $f_{cell}$ confirmed the accuracy of Raman-derived estimates of $^{13}$C-enrichments, being statistically indistinguishable to within analytical uncertainty. IRMS is known to provide highly sensitive and precise measurements of isotopic ratios and is thus considered the gold standard for validating the analytical accuracy of Raman-based $f_{cell}$ determinations. To the best of our knowledge, direct comparison of cellular isotopic enrichments by Raman and IRMS has only been previously performed with extracted proteins (11), but never using total cellular biomass. The close agreement between IRMS- and Raman-based determinations of $f_{cell}$ strongly supports our assumption that all cellular carbon pools acquire comparable $^{13}$C-signatures indicated by the reporter molecule, Phe.

Explaining why stationary phase cells were isotopically depleted relative to their media, whether measured by Raman or IRMS, is challenging. We can safely rule out that carryover of unlabeled biomass from the inoculum accounted for depressed $f_{cell}$ values, since a negligible amount (dilution 1:100) of an overnight culture was used to start the incubations. To illustrate, after exponential growth for 7 generations (3.4 h for our cultures) legacy $^{12}$C biomass can only account for 0.75% of the resulting biomass, totally independent of inoculum size. This is clearly insufficient to explain a 6-11% depletion in the $^{13}$C-biomass signature.

Alternatively, isotopic depletion of biomass might be explained by anaplerotic assimilation of ambient inorganic $^{12}$C during growth. This heterotrophic CO$_2$ fixation process can account for 1-8% of the total carbon assimilated by microorganisms (53). During anaplerotic reactions, CO$_2$ is consumed in the tricarboxylic acid cycle (TCA cycle) when phosphoenolpyruvate (PEP) or...
pyruvate is carboxylated by PEP carboxylase or pyruvate carboxylase. However, under our incubation conditions (aeration, rich complex medium, glycolytic conditions), Phe biosynthesis is decoupled from TCA cycle metabolites, deriving its precursor from the Shikimate pathway which is fed by PEP from glycolysis and erythrose 4-phosphate from the pentose phosphate pathway (54, 55). We conclude that if anaplerosis were operative in lowering $^{13}$C-fractional abundance in most cellular components except phenylalanine, then this would be reflected by higher fractional abundance of $^{13}$C-phenylalanine (= proxy for $f_{\text{cell}}$) in our Raman analysis compared to $f_{\text{cell}}$ values determined by IRMS. On the contrary, Raman and IRMS delivered almost identical values of $f_{\text{cell}}$. Furthermore, if de novo synthesis of Phe received any carbon from anaplerotic reactions, then intermediate isotopologues would be evident from Raman peaks at 989 and 978 cm$^{-1}$, but they were not. Thus, we judge anaplerosis to have played a negligible to nonexistent role in our observations.

Carbon isotopic fractionation in heterotrophic bacterial metabolism is a possible mechanism to produce light cellular biomass. The kinetic isotope effect influences specific enzymatic reaction rates resulting in isotopic compositions of certain biomolecules, such as proteins, lipids or amino acids, distinctive from the substrate utilized (34, 56–58). Although isotopic fractionation can vary considerably among individual cellular pools, Blair et al. 1985 (34) reported that E. coli proteins were only 0.11% lighter than their glucose growth substrate, effectively too small to explain observed $f_{\text{media}} - f_{\text{cell}}$ disparities. It is noteworthy that almost all previous studies examining cellular isotopic fractionation did so at natural isotopic abundances, and did not focus on bacterial metabolic fractionation of $^{13}$C-enriched tracers. While not evaluating fractionation per se, a study using SIP to follow phospholipid fatty acid synthesis found that $^{13}$C-toluene additions preferentially exerted inhibitory effects on fatty acid biosynthesis and bacterial
growth relative to $^{12}$C-toluene additions (59). In contrast, our OD-based measurements yielded statistically indistinguishable population growth curves among parallel incubations containing different isotope admixtures (Fig. S3). We also found remarkable agreement between $f_{\text{cell}}$ values determined by Raman and IRMS (Fig. 3) and between predicted and Raman measured $f_{\text{cell}}$ during the time course experiment (Fig. 4, 5). Furthermore, Raman-based growth rate estimates derived from $f_{\text{cell}}$ were statistically indistinguishable from those computed from OD measurements (Fig. 6). Nevertheless, future studies should carefully address potential biases arising from isotope fractionation and anaplerosis with different bacterial groups, carbon substrates and metabolic pathways.

