Synthesis of fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial growth *in situ*

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Fluorescent D-amino acids (FDAAs) are efficiently incorporated into the peptidoglycans (PGs) of diverse bacterial species at the sites of PG biosynthesis, allowing specific and covalent probing of bacterial growth with minimal perturbation. Here we provide a protocol for the synthesis of four FDAAs emitting light in blue (HCC-amino-D-alanine, HADA), green (NBD-amino-D-alanine, NADA, and fluorescein-D-lysine, FDL) or red (TAMRA-D-lysine, TDL) and for their use in PG labeling of live bacteria. Our modular synthesis protocol gives easy access to a library of different FDAAs made with commercially available fluorophores and diamino acid starting materials. Molecules can be synthesized in a typical chemistry laboratory in 2–3 d using standard chemical transformations. The simple labeling procedure involves the addition of the FDAAs to a bacterial sample for the desired labeling duration and stopping further label incorporation by fixing the cells with cold 70% (vol/vol) ethanol or by washing away excess dye. We discuss several scenarios for the use of these labels in fluorescence microscopy applications, including short or long labeling durations, and the combination of different labels in pure culture (e.g., for 'virtual time-lapse' microscopy) or *in situ* labeling of complex environmental samples. Depending on the experiment, FDAA labeling can take as little as 30 s for a rapidly growing species such as *Escherichia coli*.

INTRODUCTION

The PG cell wall of bacterial cells is a macromolecular polymer consisting of β-1,4-linked glycan strands that are cross-linked by short D-amino acid (DAA)-containing peptide chains. PG is an essential structure for bacterial cells that precisely determines cell size and shape, and it enables them to resist lysis. The PG biosynthetic pathway is an attractive target for antibiotic intervention, because the key polymerization and cross-linking reactions take place on the solvent-accessible surface of the bacterial cell. In addition, as animal cells do not have a PG shell, the potential for toxicity to humans is minimized[1]. Unfortunately, the resistance of pathogenic bacteria to even the newest PG-acting antibiotics (e.g., carbapenem-resistant enterobacteriaceae) has increased alarmingly, which underscores the urgent need for novel additions to our current antibiotic pharmacopeia. However, the development of novel PG-acting agents has lagged because our overall knowledge of PG biosynthesis and turnover is still very limited[2,3]. This is due, in part, to the lack of tools or methods to enable real-time spatiotemporal tracking of PG biosynthesis in live bacterial cells[4,5]. Recently, we have shown that FDAAs enable rapid and covalent detection of cell wall biosynthesis, in real time, in live cells and in a broad range of bacterial species[6]. This method has already been used to study the growth or PG synthesis mechanisms in various bacterial species[7–10].

In this protocol we describe the FDAA technology, starting with procedures for modular synthesis of four differently colored FDAAs (**Fig. 1**), namely HADA (3, emission wavelength (λ_{em}) ~450 nm), NADA (5, λ_{em} ~550 nm), FDL (8, λ_{em} ~525 nm) and TDL (10, λ_{em} ~ 575 nm), in a basic organic chemistry laboratory; this is followed by options for assays that can be performed using these FDAAs. The procedure for preparing the red FDAA (TDL) can easily be adapted for the synthesis of custom DAAs by attaching a common DAA backbone (amino-D-alanine or D-lysine) to myriad commercially available succinimidyl ester–activated dyes or other functionally activated molecules.

Strengths and limitations of existing alternative methods

The development of tools and methods to enable real-time tracking of the progression of cell wall biosynthesis and PG recycling is a problem of considerable current interest for which there was no general solution[4,5]. Methods relying on fluorescently labeled antibiotics such as vancomycin, with high affinity for nascent PGs, have had a profound effect on the field by facilitating visualization of the growth mode(s) of Gram-positive bacteria, but these methods also have some inherent limitations[11,12]. First, antibiotic concentration and treatment duration need to be carefully controlled to get acceptable labeling while avoiding extensive damage to the cell, and, second, because of the substantial size of these agents, labeling is limited to the sites of PG synthesis in bacteria with solvent-exposed cell walls (i.e., Gram-positive bacteria), or to mutants of Gram-negative bacteria with a compromised outer membrane.

Other approaches have sought to covalently modify the bacterial cell wall through incorporation of synthetically modified cell wall precursors that are metabolically incorporated by the PG biosynthetic machinery[13–17]. These methods have mostly allowed PG labeling for pulse-chase–type experiments in which the unlabeled region during the chase indicates the site of new PG synthesis. Unfortunately, these methods have provided unsatisfactory results either owing to poor substrate uptake and incorporation and/or toxicity because of the site selected for probe incorporation[13–17]. A recent report has described the use of a fluorescently modified tripeptide component of the PG stem peptide for covalent labeling of the bacterial cell wall in live *E. coli*[14]. However, as the method relies upon a specific PG recycling pathway used by this organism,

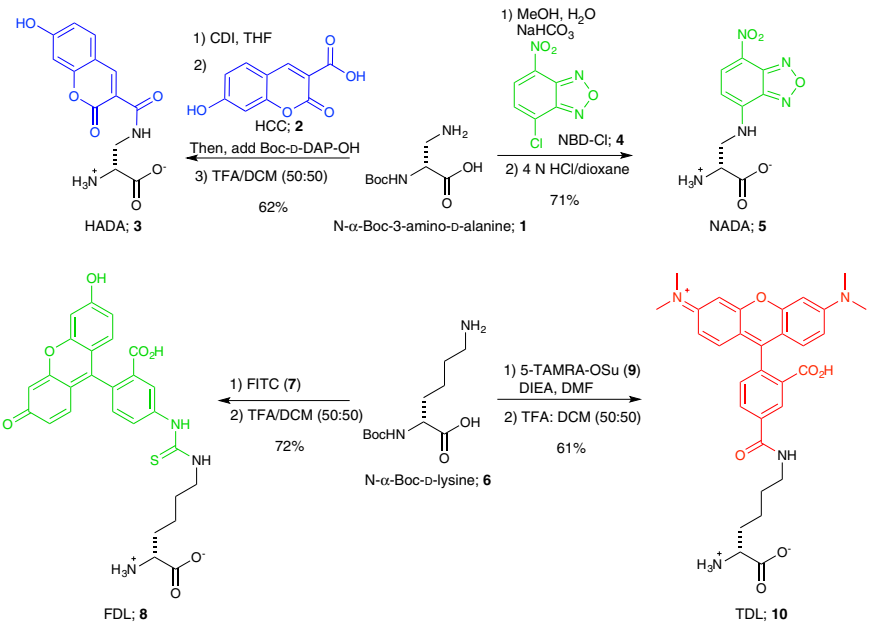

Figure 1 | Modular syntheses of FDAAs. HADA **3** and NADA **5** attaching commercially available fluorophores, 7-hydroxycoumarin-3-carboxylic acid (HCC-OH; **2**) and 4-chloro-7-nitrobenzofurazan (NBD-Cl; **4**) to the backbone N-Boc-D-2,3-diaminopropionic acid (i.e., N-α-Boc-3-amino-D-alanine; **1**); and FDL **8** and TDL **10** attaching commercially available FITC (**7**) and 5 (and 6-)-carboxytetramethylrhodamine succinimidyl ester (TAMRA-OSu; **9**) to the backbone N-α-Boc-D-lysine **6**, respectively. In our experience, HADA is the FDAA probe of choice when factors regarding ease of incorporation into the PG of diverse bacterial species, brightness, photostability and price of synthesis are considered. For example, despite its inexpensive synthesis, NADA suffers from low photostability. In contrast, although FDL and especially TDL are brighter and more photostable than HADA, the metabolic incorporation of these larger FDAAs into cell walls is particularly limited in Gram-negative bacteria. DMF, dimethylformamide; THF, tetrahydrofuran.

its application is limited to *E. coli*, and it also suffers from poor substrate analog utilization. More recently, we have shown that D-alanyl-D-alanine dipeptide analogs with small bio-orthogonal handles can efficiently and stably label PGs of a diverse set of bacteria through a similar cytoplasmic incorporation route[18]. Unfortunately, the detection of the incorporated material requires fixed and permeabilized samples, and therefore this method cannot be used to trace the growth of live bacteria.

Other efforts have used covalent incorporation of D-cysteine into PGs, either through chemoenzymatic incorporation into the Park nucleotide[19] or through live cell incorporation of D-cysteine into the stem peptide via a periplasmic exchange mechanism[12,13,17–20]. In this approach, once incorporated into the PG, the nucleophilic thiol group of D-cysteine can be used to capture an electrophilic reporter group (e.g., pyrene or biotin) and enable direct or indirect (via antibody capture) fluorescence detection. Although this method has been successfully used to label the bacterial cell wall and to monitor PG synthesis in diverse Gram-negative bacteria[13,21], it requires the laborious purification of PG sacculi away from cellular proteins, which contain reactive thiol groups. This requirement not only limits the signal detection to hollow sacculi but also precludes the application of this method for real-time detection of PG synthesis. Despite its limitations, D-cysteine labeling of PGs suggested that a similar approach using FDAAs might be generally applicable to bacteria, as evolutionary distinct species are known to produce and incorporate various DAAs into their PGs[22,23].

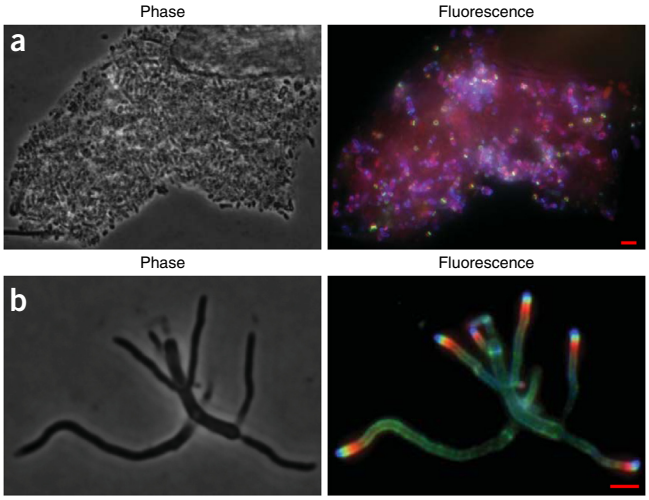

Figure 2 | Virtual time-lapse microscopy with FDAAs. (**a**) A saliva sample was pulse-labeled successively with TDL (red), FDL (green) and HADA (blue) for 15 min each. The labeling patterns on each cell provide chronological account of the areas of PG synthesis during each pulse labeling, with the red and green signals representing the oldest and the newest parts of the cell wall relative to the duration of the experiment, namely 3 × 15 min. (**b**) Virtual time-lapse microscopy *on S. venezuelae* pulse-labeled with TDL (red), FDL (green) and HADA (blue) for 5 min each. Scale bars, 2 μm.

Development of FDAAs

Our design strategy was built on the aforementioned tolerance of diverse bacterial species to incorporation of various DAAs, including the relatively small D-cysteine and the largest natural DAA, D-tryptophan, into their PGs[22–24]. These observations suggested that the mechanisms for DAA incorporation should be tolerant to modifications on the side chain of a DAA. Furthermore, DAAs have been shown to be incorporated into PGs by at least three different mechanisms, depending on the species: through the cytoplasmic steps of PG biosynthesis and via two distinct transpeptidation reactions taking place in the periplasm[24–27]. Notably, two of these possible routes, namely, the cytoplasmic route for PG biosynthesis and the periplasmic route catalyzed by the essential penicillin-binding proteins, are directly linked to PG biosynthesis and are shared by virtually all PG-synthesizing bacterial species[28–31]. Thus, we hypothesized that growing cells of a wide range of bacterial taxa exposed to fluorescent reporter groups attached to a DAA backbone would result in the incorporation of these florescent DAAs at sites of new PG synthesis. Indeed, a wide array of fluorophores sharing a common DAA carrier molecule proved to be readily and specifically incorporated into PGs at the sites of active growth in diverse bacterial species,

TABLE 1 | Basic properties of the FDAAs detailed in this protocol.

	MW	FW of HCl salt	FW of TFA salt	Excitation (nm)[a]	Emission (nm)[a]
NADA	267	304	381	~450	~555
HADA	292	359	406	~405	~460
FDL	536	572	650	~490	~540
TDL	560	596	674	~555	~595

[a]Values are for the respective fluorophores used for the synthesis of FDAAs[6]. MW (molecular weight) and FW (formula weight) are in Daltons.

regardless of the size of the fluorescent side chain (**Fig. 1**; ref. 6). Although this approach addressed the inherent limitations of the methods described above, the availability of dyes of different colors also opened the way for novel applications such as virtual time-lapse microscopy, in which the dynamics of cell growth is revealed by pulse-labeling cells with different colored dyes over time. We optimized this strategy for the polarly growing, Gram-positive species *Streptomyces venezuelae*[6] (**Fig. 2**).

An alternative to FDAAs is the use of nonfluorescent but commercially available, small and 'clickable' DAAs, which, similarly to the FDAAs, efficiently label PGs of diverse bacteria[6,26]. Once incorporated, the reactive functional groups embedded in the DAA core structure can be selectively captured in a second step, through click chemistry, including by any fluorescent dye containing the complementary functional group[32,33]. Although this two-step bio-orthogonal approach potentially introduces complications—such as dependence of the signal strength on the efficiency of the click chemistry reactions or nonspecific interaction of the probes with other cellular structures—this approach also substantially expands the utility of the DAA-mediated PG labeling method, as numerous clickable fluorophores with varied excitation and emission properties are available from commercial sources.

Labeling strategies and applications

FDAAs have been used to label sites of PG synthesis in a broad range of taxonomically diverse bacteria (**Table 1**). In addition, they have been used to address otherwise difficult-to-answer fundamental questions about bacterial growth and division[6,7,9,10,34–39]. For example, FDAAs enable visualization of distinct modes of growth by resolving sites of active growth from older cell wall[6]. Distinguishing between old and new PG can be done in two distinct ways: exposing cells to FDAAs either for long durations (1–2 generations (doublings), i.e., long labeling pulses, i in **Fig. 3**) or for shorter durations (2–5% of one generation time (doubling time), i.e., short labeling pulses (ii in **Fig. 3**); this range of times was found to be optimal for the species that we studied). Although not included in this protocol, we recommend that the user determine condition- and species-specific doubling times before the short pulses. The long pulses commonly result in cells that are uniformly labeled (**Fig. 4a**,**b**). When the excess dye is washed and the cells are allowed to grow in the absence of additional dye (i.e., a chase), the new wall synthesis commonly manifests itself as the spatiotemporal dilution of the signal. Notably, the nontoxicity of FDAAs allows this experiment to be conducted under the microscope in real time, generating single-cell time-lapse movies of cell wall growth (iii in **Fig. 3**; ref. 6). In contrast, the link of DAA incorporation to the PG biosynthetic enzymes enables FDAAs to resolve PG synthesis directly when they are added to actively growing cells for a brief labeling period. Indeed, we have observed a strong correlation between sites of FDAA labeling and previously inferred PG growth mechanisms of diverse bacterial species[6,40], indicating that FDAAs can be used to mark the regions of active growth. In addition, because of their DAA backbone, FDAAs can facilitate any study on the effects and mechanisms of DAA utilization, an area of active research with potential therapeutic applications[22,23,25,26,34]. They can also be used as substrate analogs to monitor the biochemical activity of essential PG synthetic enzymes (e.g., penicillin-binding proteins (PBPs)) in different species[24,27,41], which previously depended on the use of radioactive substrates (including radioactive DAAs) and/or suicide probes such as fluorescent β-lactams.

Owing to its modular design, the FDAA toolkit could easily be expanded to provide probes with enhanced fluorescence properties and/or emission maxima in longer wavelength regions of the visible spectrum (**Table 2**). Indeed, the synthesis protocol for the red FDAA, TDL, contains general guidelines for researchers to synthesize their own custom fluorescent or other DAA derivatives, with different user-defined features from an appropriately functionalized DAA (e.g., amino-D-alanine or D-lysine) and any suitably activated fluorescent dye.

Combined with the universality and the specificity of these probes for live bacterial cells, this modularity facilitates other applications. For example, FDAAs can be used to distinguish actively growing bacteria in a complex environmental sample (and measure bacterial viability/activity) and to assess the diversity of growth modes of natural bacterial populations in complex microbiomes, as almost all bacteria seem to share common mechanisms for incorporating FDAAs and as their incorporation does not require special treatment of the sample. Indeed, we have shown that FDAAs can be used to reveal growth

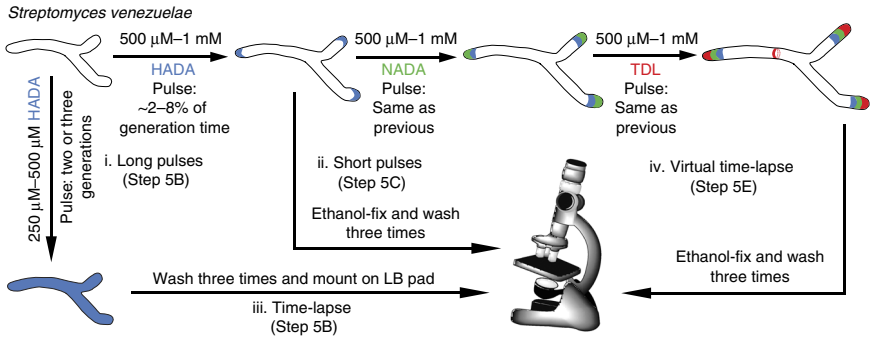

Figure 3 | Flowchart for different FDAA labeling strategies detailed in this protocol. The polarly growing Gram-positive bacterium *S. venezuelae* is used as an example.

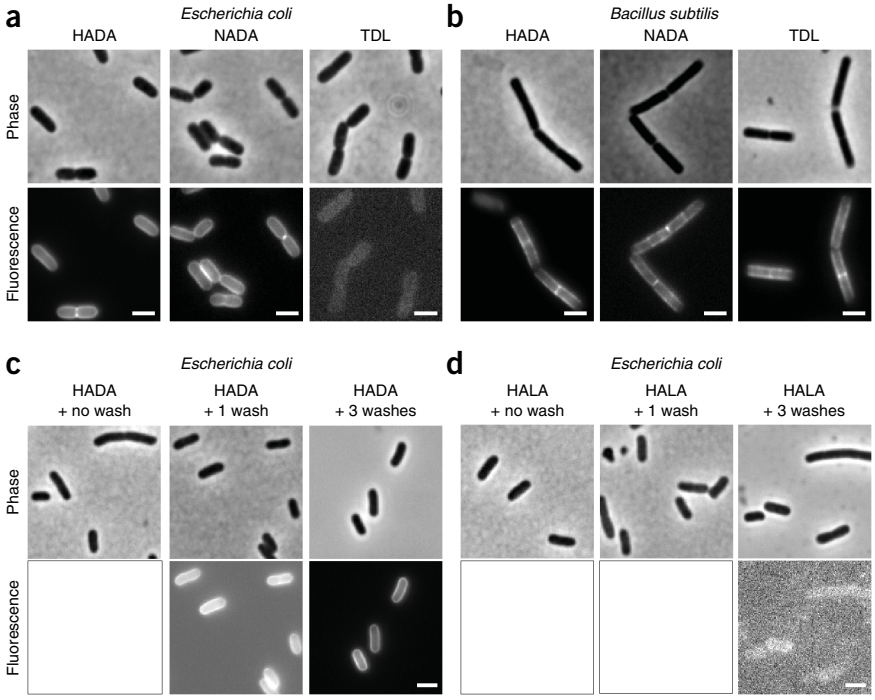

Figure 4 | The quality of the FDAA labeling depends on the choice of the dye and other experimental factors. (**a**,**b**) Although cells grown with HADA, NADA or TDL (500 µM, several generations for *E. coli* and 20 min for *B. subtilis*) show similar labeling patterns, the labeling quality, i.e., the SNR, can differ substantially. Under these experimental and imaging conditions, SNRs of HADA, NADA and TDL are 6.3, 1.9 and 1.07 (i.e., the signal is 7% above background) for *E. coli* and 2.69, 1.55 and 2.91 for *B. subtilis*. In *E. coli*, the low SNR of TDL is due to its poor outer-membrane permeability. The lack of labeling on the poles of *B. subtilis* is typical in briefly pulsed rod-shaped bacteria, and it represents the inertness of the polar cell walls. (**c**,**d**) SNR depends on other experimental factors such as effective washes. Without washes, the signal from HADA-labeled *E. coli* cells (500 µM, several generations) is obscured by the background fluorescence and therefore SNR is 1 (**c**, left, and **d**, left and middle) and the image is saturated and completely white. Each wash improves the SNR ($SNR_{HADA,wash\times1}$ =1.5; $SNR_{HADA,wash\times3}$ = 3.03). After the washes, the L-isomer (HALA)-treated cells ($SNR_{HALA,wash\times3}$ = 1.02) show ~100 times less normalized signal relative to the HADA-labeled cells. Display ranges within **c** or **d** were kept constant for visual comparison. Scale bars, 4 µm.

modes of bacteria in saliva and freshwater samples *in situ* (**Fig. 2a**; ref. 6). Therefore, FDAAs provide a valuable alternative to cell viability procedures that measure membrane integrity, such as Life Technologies' LIVE/DEAD cell viability assays, for probing bacterial activity. In addition, current FDAAs and/or near-IR derivatives may be used to diagnose bacterial activity on surfaces or in infections, and therefore may find a wide use in fields ranging from environmental microbiology and medical bacteriology to biomaterial engineering.