**Quantitative SIP-Raman applications in microbial ecology**

We demonstrate that single-cell bacterial growth rates can be derived from known fractional isotopic abundances of the growth substrate ($f_{\text{media}}$) and the cells ($f_{\text{cell}}$) measured at just two time points using Raman microspectroscopy. In a similar fashion to a recent SIP-NanoSIMS study (14), SIP-Raman approaches can also measure single-cell carbon assimilation rates, when combined with precise total carbon estimates of the organism under scrutiny (Weber et al. unpubl.). The current study used a complex medium ($^{13}$C-Bioexpress®), but specific $^{13}$C-substrates can certainly be employed to study bacterial growth response and physiological heterogeneity within and among populations. For example, in studies focusing on substrate preference, diauxic growth and carbon catabolite repression, quantitative SIP-Raman microspectroscopy used with selected labeled and unlabeled carbon substrates would permit determination of the extent to which mixed substrate are utilized on the single-cell level (27). Another elegant strategy for measuring bacterial growth in laboratory or field experiments might be provided by Raman microspectroscopic monitoring of $^{13}$C-Phe fractional abundances in cells after the addition of $^{13}$C-
labeled Phe in amounts exceeding natural concentrations. While assimilation of $^{13}$C-Phe into bacterial protein pools has been demonstrated by Raman microspectroscopy (51, 52), the practicality for studying growth dynamics has not yet been tested. If we assume that $^{13}$C-Phe additions alone do not promote growth, as assumed by the $^3$H-leucine and $^3$H-thymidine growth methods (60, 61), then estimates of growth solely supported by autochthonous organic material can be derived. Clearly, empirical testing of whether $^{13}$C-Phe additions stimulate growth in such SIP experiments is required before proceeding.

In principle, our approach can be applied to microbiome and field experiments examining bacterial growth and population dynamics based on uptake of either complex or individual labeled carbon substrates. The complex labeling medium used here (Bioexpress®) is a hydrolysate of $^{13}$C-labeled cyanobacterial biomass. Thus, to some extent it mimics the complex amalgam of dissolved organic matter released by phytoplankton in natural waters. Alternatively, using specific $^{13}$C-compounds enables studying growth responses of key players utilizing different carbon sources by determining substrate-specific growth rates. However, depending on the research question, substrate-dependent enrichments of specialized populations within the microbial community may be desired or tolerable, but are sometimes problematic. SIP studies generally depend on tracer amendments at levels exceeding the natural substrate abundances and on incubation times sufficient for microbial utilization of the labeled substrate. This potentially perturbs the natural community by selectively stimulating the activity of populations favored by the newly imposed conditions. Choosing appropriate tracer additions represents a tradeoff between detectability and perturbations to natural microbial assemblages. Therefore, the choice reflects a balance between the detection method’s sensitivity, trophic status of the sample, and quantity of cellular biomass that finally utilizes the tracer (10). Current SIP methods that can be combined with phylogenetic
identification, either by SIP-DNA/RNA or by FISH coupled to Raman or NanoSIMS, vary widely in their detection limits of isotopic tracers. Whereas NanoSIMS is the most sensitive among these, detecting as little as 0.1 atom\% (62), SIP-RNA and SIP-DNA require up to 20 atom\% (50 atom\% for high GC-content DNA) for efficient separation of heavy and light fractions (63–65). For spontaneous Raman microspectroscopy a minimum cellular isotopic content of 10 atom\% has been suggested as the threshold for discriminating between labeled and unlabeled bacterial cultures (28). Consequently, for monitoring of cellular labeling propagation to derive microbial rate measurements in environmental samples, significantly higher isotopic content may be required. However, advanced signal enhancement techniques involving colloidal metal nanoparticles, i.e. Surface-Enhanced Raman Spectroscopy (SERS) can increase the Raman signal intensity by many orders of magnitude ($10^7$-$10^{11}$) and thus potentially can improve the limit of detection of cellular isotopic labeling (9, 43). This would be transformative, because it would make the much less labor-intensive, and non-destructive Raman approach the method of choice for monitoring label propagation in preserved, frozen or live cells. SIP-SERS also has the potential to better resolve additional quantitative Raman biomarkers as recently demonstrated for adenine that exhibited strong red shifts in \textit{E. coli} cells fed with $^{13}$C-glucose (50, 66). Quantitative SIP-Raman approaches combined with methods for phylogenetic identification such as FISH or downstream sequencing of Raman sorted cells (12) represent extremely promising tools for enriching our understanding of microbial rates relevant for matter cycling in the environment.