Access to multiple color fluorophores not only simplifies any experimental design involving other fluorescent tags (e.g., fluorescently tagged proteins), but their use in concert also enables virtual time-lapse microscopy (iv in **Fig. 3**). This application records the chronological history of PG synthesis on the cell wall itself in the form of varying colors, and it enables visualization of the location and extent of growth during the respective labeling periods on individual cells *in situ* (**Fig. 2**). The length of the stripes from each labeling can be used to calculate the rate of a specific growth mode (e.g., tip growth or septation) for the respective labeling periods, which can only be inferred from conventional time-lapse data by complex analysis. Of pharmaceutical interest, this approach can also easily be adapted to probe effects of differing conditions (e.g., antibiotics, temperature, nutrient deprivation) on growth during specific periods at a single-cell level, facilitating the direct characterization of the effects of different antibiotics on bacterial growth and PG synthesis, as was recently demonstrated[38]. In such experiments, sequential labeling can reveal the effects of different conditions on growth. Typically, the test condition should be applied after pulse-labeling with a first color FDAA to measure the unperturbed growth pattern. Subsequent quantification of the labeling with additional colors, either during or after the treatment, will show the effect of the test condition at different times. The modularity of this labeling method can also allow modification of bacterial surfaces by a variety of other immunologically active molecules. For example, an immune response to a bacterial infection can be elicited by the synthetic DAA molecules coupled to haptens. Indeed, the proof of principle has recently been demonstrated[34]. Finally, FDAAs may provide a blueprint for new DAA-based antibiotics, as accumulating evidence suggests that a DAA core is sufficient to direct a molecule into the active sites of essential and high-value targets, PBPs, *in vitro*[24,27] and *in vivo*[22] in diverse bacterial species.

Experimental design and crucial parameters

The synthetic routes for FDAA synthesis (**Fig. 1**) are simple and the labeling protocol is straightforward. HADA (**3**) and NADA (**5**), which are currently the most robust DAA derivatives, can be prepared in good yields from N-α-Boc–protected 3-amino-D-alanine (**1**), via nucleophilic capture of an activated ester of 7-hydroxycoumarin-3-carboxylic acid (HCC-OH; **2**) or by nucleophilic aromatic substitution of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl; **4**), respectively. The D-lysine–based probes, FDL (**8**) and TDL (**10**), are readily prepared via acylation of the ε-amino group of N-α-Boc–protected D-lysine with FITC (**7**) and 5-carboxytetramethylrhodamine succinimide ester (5-TAMRA-OSu; **9**), respectively. Although we did not observe substantial labeling with enantiomeric fluorescent L-amino acid negative controls[6], if necessary, these molecules can be synthesized by following the same protocols for the FDAAs using either N-α-Boc–protected 3-amino-L-alanine or N-α-Boc–protected L-lysine as the amino acid backbones. Freeze-dried FDAAs should be stored at −20 °C or below.

The probes are highly soluble in DMSO (up to 1 M). Thus, stock solutions should be made with DMSO, and these solutions should

TABLE 2 | Applications and conditions of FDAA labeling in different bacteria reported to date.

Application	Bacteria	FDAAs	FDAA concentration (mM)	Labeling duration	Temp (°C)	Growth medium	Ref.
Introduces the concept that custom hapten-containing D-amino acids may be used to elicit an immune response	*B. subtilis*	NDL	0.25	4 h	37	LB	34
Detection of PG localization in a mutant that loses its periplasmic integrity	*E. coli*	HADA	0.5	2.5 h	37	MOPS	35
Role of various members of PG assembly complex on cell morphology	*S. pneumoniae*	BADA	0.5	4 min	37	THY	44
Validation of a new cell-growth marker	*E. coli*	HADA	1	1 h	37	LB	36
Labeling active sites of PG synthesis in three Gram-negative α-proteobacteria	*C. crescentus*	HADA	0.25	5 min	30	PYE	37
	Asticcacaulis excentricus				26		
	A. biprosthecum				26		
Revealing the presence of a functional sacculus in environmental Chlamydiae	*Acanthamoeba castellanii* infected with *Protochlamydia amoebophila* or *Simkania negevensis*	HADA BADA	1.5	6 h	20	TSY	7
Characterization of divin, a new and specific inhibitor of bacterial cell division	*C. crescentus*	HADA	0.5	10 min	30	PYE	38
Testing the role of concerted cell elongation and division factors for proper cell division	*E. coli*	HADA	1.1	30 s	30	M9	39
Quantification of the presence of PG in wall-less spheroplasts	*E. coli*	HADA	0.5	1 h	37	LB	9
Probing PG synthesis during spore formation	*B. subtilis*	FDL	0.5	5 min	37	SM	10
Introduction of FDAAs as a tool to label sites of PG synthesis in diverse bacteria. Condition for short pulses with HADA is shown. As a starting point, half the shown FDAA concentration can be used for pulses longer than one generation	*B. subtilis*	HADA (NADA, FDL, TDL)	1	30 s	37	LB	6
	Brachybacterium conglomeratum		1	8 min	30	LB	6
	Lactococcus lactis		1	2 min	37	LB	6
	S. aureus		1	2 min	37	LB	6

(continued)

TABLE 2 | Applications and conditions of FDAA labeling in different bacteria reported to date (continued).

Application	Bacteria	FDAAs	FDAA concentration (mM)	Labeling duration	Temp (°C)	Growth medium	Ref.
	S. pneumoniae		0.5	4 min	37	BHI	6
	Streptomyces venezuelae		0.5	2 min	30	LB	6
	Agrobacterium tumefaciens		1	2 min	26	LB	6
	Burkholderia phytofirmans		0.5	20 min	30	LB	6
	C. crescentus		0.5	5 min	30	PYE	6
	E. coli		1	30 s	37	LB	6
	Synechocystis sp. PCC 6803		1	1 h	26	BG11	6
	Verrucomicrobium spinosum		0.25	10 min	30	VM	6

BHI, brain-heart infusion broth; BG11, BlueGreen medium; LB, Luria broth; MOPS, morpholinepropanesulfonic acid minimal medium; ; M9, M9 salts minimal media; PYE, peptone yeast extract; SM, sporulation medium; THY, Todd-Hewitt yeast broth; TSY, tryptic soy medium; VM, verrucomicrobia medium. BADA (first introduced in Pilhofer[7]) is a brightly green fluorescing Gram-negative accessible FDAA that can be synthesized by coupling Bodipy-Fl-NHS ester to amino-D-alanine according to the principles of Step 1D.

be stored in a freezer at −20 °C or below. When they are in solution, we have observed slow decomposition of NADA and FDL[6]. Therefore, lengthy storage times of the FDAA stock solutions, in particular at room temperature (24–26 °C), should be avoided. Details regarding storage, quality control and determination of photostability have been included in the Reagent Setup.

For a controlled and reproducible labeling experiment, a pure and exponentially growing bacterial culture should be used, preferably started from a single colony or from a −80 °C freezer stock. In addition, the introduction of any contaminants should be avoided by using sterilized growth media and by performing experiments using sterile techniques and procedures. Although we did not observe a clear correlation between the extent of probe incorporation and the richness of the growth medium, we recommend the use of a medium (and other conditions such as temperature and aeration) that supports the optimal growth for the given species. We recommend performing labeling experiments using exponentially growing cultures. Although the duration of a labeling experiment will depend on the growth rate of the bacterium and the type of the experiment (compare the labeling durations in **Table 1**), the total time required to complete a labeling experiment from an exponentially growing culture is as little as 30 min. For example, sample processing involves ethanol fixation (10–12 min), followed by washing (three washes; 3–5 min each) and mounting of the sample(s) for imaging (~10 min). We have found that as many as four distinct labeling conditions can be sampled simultaneously without substantially extending the sample processing time. Finally, we recommend allowing at least 10 min of imaging time per sample for qualitative analyses (e.g, imaging multiple fields, 20–40 cells). If quantitative comparisons between different growth conditions are required, we recommend analyzing images of no fewer than 100 cells and performing experiments in triplicate.

Although FDAA labeling is applicable to a wide range of taxonomically diverse bacteria[6], for initial experiments involving an untested strain (or for testing the quality of the synthesized FDAAs, see below), the user should label a strain that is known to incorporate FDAA well as a positive control. More specifically, we recommend *Bacillus subtilis* PY79 ATCC 55567 and *E. coli* MG1655 ATCC 700926 as Gram-positive and Gram-negative control strains, respectively (**Fig. 4a,b**). We have found that the signal-to-noise ratio (SNR) of FDAA labeling is different for different bacterial species, with Gram-positive bacteria usually providing better SNRs (E.K., unpublished results). This observation could be attributable to the outer-membrane permeability barrier present in Gram-negative bacteria, to the fact that Gram-positive bacteria have a thicker PG layer, or to species-specific efficiency of FDAA incorporation or turnover.

In a given strain, although the labeling patterns are similar, if not identical (**Fig. 4a**,**b**), and the labeling is D-isomer-specific (**Fig. 4c**,**d**), the labeling efficiency and the SNR show a strong dependence on the nature of the probe, probe concentration, duration of incubation and medium (see **Box 1**). Although HADA is dimmer and less photostable than FDL or TDL, it most reproducibly and robustly labels the PG of most bacterial species, typically without the need for extensive optimization. Therefore, we recommend the use of HADA, especially for endpoint applications. Imaging of the HADA-labeled cells should be done in neutral or slightly basic buffers (e.g., 1× PBS, pH 7.4), as the deprotonation of the core fluorophore (7-hydroxycoumarin) at ~pH 7 is essential for its fluorescence. For time-course experiments, TDL is recommended over HADA, as TDL is the most photostable FDAA and its higher excitation wavelength is expected to cause the least photodamage to the cells; however, it should also be noted that TDL does not usually label Gram-negative bacteria owing to poor outer-membrane permeability (**Fig. 4a**).

FDAAs are nontoxic to cells at the typical concentrations used for labeling. Indeed, growth curves of *E. coli*, *B. subtilis* and *A. tumefaciens* indicated that 500 μM of HADA had no effect on cell shape, lag phase, growth rate and growth yield[6]. In general, increasing the probe concentration resulted in a noticeable increase in SNR while remaining neutral to bacterial growth and morphology, at least until probe concentrations reached ~500 μM–1 mM (ref. 6). Thus, the user should

Box 1 | Evaluation of labeling

In practice, the quality of the FDAA labeling can be defined as the higher SNR of fluorescence on imaged cells compared with the untreated or with the fluorescent L-amino acid–treated controls (**Fig. 4c**,**d**). A higher SNR not only indicates that the labeling is specific, but it also means that the contrast (or quality) of the images is higher (**Fig. 4c**). Although SNR depends on a variety of instrumental factors such as optics, camera sensitivity, filters, strength of excitation light and so on, the user can increase the specific SNR on cells by following any of the following suggestions (detailed in the introduction): (i) use the brightest FDAA possible; (ii) minimize background fluorescence by thoroughly washing away any excess and unbound FDAA (**Fig. 4c**,**d**); (iii) use the excitation and emission filter sets that are matched to the FDAA used; and (iv) increase the strength of the excitation light or the exposure time. Because of numerous difficult-to-control experimental parameters, relating fluorescence intensity to the actual number of FDAAs on a cell is challenging. However, one can still make reliable, quantitative comparisons of labeling efficiencies between different conditions or bacterial species as long as the used FDAA and all the other sample handling and imaging conditions are identical.

titrate the FDAA concentration until a desired SNR is achieved, while also watching for adverse effects on growth rate or morphology. We note that cells tolerate higher FDAA concentrations for short labeling times—presumably because of the shorter perturbation to the system—which can be exploited to increase the SNR on PGs labeled by relatively dimmer FDAAs such as HADA. However, longer exposure times to FDAA concentrations that are substantially higher than the optimal FDAA concentrations should be avoided, as reduced growth rates and cell shape changes might occur. In addition, the overall SNR increases as a function of incubation time, indicating that FDAA incorporation is strongly correlated with growth, but it also appears to plateau after one or two generations (1–2% of all muropeptides are labeled at the optimal probe concentration used for long pulses)[6]. Accordingly, heat-killed cells[6] or dormant cells (E.K., unpublished results) did not incorporate FDAAs. Finally, for applications that require sparse labeling, e.g., single-molecule tracking, concentrations far lower than 500 µM, e.g., ~0.1 µM for *E. coli*, are sufficient to decorate the cell wall with well-separated single FDAA molecules (E.K., unpublished results).

Limitations

In contrast to fluorescently modified vancomycin derivatives, which bind noncovalently to newly synthesized PGs with high affinity, our data suggest that FDAA probes are covalently incorporated into PGs via periplasmic exchange mechanisms involving bacterial transpeptidases. Procedurally, this means that the user needs to expose cells to relatively high concentrations of the probe (250 µM–1 mM). Thus, before imaging, the overwhelming signal from the excess free dye needs to be removed by washing (**Fig. 4c**,**d**). Depending on the time required for washing, cells may continue to grow, thereby diluting the FDAA signal, especially if a growth medium is used for washes. Therefore, the user should either perform washes in buffer (e.g., cold 1× PBS) or use an appropriate fixative (such as ethanol or paraformaldehyde) before washing. Of course, washing with nontoxic buffers is essential for time-course experiments.

The close correlation between known growth modes of different bacterial species with the labeling patterns of cells that are briefly pulsed with FDAAs led to the conclusion that short pulses with FDAAs mark sites of new PG synthesis[6]. However, PG turnover might diminish signal intensity at sites of PG synthesis, especially with experiments conducted for longer labeling periods. A body of accumulating *in vivo* and *in vitro* evidence indicates that noncanonical DAAs and FDAAs are incorporated predominantly by periplasmic L,D- and/or D,D-transpeptidases in certain species (e.g., *E. coli*, *Vibrio cholerae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *B. subtilis*)[6,24,25,27](and E.K., unpublished data). D-Cycloserine inhibition experiments have suggested that the cytoplasmic pathway might have a role in the incorporation of some small noncanonical DAAs (including the synthetic ones with bio-orthogonal handles, e.g., alkynes or azides) in other species such as *Caulobacter crescentus* and *Listeria monocytogenes*[25,26,42,43]. However, as the cytoplasmic route requires tolerance of 5–7 additional enzymatic steps in addition to the uptake of the DAAs through the inner membrane, the highly tolerable periplasmic transpeptidations for the incorporation of abnormally large and charged FDAAs appear to be a more likely route. Nonetheless, the FDAA incorporation mechanism(s) will need to be established for rigorous interpretation of the labeling data in a given species. For example, in a species that incorporates FDAAs solely through periplasmic PBPs, the short pulse data will be more indicative of the spatiotemporal distribution of the active PBPs than of the nascent PG introduction into the wall by transglycosylation of the lipid II PG precursors.

The vast majority (>90%) of the bacterial strains we have studied thus far were labeled with FDAAs on the first attempt. A few strains required optimization for significant labeling (e.g., *Myxococcus xanthus*) or could not be labeled (e.g., *Planctomyces limnophilus*). When a strain cannot be labeled, the user needs to consider several possibilities: (i) the strain might turn over FDAA-modified muropeptides effectively (e.g., high D,D-carboxypeptidase and D,D-transpeptidase activity has the potential to cleave the FDAAs of the modified muropeptides); (ii) the strain might lack a mechanism for FDAA uptake and/or incorporation; or (iii) the strain might lack a conventional DAA-rich cell wall. Although the first two possibilities could be addressed by the use of alternative metabolic labeling strategies such as fluorescent tripeptides[14] or D-Ala-D-Ala dipeptide analogs[18], the lack of a conventional PG biosynthetic pathway in a given species might evade any of the currently available PG detection methods.

DAAs are known to modify and strengthen the PG layer[23,25]. Although it is clear that FDAAs modify PG, it is still not known whether their incorporation under experimental concentrations affects PG properties. Nevertheless, the user is advised to determine and use the lowest probe concentration that will result in an acceptable SNR. Finally, it should be noted that the unique molecular structure of each probe presents the possibility for probe-dependent interactions with macromolecules within the

cell that may result in differences in their labeling efficiencies or in labeling patterns. For example, in some bacteria, labeling with NADA required a probe concentration that was four times higher than that required for the HADA probe in order to achieve a similar SNR (E.K., unpublished results). In addition, the size of the probe can affect its efficient uptake and incorporation. Indeed, although the patterns of labeling with HADA, NADA and TDL were similar within *E. coli* or *B. subtilis* (**Fig. 4a**,**b**), TDL does not strongly label *E. coli*[6] (**Fig. 4a**) unless outer-membrane permeability is increased (E.K., unpublished data).

MATERIALS

REAGENTS

! CAUTION Use appropriate safety measures when you are performing the procedure (fume hood, lab coat, safety goggles and safety gloves).

- Anhydrous dimethylformamide (EMD Chemicals, cat. no. DX1726)
- Anhydrous sodium sulfate (Macron Chemicals, cat. no. SX0760E)
- Argon
- Aqueous hydrochloric acid, 1 M (HCl; Macron Chemicals, cat. no. 7-10108)
  ! CAUTION It is corrosive and an irritant; avoid contact with skin and eyes.
- Carbonyldiimidazole (CDI; Sigma-Aldrich, cat. no. 115533)
- 5 (and 6-)-Carboxytetramethylrhodamine succinimidyl ester (TAMRA-OSu; Anaspec, cat. no. 81124)
- 4-Chloro-7-nitrobenzofurazan (NBD-Cl; Sigma-Aldrich, cat. no. 25455)
- Dichloromethane (DCM; Macron Chemicals, cat. no. 1-19590)
- Diisopropylethylamine (DIEA; Sigma-Aldrich, cat. no. 387649)
- Ethyl acetate (Macron Chemicals, cat. no. 1-19950)
- FITC (Anaspec, cat. no. 20151)
- HPLC-grade acetonitrile (Sigma-Aldrich, cat. no. 34851)
- HPLC-grade water (EMD Chemicals, cat. no. WX0008)
- Hydrochloric acid solution in dioxane, 4 M (Sigma-Aldrich, cat. no. 345547)
  ! CAUTION It is corrosive and an irritant; avoid contact with skin and eyes.
- 7-Hydroxycoumarin-3-carboxylic acid (HCC; Anaspec, cat. no. 81205)
- Methanol (Macron Chemicals, cat. no. 1-12108)
- (Nα-tert-butoxycarbonyl)-D-2,3-diaminopropionic acid (Boc-D-Dap-OH; Chem-Impex, cat. no. 06293)
- (N-α-tert-butoxycarbonyl)-D-Lysine (Boc-D-Lys-OH; Chem-Impex, cat. no. 05507)
- Saturated aqueous sodium chloride (NaCl) solution (brine)
- Sodium bicarbonate (Macron Chemicals, cat. no. 1-30850)
- Trifluoroacetic acid (TFA; J.T. Baker, cat. no. W729) **! CAUTION** TFA is highly corrosive; avoid contact with skin and eyes.
- High-quality anhydrous DMSO (Sigma-Aldrich, cat. no. 276855)
  ! CAUTION DMSO is inflammable, an irritant and a permeator. Avoid contact with skin and eyes.
- Ethanol, 200 proof (100% (vol/vol); Fisher Scientific, cat. no. 04-355-222)
  ! CAUTION Ethanol 200 proof is flammable.
- PBS (Sigma-Aldrich, cat. no. P4417)
- Growth medium appropriate for the growth of the bacteria under investigation such as Luria broth (LB) medium sterilized by autoclaving (Sigma-Aldrich, cat. no. L3397)
- Solid growth medium appropriate for the growth of the bacteria under investigation, such as pre-poured LB agar plates (Sigma-Aldrich, cat. no. L5542)
- Agarose (SeaKem LE agarose, Lonza, cat. no. 50000)
- Vaseline (also called petrolatum)
- Lanolin
- Paraffin wax

EQUIPMENT

- Aluminum foil
- Argon tank
- Balloons
- C_{18} column for HPLC
- Cotton
- Erlenmeyer flasks
- Exhaust flow hood
- ^{1}H-NMR and ^{13}C-NMR spectrometers
- HPLC apparatus with preparatory reversed-phase C_{18} column
- Luna C_{18} reverse-phase HPLC column (10-µm pore size, 30-mm internal diameter and a length of 250 cm)
- Luna C_{18} reverse-phase HPLC column (5-µm pore size, 21.2-mm internal diameter and a length of 250 cm)
- Magnetic stir bars
- Microsyringe, 10 µl
- Lyophilization flasks
- Lyophilizer
- Propane torch
- Reflux condenser
- Rotary evaporator
- Round-bottomed flasks
- Rubber septa
- Separatory funnels
- Syringe needles (BD PrecisionGlide, 21-gauge × ¼ inch)
- High-vacuum pump
- Eppendorf polypropylene microcentrifuge tubes, 1.5 ml (Fisher Scientific, cat. no. 05-402-24B), sterilized by autoclaving
- Tubes appropriate for the growth of the bacteria under investigation such as glass culture tubes (rimless 18 mm × 150 mm, Fisher Scientific, cat. no. 11587403) and culture tube closures (20 mm, Fisher Scientific, cat. no. 05-888-1D), sterilized by autoclaving
- Incubator, appropriate for the optimum growth of the desired bacterial species
- Temperature-controlled shaker suitable for bacterial growth in microcentrifuge tubes, such as a Thermomixer (Fisher Scientific, cat no. 05-400-200)
- Vortex mixer
- Microcentrifuge
- Nylon membrane, 25 mm, 0.2-µm-pore DMSO-safe Acrodisc syringe filters (Pall, part no. 4433)
- Cellulose acetate filters, 0.2-µm pore
- Sterile Luer lock syringes, 3 ml (BD, product no. 309657)
- A fluorescence microscope equipped with appropriate excitation/emission filters that cover the excitation/emission maxima of FDAAs (**Table 2**). For example, a Nikon 90i fluorescence microscope equipped with a Plan Apo 100×/1.40 Oil Ph3 DM objective and a Chroma 83700 triple filter cube
- Glass slides (25 × 75 mm, 1.0 mm thick; VWR, cat. no. 48300-025)
- Glass coverslips (22 × 22 mm, no. 1.5; VWR, cat. no. 48366-227)
- Spectrophotometer suitable for measuring at 600 nm or McFarland turbidity standards (set of 1–10, Northeast Laboratory Services, cat. no. M1100)
- Sterile cryogenic vials for bacterial freezer stocks (2 ml, polypropylene; VWR, cat. no. 89094-806)
- Sterile inoculating loops (1 µl, VWR, cat. no. 12000-808)
- Bunsen burner
- Micropipettors P2 (Fisher Scientific, cat. no. F144801G Gilson), P20 (cat. no. F123600G), P200 (cat. no. F123601G) and P1000 (cat. no. F123602G)
- Sterile pipette tips for P2 and P20 (Fisher Scientific, cat. no. F171100G Gilson), for P200 (cat. no. F171300G) and for P1000 (cat. no. F171500G).
- Propipettors; Fisher Scientific, cat. no. 03-391-250 Argos Technologies Omega single-channel pipet controller
- Sterile serological pipettes, 10 ml (Fisher Scientific, cat. no. 13-678-12E Fisherbrand) and 50 ml (cat. no. 13-678-14C Fisherbrand)