Conclusion and future perspectives
Our proof of concept study demonstrates that the SIP-Raman approach enables reliable bacterial growth rate measurements based on assimilation of $^{13}$C-labeled organic matter by quantitation of the fractional abundance of Phe isotopologues over time. This approach is highly applicable to many questions in biomedical, applied and environmental microbiology that aim to assess bacterial growth, substrate preference, and metabolic fluxes. Further, it allows analysis at both single-cell and population levels, without prior isolation. When the nature of the sample limits application of traditional methods (e.g., cell counts, most probable number, optical density, respiration rates), such as in complex matrices (e.g. soil, sediment, biofilms) or mixed microbial assemblages, SIP-Raman is an especially elegant alternative.

Several studies have shown the compatibility of SIP-Raman with fluorescence in situ hybridization (FISH) or with molecular analysis of marker genes within libraries constructed from cells that were Raman sorted according to isotopic signatures (12, 13, 24, 28, 31). Combining these tools enables investigators to directly link genetic identity to functional traits of single cells in mixed microbial assemblages. However, these combined approaches have thus far been performed largely in a qualitative manner, i.e., cataloguing active and non-active cells. Further refinement of quantitative SIP-Raman approaches has the potential to accurately measure microbial metabolic rates. Quantitative SIP-Raman approaches complemented by phylogenetic identification tools overcome a major technical constraint; linking phylogenetic identity with functions and metabolic rates of cells in environmental samples. SIP-Raman approaches offer tremendous potential to study trophodynamics in microbial interactions, such as the interspecies carbon transfer in predator-prey relationships (Weber et al. unpubl.) and among symbiotic partners. We believe that this approach will pave the way towards a deeper understanding of numerous microbial processes.
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Table and Figures

Table 1: Wavenumber assignment to $^{12}$C-parent and “red shifted” spectral peaks of macromolecules and chemical moieties reported in the literature (35–38).

<table>
<thead>
<tr>
<th>Peak Nr.</th>
<th>Raman shift (cm$^{-1}$)</th>
<th>Assignment</th>
<th>Explanation</th>
</tr>
</thead>
</table>
| 1        | 620, 641                 | Phe $\tau$(C-C)  
Tyr $\tau$(C-C) | During labeling, Tyr peak moves to the Phe position. Phe red shift is not evident, due to weak signal. |
| 2        | 723  
712 red shift | A ring breathing mode (DNA) | Adenine breathing mode. Also may include C-S stretching from protein, and other vibrations that broaden feature disqualifying it for quantitative analysis. |
| 3        | 779  
761 red shift | C, T ring breathing mode, $\nu$(O=P=O) DNA backbone | Pyrimidines ring breathing, also O-P-O symmetrical and asymmetrical stretching |
| 4        | 1002  
966 red shift | Phe ring breathing mode (Proteins) | Phenylalanine ring breathing – single sharp peak, making it amenable to quantitative analysis. |
| 5        | 1090 | $\nu$(P=O$^2$) (DNA),  
$\nu$(C-O), (saccharides),  
$\nu$(C-C) (saccharides, lipids) | Red shifts not evident perhaps due to low relative amounts of lipids and carbohydrates. |
| 6        | 1225  
1206 red shift  
1177 red shift | C, A, G (DNA), Amide III (Proteins), $\nu$(P=O$^3$). (DNA) | Too many overlapping vibrations for quantitative analysis. |
| 7        | 1322  
1307 red shift  
1361 | $\tau$(CH$_2$) (proteins and nucleic acids), $\delta$(C=H) (unsaturated fatty acids) | Too many overlapping vibrations for quantitative analysis. Ring mode of DNA bases at 1361 shifted toward proteins and fatty acids bands. |
| 8        | 1450  
1478 | $\alpha$(CH$_3$/CH$_2$) $\beta$(CH$_3$/CH$_2$) (proteins and lipids)  
A, G (N7 ring mode) (DNA) | Red shift not evident at 1450, 1478 peak red-shifted toward 1450, but difficult to resolve. |
| 9        | 1581  
1526 red shift | G, A $\nu$(ring breathing mode) (DNA) | Vibration from purine bases shows significant and predictable red shifts. |
| 10       | 1668  
1630 red shift | Amide I (proteins), T $\nu$(C=O) (DNA) C $\nu$(C=O) (DNA), Glu, Asp $\nu$(C=O)OH (proteins) | Red shifts are evident, but poor candidate for quantitation due to peak broadness and overlap with neighboring vibrations. |

Modes of vibration: $\alpha$, scissoring; $\beta$, bending; $\delta$, deformation; $\tau$, twisting; $\nu$, stretching

Abbreviations: Phe (Phenyalanine), Tyr (Tyrosine), A (Adenine), G (Guanine), T (Thymine), C (Cytosine)
Figure 1. Stacked Raman spectra of *E. coli* Bs-1 illustrating peaks emanating from macromolecules and their respective red shifts due to $^{13}$C enrichment during the time course experiment (B). Amplified views of original and red-shifted DNA and Phe peaks appear in panels (A) and (C), respectively. Lines and shaded areas represent the average and standard deviation computed at every wavenumber from 30-40 spectra obtained from individual cells. Peak numbers 1-10 (top) correspond to wavenumber assignments of vibrational modes in Table 1. Composite spectrum of cells grown for 59 min at natural isotopic abundances is shown in a). Composite spectra b) to h) were acquired from cells grown in the most isotopically enriched media ($f_{\text{media}} = 0.985$) with incubation times and Raman-derived $f_{\text{cell}}$ values as follows: b) 0 min; $f_{\text{cell}} = 0.02$, c) 30 min; $f_{\text{cell}} = 0.19$, d) 59 min; $f_{\text{cell}} = 0.49$, e) 88 min; $f_{\text{cell}} = 0.68$, f) 119 min; $f_{\text{cell}} = 0.84$, g) 180 min; $f_{\text{cell}} = 0.89$, and h) 270 min; $f_{\text{cell}} = 0.93$.

Figure 2. Relationship between cellular fractional $^{13}$C-labeling ($f_{\text{cell}}$) and growth media with varying $^{13}$C enrichments ($f_{\text{media}}$) determined in stationary phase *E. coli* Bs-1 cells. Mean $f_{\text{cell}}$ (± SD) for cells grown for 4.5 (closed circles) and 18 hours (open circles) were determined from Phe isotopologue peaks of ~800 Raman spectra acquired for each $f_{\text{media}}$ treatment. Dashed line represents 1:1 relationship between $f_{\text{media}}$ and $f_{\text{cell}}$. Linear regression results are given in the plot. Difference between the $f_{\text{cell}}$ slopes at 4.5 and 18 h was insignificant (p-value = 0.44; Significance test (63)).