REAGENT SETUP

Wash buffer For washes, 1× PBS can be used. 1× PBS (pH 7.4) can either be made by dissolving commercially available tablets in water or from scratch. To make it from scratch, dissolve 8 g of NaCl, 0.2 g of KCl, 1.78 g of $Na_2HPO_4{\cdot}2H_2O$ and 0.27 g of KH_2PO_4 in 1 liter of distilled water, and filter-sterilize the mixture using 0.2-µm-pore cellulose-acetate filters. The buffer is stable at room temperature for at least 1 year. Acidic, basic or strong redox buffers should be avoided, as they can modify dye molecules irreversibly.

Sterile technique For consistent labeling results, the bacterial culture under investigation should be kept free of contaminants. Toward this end, 'sterile technique' must be used throughout the labeling experiments. Before starting an experiment, the relevant surfaces (the laboratory bench, the pipettes and so on) should be wiped with 70% (vol/vol) ethanol. Furthermore, transfer of cultures or buffers should be done close to a lit Bunsen burner, which provides an updraft of heated air that helps prevent contamination. Finally, presterilized disposables, such as inoculating loops, tips, pipettes, plates and tubes must be used throughout, and their exposure to open air must be limited when not in immediate use. Once the labeling experiment is completed, the fixation, washing steps and imaging can be performed under nonsterile conditions.

Preparation of sterile liquid medium For reproducible results, the labeling experiments must be done with pure cultures. This requires the use of a sterile growth medium. As a common nutrient-rich liquid medium, LB medium supports the growth of a variety of different species. To prepare 1 liter of sterile LB medium, add 15.5 g of LB medium powder into 1 liter of distilled water and stir using a magnetic stirrer with warming until the powder dissolves. Autoclave the medium for 30 min at 121 °C to sterilize it. For other species that may require more specialized growth media, follow the recipes available at http://www.atcc.org/.

Inoculation and growing bacteria in liquid and on solid medium Cells may be cultured in any growth-supporting medium. The only requirement for labeling is active cell growth at the time of FDAA addition. A liquid culture can be started by transferring some cells from freezer stocks (by scraping the frozen surface with a sterile loop or pipette) or from a single colony formed on a nutrient agar plate into the liquid medium. This can be done by scratching a freezer stock or by touching a colony with the tip of a sterile inoculating loop. Grow the bacteria using the optimal conditions, and measure the increase in optical density (OD) (see 'Tracking bacterial growth' below). For example, a well-aerated *E. coli* culture in LB medium can show growth in the form of a visible cloudiness of the liquid medium within 3–5 h if it is incubated at 37 °C.

Many types of bacteria can form colonies on solid nutrient agar plates, which can be used as a short-term (1–2 weeks) strain storage method. Premade LB agar plates are commercially available. Single colonies can be obtained by the streaking technique after some cells are transferred as a small spot on the plate using a sterile inoculation loop. The large concentration of cells on this spot can be diluted by dragging a new sterile loop from this spot and by zig-zagging until ~25% of the plate's surface is covered. To get well-isolated, single colony-forming units, this should be repeated three more times, each time with new sterile loops by dragging from the previous section and covering the next untouched ~25% of the plate's surface. Once the streaking is done, the plates should be incubated lids down under optimal growth conditions until visible colonies form. For example, *E. coli* can form visible colonies on LB agar within 10–12 h if it is incubated at 37 °C. For other species that may require more specialized growth media and conditions, follow the instructions available at http://www.atcc.org/.

Tracking bacterial growth The labeling experiments should be performed with exponentially growing bacteria to get optimal labeling results. The exponential phase (or the log phase) follows the lag phase and precedes the stationary phase, and it is defined as the period in which the net increase in the number of bacterial cells per unit time is proportional to the current number of cells. Generally, the OD of liquid bacterial cultures is proportional to the concentration of bacterial cells. Thus, by following the increase of the OD over time, one can determine whether a culture is in the exponential phase. The OD of a culture can be quantified by measuring OD at 600 nm (OD_{600}) using a spectrophotometer, and by following the manufacturer's instructions. Alternatively, one can estimate OD of a culture by comparing it to commercially available McFarland standards (roughly, the McFarland standards numbers 1, 2, 3 and 4 correspond to OD_{600} values of 0.25, 0.4, 0.6 and 0.7). More specialized methods may be required for other species, such as obligate intracellular bacteria.

Making a freezer stock of bacteria In a sterile, cryogenic, 2-ml vial, combine 900 µl of pure liquid cultures from late exponential phase with 100 µl of sterile DMSO or with 100 µl of sterile glycerol, and mix well. Without delay, transfer the tube to a freezer at −80 °C. To start a new culture, working aseptically, transfer a small volume (~1 µl) of the frozen stock into 2–3 ml of sterile liquid medium.

Stock solution of FDAAs The FDAA salts prepared as described in Step 1 of the PROCEDURE are poorly soluble in water. For example, at room temperature, the water solubility (S_W) of HADA is ~3 mM. Thus, stock solutions should be made in DMSO (referred to as $FDAA_{DMSO}$ throughout). Use the following formula to determine the right volume of DMSO in which to dissolve your preweighed FDAA.

$$V = \frac{W \times 100{,}000}{M \times C}$$

where V = volume of DMSO to be added in µl; W = mass of the FDAA aliquot in mg; M = molecular mass of the FDAA salt (**Table 2**) in g mol^{-1}; C = desired concentration of the stock solution in mM.

The concentration of the stock solution should be determined on the basis of the desired final DMSO concentration in the culture (usually between 0.1–1%). For example, for final concentrations of 1 mM FDAA and 1% (vol/vol) DMSO, prepare a 100 mM (100×) FDAA stock solution. All of the FDAAs are soluble in DMSO at least up to 1 M concentrations. Sterilization of stock solutions is not required, but it could be necessary for applications that require extended labeling times (>1 d). When required, the stock solutions should be filter-sterilized using 0.2-µm-pore DMSO-resilient nylon filters.

Chemical stability of FDAA stock solutions Powdered FDAAs (in dry compartments) and stock solutions should be stored at −20 °C or lower. The stock solutions should be thawed rapidly by bringing the frozen stock to room temperature. In general, excessive thawing and refreezing, or extended exposure and storage at room temperature, should be avoided. Therefore, it is recommended to divide a fresh stock solution into smaller multiple-use aliquots. We have not observed decomposition of powder FDAAs over several months when they are kept dry and at −20 °C or lower. Similarly, in our hands the potency of stock solutions remained unchanged when they were frozen and thawed approximately ten times; however, we detected marked decomposition of the material when stock solutions were kept at room temperature for 30 d in the dark (E.K., unpublished results).

Quality control The effective concentration of an FDAA in a stock solution can be determined in three ways: first, the stock solution can be analyzed by HPLC and the HPLC profile of the current stock solution can be compared with that of the original stock solution. This will give both the effective FDAA concentration and the distribution of any decomposition products. Preparative HPLC can be used to re-purify FDAA recovered from an old stock solution. Second, a standard curve of concentration versus absorbance can be made from a fresh stock solution and then be used to estimate the effective FDAA concentration of an unknown stock solution. For example, HADA has a strong linear correlation of concentration and absorbance (at 400 nm) between 1–50 µM concentrations in 1× PBS (pH 7.4) and 1% (vol/vol) DMSO. Finally, one can compare the cell signal obtained with an old stock solution in parallel with a freshly prepared one, in a microscopy experiment on a model bacterium (e.g., *B. subtilis*). The old stock should be discarded or the FDAA should be re-purified, if any decrease of signal or changes in labeling patterns is noted.

Photostability Despite the photolability of the smaller FDAAs (NADA, and to a lesser extent, HADA) during fluorescence microscopy applications, ambient light in a typical laboratory does not noticeably affect the brightness of labeled samples. Still, avoiding prolonged exposure of the samples to incident light is recommended. For experimental purposes, the photostability of an FDAA can be estimated by repeatedly exposing a field of labeled cells with typical microscopy and image acquisition setups. Cells that are labeled with a photosensitive FDAA (e.g., NADA) will exhibit a reduction of fluorescence signal with each exposure. The rate of photobleaching can be determined by calculating the percentage of fluorescence decrease on a cell over repeated exposures. Among the four FDAAs reported here, TDL is the photophysically most impressive FDAA. It is bright, extremely photostable and its red-shifted excitation helps reduce photodamage to the cells during time-lapse microscopy.

Although FDL and NADA are seemingly brighter than TDL, they are also prone to extensive photobleaching. HADA is moderately bright, but it is much more photostable than NADA. If constant exposure of a single cell is required (e.g., time-lapse or structured illumination microscopy), then the use of TDL is recommended (followed by HADA, FDL and NADA). The choice of FDAA is more forgiving for endpoint experiments (e.g., short pulses or virtual time-lapse experiments).

Fixation To quench labeling reactions, we recommend the generic 70% (vol/vol) ethanol or paraformaldehyde fixation techniques. Given its convenience and its chemical inertness toward the PG cell wall, ethanol fixation can be followed as the starting point. One should note that ethanol fixation causes an apparent loss of the cell pellet once the fixed cells are rehydrated in an aqueous solution (e.g., 1× PBS). Furthermore, in some Gram-positive species such as *B. subtilis,* ethanol fixation results in partly phase-transparent (i.e., hollow) cells, which does not seem to affect the cell wall structure. In *S. pneumoniae*, ethanol fixation causes complete autolysis of the cells upon rehydration, which can partly be overcome by heat-killing the cells before the ethanol fixation. Glutaraldehyde fixation should be avoided, as it causes high background signal, presumably via nonspecific cross-linking of free FDAAs.

EQUIPMENT SETUP

HPLC FDAAs are purified by HPLC. The HPLC instrument should be set up according to the manufacturer's recommendations and specifications.

Mass spectrometry analysis Mass spectral data are recorded. The following instruments have been used in our laboratory: Waters LCT Classic electrospray time-of-flight analyzer with an Agilent capillary HPLC inlet; Sciex API III electrospray quadrupole with a direct infusion inlet; or Finnigan MAT-95. Sample ionization is achieved through the use of chemical ionization (CI) or electron impact (EI).

Vacuum pump aspirator Although excess FDAAs can be removed by pipetting the supernatant of pelleted cells (1 min, maximum speed in a microcentrifuge, room temperature), we recommend removing the supernatant using a vacuum pump aspirator, as the labeling protocols involve several washes. In our hands, aspirating supernatants of multiple samples with the same pipette tip significantly reduces the dead-time between washes and does not cause a noticeable cross-contamination.

Wide-field fluorescence microscope HADA can be imaged in the DAPI channel, NADA and FDL can be imaged in the FITC channel, and TDL can be imaged in the TAMRA channel on a conventional wide-field fluorescence microscope. See also **Table 2**.

PROCEDURE

▲ **CRITICAL** With FDAAs in hand, start at Step 2 of the PROCEDURE.

Synthesis of FDAAs

1| To prepare the FDAAs used in these studies (HADA, NADA, FDL and/or TDL), follow the procedures described in options A–D, respectively. Option D can also be used for the custom synthesis of novel FDAAs. One can follow these steps and simply change which commercially available succinimidyl ester activated fluorophore and/or which Boc-protected D-diamino acid linker is used in Step 1D(ii) as long as the molar ratios are kept constant. The HPLC profile in Step 1D(xi) will change depending on the product.

(A) Synthesis of HADA ● **TIMING ~51 h**

(i) Seal a 100-ml round-bottomed flask containing a magnetic stir bar with a rubber septum.

(ii) Pierce the septum with a syringe needle attached to a high vacuum line and place the flask under vacuum.

(iii) By using a propane torch, carefully heat the flask evenly across the rounded surface for only 1 min to dry it.
■ **PAUSE POINT** The round-bottomed flask may also be oven-dried.

(iv) Allow the flask to cool to room temperature while still under vacuum for 10–15 min.

(v) Fill a balloon with argon gas and affix a syringe with a two-way tap and a needle. Nitrogen gas may be used in place of argon.

(vi) Pierce the septum with the argon balloon needle and then remove the vacuum line.

(vii) Remove the septum and add 300 mg (1.455 mmol) of HCC and 236 mg of carbonyldiimidazole (1.455 mmol) to the flask, and then reseal the flask with the septum and argon balloon.
▲ **CRITICAL STEP** Carbonyldiimidazole is moisture-sensitive. Quickly weigh the solid and transfer it to the reaction flask. Store the reagent in a dry desiccator for future use.

(viii) Add anhydrous dimethylformamide (14.5 ml) via an oven-dried syringe, and stir it at room temperature for 2 h.

(ix) Remove the septum and add 297 mg of N-α-Boc-2,3-diaminopropionic acid (1.455 mmol) in one portion, reseal the flask with the septum and argon balloon, and stir it overnight (17 h).

(x) Remove the solvent on a rotary evaporator under vacuum and dilute the product with ethyl acetate (100 ml).

(xi) Transfer the solution to a 250-ml separatory funnel.

(xii) Wash with 1 M aqueous HCl (50 ml).

(xiii) Wash with distilled water (100 ml).

(xiv) Wash with saturated aqueous NaCl (50 ml).

(xv) Transfer the organic layer to a 200-ml Erlenmeyer flask, dry it over anhydrous sodium sulfate for 15 min and filter it into a 250-ml round-bottomed flask.

(xvi) Remove the solvent on a rotary evaporator under vacuum.

(xvii) To remove any traces of ethyl acetate, re-dissolve the product in dichloromethane (DCM) and dry it on a rotary evaporator three times.
■ **PAUSE POINT** The product can be stored in a freezer overnight at –20 °C.

(xviii) Without further purification, add a magnetic stir bar to the flask and treat the product with a 1:1 solution of TFA/DCM (10 ml).
(xix) Stir it at room temperature for 30 min, remove the solvent on a rotary evaporator and dry it under high vacuum for an additional 30 min.
(xx) Dissolve the product in 15 ml of acetonitrile/water (1:1).
(xxi) Purify HADA by reversed-phase HPLC with a gradient of acetonitrile in water with 0.1% (vol/vol) TFA (10–90% (vol/vol) acetonitrile for 10 min, eluting at 40 ml min^{-1}). The product elutes at 6.0 min on a 10-μm Luna C_{18} column with a 30-mm internal diameter and a length of 250 cm.
(xxii) Combine the product fractions in a 500-ml round-bottomed flask.
(xxiii) Concentrate the solution by rotary evaporation until it reaches approximately one-third of the initial volume.
! CAUTION Mixtures of acetonitrile/water will bump easily. Slowly increase the vacuum pressure (30–50 mm Hg) at 25 °C to avoid this problem.
(xxiv) Transfer the remaining solution to a 300-ml lyophilization flask to lyophilize the final product. The powder will be the TFA salt of HADA.
■ PAUSE POINT Store the product at –20 °C. In our hands, no decomposition was detected in at least 6 months.

(B) Synthesis of NADA ● TIMING ~45 h

(i) Preheat an oil or water bath to 55 °C, and add a magnetic stir bar to a 25-ml round-bottomed flask.
(ii) Add 100 mg of N-α-Boc-D-2,3-diaminopropionic acid (0.49 mmol).
(iii) Add 123 mg of sodium bicarbonate (1.47 mmol).
(iv) Add 1.8 ml of water and partially dissolve the mixture in the preheated 55 °C oil (or water) bath.
(v) In a test tube, prepare a solution of 108 mg of NBD-Cl (0.539 mmol) in 8.5 ml of methanol.
(vi) Add the methanolic solution dropwise over 10 min to the reaction flask.
(vii) Affix a reflux condenser with cold water running continuously to the reaction flask.
(viii) Stir the reaction at 55 °C for 1 h.
(ix) Remove the solvent on a rotary evaporator under vacuum.
(x) Acidify the product residue with 1 M aqueous HCl (pH 3–4).
(xi) Transfer the mixture to a 250-ml separatory funnel, and rinse the round-bottomed flask with DCM to completely recover the remaining product.
(xii) Extract with DCM (50 ml) three times.
(xiii) Combine the organic extracts and wash with saturated aqueous NaCl (50 ml).
(xiv) Transfer the organic layer to a 200-ml Erlenmeyer flask, dry it over anhydrous sodium sulfate for 15 min and filter it into a 250-ml round-bottomed flask.
(xv) Concentrate the solution by rotary evaporation until it reaches approximately one-third of the initial volume.
■ PAUSE POINT The product can be stored in a freezer overnight at –20 °C. Extended storage times at temperatures higher than –20 °C are not recommended.
(xvi) Without further purification, add a magnetic stir bar to the flask and treat the solid with 4 M HCl in dioxane (10 ml).
(xvii) Stir the reaction at room temperature for 30 min.
(xviii) Concentrate the solution by rotary evaporation until it reaches approximately one-third of the initial volume.
▲ CRITICAL STEP Purification must be performed as soon as possible. The reaction product is prone to slow decomposition at room temperature.
(xix) Immediately dissolve the product in a 1:1 solution of HPLC-grade acetonitrile/water (10 ml).
? TROUBLESHOOTING
(xx) Purify NADA by reversed-phase HPLC with a gradient of acetonitrile in water with 0.1% (vol/vol) TFA (20–90% (vol/vol) acetonitrile for 10 min, eluting at 40 ml min^{-1}). The product elutes at 5.0 min on a 10-μm Luna C_{18} column with a 30-mm internal diameter and a length of 250 cm.
(xxi) Combine the pure fractions in a 250-ml round-bottomed flask and remove the majority of the acetonitrile.
(xxii) Transfer the remaining solution to a lyophilization flask and lyophilize it overnight.
(xxiii) Re-dissolve the solid in a 1:3 mixture of acetonitrile/0.5 M aqueous HCl and again lyophilize overnight. The powder will be the HCl salt of NADA.
▲ CRITICAL STEP The TFA salt of the product is not stable. It must be converted to and stored as the HCl salt.
■ PAUSE POINT Store the product at –20 °C. In our hands, no decomposition was detected in at least 6 months.

(C) Synthesis of FDL ● TIMING ~34 h

(i) Flame-dry a 10-ml round-bottomed flask containing a magnetic stir bar and fill it with argon, as described in Step 1A(i–vi).
(ii) Remove the septum and add N-α-Boc-D-Lys-OH (19.3 mg, 0.078 mmol), add FITC (25 mg, 0.065 mmol), and then reseal the flask with the septum and argon balloon.

(iii) Add anhydrous dimethylformamide via an oven-dried syringe and stir the reaction at room temperature for 4 h.
(iv) Remove the solvent on a rotary evaporator.
(v) Dissolve the residue in ethyl acetate (10 ml).
(vi) Transfer the solution to a 50-ml separatory funnel.
(vii) Wash with 1 M aqueous HCl (10 ml).
(viii) Wash with saturated aqueous NaCl (10 ml).
(ix) Collect the organic layer in a 50-ml Erlenmeyer flask.
(x) Dry the organic layer over anhydrous sodium sulfate for 15 min.
(xi) Filter the solution into a 100-ml round-bottomed flask and remove the solvent on a rotary evaporator.
■ **PAUSE POINT** The product can be stored in a freezer overnight at –20 °C.
(xii) Without further purification, add a magnetic stir bar to the flask and treat the product with a 1:1 solution of TFA/DCM (10 ml).
(xiii) Stir the reaction at room temperature for 30 min.
(xiv) Remove the solvent on a rotary evaporator.
(xv) Dissolve the crude product in a 2:3 solution of HPLC-grade acetonitrile/water (10 ml).
(xvi) Purify FDL by reversed-phase HPLC with a gradient of acetonitrile in water with 0.1% (vol/vol) TFA (30–45% (vol/vol) acetonitrile for 10 min, eluting at 40 ml min^{-1}). The product elutes at 5.1 min on a 10-µm Luna C_{18} column with a 30-mm internal diameter and a length of 250 cm.
(xvii) Combine the product fractions in a 250-ml round-bottomed flask.
(xviii) Remove the majority of the acetonitrile on a rotary evaporator.
(xix) Transfer the remaining solution to a 300-ml lyophilization flask to lyophilize the final product. The powder will be the TFA salt of FDL.
■ **PAUSE POINT** Store it at –20 °C. In our hands, no decomposition was detected in at least 3 months.