Figure 3. Comparisons of isotopic enrichments measured by Raman microspectroscopy and isotope-ratio mass spectrometry (IRMS) for *E. coli* Bs-1 grown for 4.5 h. (a) Mean (± SD) of $f_{\text{cell}}$ determined by Raman microspectroscopy ($\lambda$) and IRMS (○) as a function of $f_{\text{media}}$. $^{13}$C enrichment was determined by IRMS in samples of dried media and *E. coli* cells and was measured in triplicate. Raman-derived $f_{\text{cell}}$ values were based on spectra from 500± 45 cells in three different mapping regions per treatment. (b) Direct comparison of Raman microspectroscopic and IRMS determinations of $f_{\text{cell}}$ using same data as in (a). Difference between the two slopes of $f_{\text{cell}}$ (Raman & IRMS) in (a) was insignificant (p-value = 0.99; Significance test (67).
Figure 4. Population growth-dependent changes in cellular $^{13}$C fractional abundances ($f_{\text{cell}}$) measured by Raman microspectroscopy and predicted from OD-based growth curves (absorbance at 600 and 400 nm) and equations 2-5. Results are for *E. coli* Bs-1 grown in labeled medium with $f_{\text{media}}$ of 0.348 (a) and 0.985 (b). Insets show linear regression results of comparison between Raman measured and predicted (OD$_{600}$) $f_{\text{cell}}$ values. Linear regression results of comparison between Raman measured and predicted (OD$_{400}$) $f_{\text{cell}}$ values (not shown in graph) were as follows: for *E. coli* Bs-1 grown at $f_{\text{media}}$ of 0.348 (a) and 0.985 (b) $f_{\text{cell}}$ (Raman) = -0.03 + 1.12 * $f_{\text{cell}}$ (OD$_{400}$); $R^2 = 0.97$; S.E. = ±0.027 and $f_{\text{cell}}$ (Raman) = -0.089 + 1.006 * $f_{\text{cell}}$ (OD$_{400}$); $R^2 = 0.95$; S.E. = ±0.084, respectively.

Figure 5. Bland-Altman difference plots comparing OD$_{600}$, OD$_{400}$, and Raman derived $f_{\text{cell}}$ estimates. Results of the three time course labeling experiments *E. coli* K in $f_{\text{media}}$ = 0.985 (n=8), *E. coli* Bs-1 in 0.348 (n=8) and *E. coli* Bs-1 in $f_{\text{media}}$ = 0.985 (n=8) are combined (n=24). Comparisons of predicted $f_{\text{cell}}$ from OD$_{600}$, OD$_{400}$, and Raman $f_{\text{cell}}$ are shown in panels A) and B), respectively. Panel C shows the comparison of the two $f_{\text{cell}}$ values predicted from OD$_{600}$ and OD$_{400}$. Solid horizontal line marks the mean difference between $f_{\text{cell}}$ values derived by the two methods. Dashed horizontal lines represent the 95% confidence intervals (±1.96 standard deviations). One way ANOVA found no statistical difference between $f_{\text{cells}}$ by Raman, OD$_{600}$ and OD$_{400}$ (P = 0.849).

Figure 6. Comparison of mean (± 1 SD) growth rates derived from OD$_{600}$, OD$_{400}$, and Raman results for *E. coli* Bs-1 (a) and *E. coli* K (b) during exponential phase. OD-derived growth rates are based on population growth of three and two parallel incubations of *E. coli* Bs-1 (a) and *E. coli* K (b), respectively. Raman-derived growth rates are based on approximately 2400 interrogated cells per time point in each labeling treatment ($f_{\text{media}}$ = 0.348 and 0.985 for *E. coli* Bs-1 and $f_{\text{media}}$ = 0.985 for *E. coli* K). P-values of a single factor ANOVA are given in the upper right corner of each plot.
$f_{\text{cell}}(4.5\,\text{h}) = -0.007 + 0.928\,f_{\text{media}}$
$R^2 = 0.99;\,\text{S.E.} = \pm 0.017$

$f_{\text{cell}}(18\,\text{h}) = -0.014 + 0.908\,f_{\text{media}}$
$R^2 = 0.99;\,\text{S.E.} = \pm 0.019$
**a**

\[ f_{cell} \text{ (Raman)} = 0.012 + 0.897 f_{media}; \]
\[ r^2 = 0.99; \text{ S.E.} = \pm 0.007 \]

\[ f_{cell} \text{ (IRMS)} = 0.008 + 0.896 f_{media}; \]
\[ r^2 = 0.99; \text{ S.E.} = \pm 0.052 \]

**b**

\[ f_{cell} \text{ (Raman)} = 0.005 + 0.997 f_{cell} \text{ (IRMS)}; \]
\[ r^2 = 0.99; \text{ S.E.} = \pm 0.066 \]