(D) Synthesis of TDL ● **TIMING ~32 h**

(i) Flame-dry a 5-ml round-bottomed flask containing a magnetic stir bar and fill it with argon, as described in Step 1A(i–vi). In addition, oven-dry all syringes to be used for the transfer of solvents and reagents.
(ii) Remove the septum from the round-bottomed flask and add 5 mg (0.0095 mmol) of 5 (and 6-)-carboxytetramethylrhodamine succinimidyl ester and 3.3 mg of N-α-Boc-D-Lys-OH (0.0134 mmol), and reseal the flask with the septum and argon balloon.
(iii) Add 0.2 ml of anhydrous dimethylformamide via an oven-dried syringe.
(iv) Add 2.5 µl of diisopropylethylamine via a 10-µl microsyringe.
(v) Stir the reaction at room temperature overnight (15 h).
(vi) Remove the solvent in a rotary evaporator.
■ **PAUSE POINT** The product can be stored in a freezer overnight at –20 °C.
(vii) Without further purification, add a magnetic stir bar to the flask and treat the product with 1:1 TFA/DCM (2 ml).
(viii) Stir the reaction at room temperature for 30 min.
(ix) Remove the solvent on a rotary evaporator.
(x) Dissolve the product in a 1:4 solution of HPLC-grade acetonitrile/water (3 ml).
(xi) Purify TDL by reversed-phase HPLC with a gradient of acetonitrile in water with 0.1% (vol/vol) TFA (20–40% (vol/vol) acetonitrile for 10 min, eluting at 20 ml min^{-1}). The first product isomer elutes at 7.9 min and the second elutes at 9.1 min on a 5-µm Luna C_{18} column with a 21.2-mm internal diameter and a length of 250 cm. (Note: as the carboxytetramethylrhodamine starting material is available as a mixture of two carboxyl regioisomers, two regioisomeric reaction products are obtained upon coupling with N-α-Boc-D-Lys-OH. We have found that both regioisomers perform similarly in all labeling applications).
(xii) Transfer the remaining solution to a 60-ml lyophilization flask to lyophilize the final product. The powder will be the TFA salt of TDL.
■ **PAUSE POINT** Store it at –20 °C. In our hands, no decomposition was detected in at least 3 months.

Preparation before FDAA labeling ● **TIMING ~18 h 30 min**

2| From a –80 °C bacterial freezer stock or a single colony on a Petri plate, inoculate the cells into sterile liquid medium and grow them in optimal, species-specific conditions at least until exponential growth is achieved.

3| Dilute the culture to an OD_{600} of 0.05 in fresh medium, and grow the cells further until they reach an OD_{600} of ~0.4.
▲ **CRITICAL STEP** FDAA incorporation is best characterized in exponentially growing cultures. The three doublings in OD ensure that exponential phase growth has been achieved, even in the case of a starting stationary-phase culture.

4| Prepare desired stock solution(s) of FDAA in DMSO, as described in Reagent Setup.
▲ CRITICAL STEP DMSO stock solutions of FDAAs should be stored at –20 °C or at lower temperatures, as they have been observed to decay at prolonged exposure to room temperature. The stocks can be kept frozen at –20 °C for many months and thawed at least 10–15 times without decomposing.

Labeling with FDAAs

5| If the species under investigation has not been stained by FDAA before, follow the steps in option A using HADA as the FDAA. Options B–F are labeling procedures for specific applications. Long pulses for endpoint or time-lapse microscopy experiments are performed as described in option B using any FDAA at its optimal concentration (**Table 2**, ref. 6, or as determined by performing Step 5A). To simplify the procedure, we have used the conditions optimized for *E. coli* as an example.

For option C, we define short-pulse labeling as the growth of cells in the presence of FDAAs for durations that lie between 2–8% of the doubling time (ii in **Fig. 3**). In short pulses, higher FDAA and DMSO concentrations can be used without any signs of growth defects, because the cells are exposed for less time; for a higher SNR during microscopy, we recommend FDAA concentrations at least two times higher than those determined in Step 5A or used in Step 5B.

Short pulses can also be performed on about ten times more concentrated cells without causing a noticeable change in labeling patterns (option D), substantially reducing the amount of FDAA required per experiment. Reduction in sample volume also makes handling of multiple samples easier. Larger numbers of conditions (e.g., different FDAAs) can be sampled at the same time using, for example, multiple wells of a 48-well plate and a plate incubator.

For option E, we define a virtual time-lapse microscopy experiment as the consecutive exposure of the cells to differently colored FDAAs (iv in **Fig. 3**) in a series of short pulses. This can be used either to probe growth dynamics or as a diagnostic tool to probe the effects of different conditions (e.g., antibiotics and temperature) during the respective labeling periods on growth. Washing cells before, between and after each pulse with prewarmed medium normalizes the effect of the multiple pulses and minimizes bleed-over of the signals caused by the incorporation from the excess FDAA left from the previous round. Step 5E was optimized for *S. venezuelae*[6]; the labeling duration and the starting OD should be adjusted for different growth rates so that the cells are kept in the exponential phase throughout a given labeling period.

Option F describes how to use FDAAs to probe active bacteria in a saliva sample, and it could be adapted for the analysis of other environmental samples.

(A) Determination of species-specific optimum FDAA concentration with a long pulse ● **TIMING ~3 h**

(i) Serially dilute a 400 mM stock solution of HADA six times at a 1:2 ratio in DMSO.
(ii) Add 20 µl of each dilution to six sterile culture tubes; add 20 µl of pure DMSO into a seventh tube as the DMSO control.
(iii) Dilute the culture to an OD_{600} of 0.05 in fresh LB medium.
(iv) Add 1,980 µl of the culture to each of the seven tubes, and add 2 ml of the culture into the eighth tube as the growth control. This dilutes the FDAA and DMSO in each tube 100 times and gives twofold dilutions of FDAA, with final concentrations ranging from 4 mM–125 µM, each with the final DMSO concentration of 1%.
? TROUBLESHOOTING
(v) Incubate the culture in the desired conditions (e.g., in species-specific rich medium at optimal growth temperature). Monitor the effects of different HADA and DMSO (final 1%) concentrations on growth and morphology by measuring OD_{600} and checking cell morphology under light microscopy after each doubling.
? TROUBLESHOOTING
(vi) When the culture reaches an OD_{600} of 0.4–0.6, pellet 1 ml of the cells (1 min, maximum speed in a microcentrifuge, room temperature).
(vii) Resuspend the cells in 1 ml of 70% (vol/vol) ice-cold ethanol and fix the cells on ice for 10–15 min.
▲ CRITICAL STEP Proceed to Step 6 immediately.
(viii) In the meantime, continue to grow the remaining culture until the cells reach stationary phase, and continue to monitor for growth and morphology effects as in Step 5A(v).

(B) Long pulses for endpoint or time-lapse microscopy experiments ● **TIMING 1 h 15 min**

(i) To a culture tube, add 10 µl from a 50 mM $FDAA_{DMSO}$ stock solution. Alternatively, add 2.5 µl of $FDAA_{DMSO}$ from a 200 mM stock and 7.5 µl of DMSO.
(ii) Dilute the culture to an OD_{600} of 0.05 in fresh LB medium.
(iii) Add 990 µl of the culture to the culture tube containing FDAA to get a final FDAA concentration of 500 µM and a DMSO concentration of 1% (vol/vol) in LB medium, and then incubate the culture with shaking at 37 °C.
(iv) When the culture reaches an OD_{600} of 0.4–0.6, pellet the cells (1 min, maximum speed in a microcentrifuge, room temperature).
▲ CRITICAL STEP Proceed to Step 6 immediately.

(C) Short pulse for detecting the site of active growth ● TIMING 30 min

(i) To a culture tube, add 10 µl from a 100 mM $FDAA_{DMSO}$ stock solution. Alternatively, add 5 µl of $FDAA_{DMSO}$ from a 200 mM stock and complement it with 5 µl of DMSO.

(ii) Prewarm the culture tube and immediately add 990 µl of the exponentially growing culture with an OD_{600} of ~0.4 to get a final FDAA concentration of 1 mM and a DMSO concentration of 1% (vol/vol) in LB medium.

(iii) Incubate the culture with shaking at 37 °C for 15–30 s.

(iv) Pellet the cells (1 min, maximum speed in a microcentrifuge, room temperature) and resuspend the cells in 1 ml of 70% (vol/vol) ice-cold ethanol.

▲ **CRITICAL STEP** To increase the temporal resolution of the pulse, the user should consider fixing cells by directly adding 2.3 ml of ice-cold 100% ethanol into the 1-ml culture to get a final ethanol concentration of 70% (vol/vol).

(v) Fix the cells on ice for 10–15 min.

▲ **CRITICAL STEP** Proceed to Step 6 immediately.

(D) 'FDAA-saver' short pulse for detecting the site of active growth ● TIMING 40 min

(i) To a 1.7-ml Eppendorf tube, add 1 µl from a 100 mM $FDAA_{DMSO}$ stock solution.

(ii) Pellet 1 ml of the exponential culture with an OD_{600} of ~0.4 (1 min, maximum speed in a microcentrifuge, room temperature).

(iii) Resuspend the pellet in 100 µl of prewarmed LB medium, and add this concentrated culture to the prewarmed, FDAA-containing Eppendorf tube to get a final FDAA concentration of 1 mM and a DMSO concentration of 1% (vol/vol) in LB medium.

(iv) Incubate the tube with rocking at 37 °C for 15–30 s.

▲ **CRITICAL STEP** Avoid using this for pulses longer than 20% of the generation time. Some species such as *B. subtilis* show morphological defects with longer pulses, presumably because of poor aeration of the high-density cell suspension.

(v) Add 230 µl of ice-cold 100% ethanol into the 100-µl culture to get a final ethanol concentration of 70% (vol/vol).

(vi) Add an additional 1 ml of cold 70% (vol/vol) ethanol into the tube, contributing to the removal of the excess dye.

(vii) Fix the cells on ice for 10–15 min.

▲ **CRITICAL STEP** Proceed to Step 6 immediately.

(E) Virtual time-lapse using short pulses of multiple FDAAs ● TIMING 3–7 h

(i) Pellet 1 ml of exponentially growing *S. venezuelae* culture (optimally in LB medium with shaking at 30 °C and an OD_{600} of ~0.4) in an Eppendorf tube (1 min, maximum speed in a microcentrifuge, room temperature).

(ii) Discard the supernatant and resuspend the cells in 1 ml of prewarmed LB medium.

(iii) Pellet the cells again (1 min, maximum speed, at room temperature), discard the supernatant and resuspend the cells in 990 µl of prewarmed LB medium.

(iv) To a culture tube, add 10 µl from a 100 mM TDL_{DMSO} stock solution. (Alternatively, add 5 µl of TDL_{DMSO} from a 200 mM stock and complement with 5 µl of DMSO.)

(v) Prewarm the culture tube to 30 °C and immediately add the washed *S. venezuelae* culture to get a final TDL concentration of 1 mM and a DMSO concentration of 1% (vol/vol) in LB medium.

(vi) Incubate the culture with shaking at 37 °C for 3–6 min.

(vii) Pellet the cells and wash the cells once with LB medium and resuspend them in 990 µl of prewarmed LB medium, as described in Step 5E(i–iii).

(viii) To a culture tube, add 10 µl from a 100 mM FDL_{DMSO} stock solution. (Alternatively, add 5 µl of FDL_{DMSO} from a 200 mM stock and complement it with 5 µl of DMSO.)

(ix) Prewarm the culture tube to 30 °C and immediately add the washed *S. venezuelae* culture to get a final FDL concentration of 1 mM and a DMSO concentration of 1% (vol/vol) in LB medium.

(x) Incubate the culture with shaking at 37 °C for 3–6 min.

(xi) Pellet the cells and wash the cells once with LB medium and resuspend them in 990 µl of prewarmed LB medium, as described in Step 5E(i–iii).

? TROUBLESHOOTING

(xii) To a culture tube, add 10 µl from a 100 mM $HADA_{DMSO}$ stock solution. (Alternatively, add 5 µl of $HADA_{DMSO}$ from a 200 mM stock and complement with 5 µl of DMSO.)

(xiii) Prewarm the culture tube to 30 °C and immediately add the washed *S. venezuelae* culture to get a final HADA concentration of 1 mM and a DMSO concentration of 1% (vol/vol) in LB medium.

? TROUBLESHOOTING

(xiv) Incubate the culture with shaking at 37 °C for 3–6 min.

(xv) Pellet and wash the cells once with prewarmed LB medium (1 min, maximum speed in a microcentrifuge, room temperature).

(xvi) Fix the cells by resuspending and incubating them in 1 ml of 70% (vol/vol) cold ethanol on ice for 10–15 min.
▲ CRITICAL STEP Proceed to Step 6 immediately.

(F) Labeling environmental samples (using saliva as an example) ● TIMING **30 min–4 h 30 min**

(i) Collect ~1 ml of saliva in a culture tube.
(ii) To this sample, directly add 10 µl of $HADA_{DMSO}$ from a 50 mM stock solution to get a final HADA concentration of 500 µM and a DMSO concentration of 1% (vol/vol).
(iii) Incubate the culture tube at 37 °C (+ 5% CO_2, optional) for any duration between 5 min (for short pulses) and 4 h (for long pulses).
(iv) Pellet the cells (1 min, maximum speed in a microcentrifuge, room temperature).
(v) Resuspend the cells in 1 ml of 70% (vol/vol) ice-cold ethanol and fix the cells on ice for 10–15 min.
▲ CRITICAL STEP Proceed to Step 6 immediately.

6| Wash the cells with 1 ml of 1 × PBS at least two or three times until free FDAA is removed. Resuspend the cells in 20–100 µl of PBS or LB medium.
▲ CRITICAL STEP If this wash step follows a 70% (vol/vol) ethanol fixation step, after the first wash the pellet becomes noticeably transparent.
▲ CRITICAL STEP If this wash step is followed by a time-lapse microscopy experiment (i.e., Step 5B), the suspension should be well mixed and the cell density should be adjusted to an OD_{600} of 0.05 to get well-separated single cells.
■ PAUSE POINT If the imaging of the fixed cells will not be performed immediately, the cells can be stored at –20 °C after they are resuspended in 20–100 µl of PBS + 10% (vol/vol) DMSO.

Microscope setup ● TIMING **1 h**

7| If the viability and continued growth of the cells is not important during the imaging, follow the steps in option A. If imaging of the cells involves time-lapse microscopy as the continuum of Step 5B, follow the steps in option B.
▲ CRITICAL STEP HADA is virtually nonfluorescent in acidic pH or nonbuffered water. For the maximum brightness of HADA-labeled cells, imaging in mounting medium that is buffered above pH 7.0 (e.g., 1× PBS or LB medium) is recommended.

(A) Preparation of imaging agar pads ● TIMING **10 min**

(i) Dissolve 0.2 g of agarose in 20 ml of 1× PBS by microwaving to get a final concentration of 1% (wt/vol) agarose, and then bring the temperature of the solution to ~70 °C.
(ii) Spot ~200 µl of the solution on the middle of a glass microscopy slide, swiftly place a coverslip on top of this drop and gently press down before the agarose solidifies in order to make a flat, thin layer of agarose pad.
(iii) Once the pad is solidified, carefully remove the coverslip and air-dry the pad for no longer than 1 min.
▲ CRITICAL STEP When the slide is not dried properly, the cells do not settle on the agarose pads, making the imaging challenging.

(B) Preparation of nutrient agar pads for time-lapse microscopy of live cells prepared in Step 5B ● TIMING **10 min**

(i) Dissolve 0.2 g of agarose in 20 ml of LB medium (or appropriate liquid growth medium) by microwaving to get a final concentration of 1% (wt/vol) agarose, and then bring the temperature of the solution to ~70 °C.
! CAUTION Agarose solutions with nutrient media tend to boil over quickly.
(ii) Spot ~200 µl of the LB medium-agarose solution on the middle of a glass microscopy slide, swiftly place a coverslip on top of this drop and gently press down before the agarose solidifies in order to make a flat, thin layer of agarose pad.
(iii) Once the pad is solidified, carefully remove the coverslip and air-dry the pad for no longer than 1 min.
? TROUBLESHOOTING

8| Spot ~1 µl of cells on the agarose pad, gently place a new coverslip on top of the cells and seal the edges of the coverslip with a 1:1:1 mixture of melted Vaseline, lanolin and paraffin.

9| Image cells using a fluorescence microscope and FDAA-specific excitation and emission filter sets (**Table 2**). For the time-lapse microscopy of unfixed, live cells (prepared following Step 5B), continue acquiring images at constant intervals up to five or six generations.
! CAUTION Do not overexpose labeled cells, as it may cause loss of details in labeling patterns on the acquired micrograph.
? TROUBLESHOOTING

10| Evaluate labeling efficiency as discussed in **Box 1** and **Figure 4**.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1B(xix)	Instability of NADA before purification	Cyclization of the free amino group with the NBD ring	Decomposition of NADA is the only potential problem in any of the syntheses. Smaller-scale reactions tend to give lower amounts of the decomposed product. To maximize yields, the final product must be purified immediately and converted to the corresponding HCl salt as soon as possible
5A(iv)	At certain dilutions, temperatures and media, a precipitation of FDAAs forms	The FDAA under investigation is too hydrophobic and not sufficiently water-soluble	Check the labeling with a dilution that does not give any precipitation Increase the final DMSO in the medium. Many bacterial species will tolerate final DMSO concentrations up to 10% (vol/vol) Warm the medium until the precipitate dissolves Use a more hydrophilic FDAA (e.g., TDL over HADA)
5A(v)	The FDAA and/or DMSO is toxic or changes cell morphology within the experimental concentration range	High levels of FDAA incorporation interfere with cell wall synthesis. High levels of DMSO interfere with cell growth	Titrate either FDAAs and/or DMSO down and determine the minimal concentrations of FDAA and/or DMSO that are innocuous to growth and morphology but that still give detectable labeling
5E(xi)	In the virtual time-lapse microscopy experiment, the signal from the first FDAA is low or absent	In some species, turnover of the PG results in a substantial reduction of the signal from an incorporated FDAA during growth in the absence of that FDAA	Use inherently dimmer FDAAs for the last pulses (as done with *S. venezuelae* example in Step 5E), and consider designing and synthesizing FDAAs using brighter organic dyes and following the principles for the synthesis of TDL (Step 1D). Such FDAAs are expected to be well tolerated especially by Gram-positive or outer membrane–permeable Gram-negative species
5E(xiii)	In virtual time-lapse microscopy experiments involving multiple FDAAs, there is slower cell growth (i.e., lower incorporation efficiency of the most recent FDAA)	The cells will inevitably accumulate stress with every additional round of pulse labeling owing to exposure to high FDAA concentrations and vigorous washing steps	Reduce each of the FDAA concentrations and skip washes between steps. The effective concentration of the previous FDAA in the medium can still be reduced 100–1,000 times by simply pelleting the cells, carefully discarding the supernatant and resuspending the cells in fresh medium containing the next FDAA
7B(iii)	The SNR is too low	The nutrient agarose pad is autofluorescent	Increase the FDAA concentration to increase the overall signal of labeled cells Try a different growth medium (e.g., M9minimum medium for *E. coli*) that is less autofluorescent in a given channel
9	For a particular application or species, the FDAAs reported here fail to give a satisfactory SNR, despite all the labeling optimization attempts	High background fluorescence owing to insufficient washes	Increase the number of washes
		The labeling is enzymatic and is susceptible to cell wall turnover mechanisms	Mutate the genes for key turnover enzymes starting with the most active D,D-carboxypeptidase, e.g., DacA in *B. subtilis*[6]
		The FDAAs reported here are not bright or photostable enough	Design and synthesize FDAAs using brighter organic dyes according to the principles for the synthesis of TDL (Step 1D). FDAA MWs up to ~900 Da are expected to be well tolerated especially by Gram-positive or outer membrane–permeable Gram-negative species Use the D-Ala-D-Ala dipeptide labeling approach[18]

● TIMING

Step 1A, synthesis of HADA

i–vi: 30 min

vii,viii: 2 h 15 min

ix: 17 h 15 min

x–xvii: 1 h 15 min

xviii,xix: 1 h

xx–xxiii: 3 h

xxiv: 24 h

Step 1B, synthesis of NADA

i–vii: 30 min

viii: 1 h

ix–xviii: 1 h 30 min

xix–xxi 3 h

xxii: 24 h

xxiii: 15 h

Step 1C, synthesis of FDL

i: 30 min

ii: 15 min

iii: 4 h

iv–xi 1 h 30 min

xii–xiv: 1 h

xv–xviii: 3 h

xix: 24 h

Step 1D, synthesis of TDL

i: 30 min

ii–iv: 15 min

v: 15 h

vii–ix: 1 h

x,xi: 3 h

xii: 12 h

Preparing before FDAA labeling

Steps 2–4: 18 h 30 min

Step 5A, determination of species-specific optimum FDAA concentration with a long pulse

i,ii: 30 min

iii,iv: 10 min

v,vi: determined by growth rate of the species

vi,vii: 20 min

viii: determined by growth rate of the species

Step 5B, long pulses for endpoint or for time-lapse microscopy experiments

i–iii: 15 min

iv: 1 h

Step 5C, short pulse for detecting the site of active growth: 30 min

Step 5D, 'FDAA-saver' short pulse for detecting the site of active growth: 40 min

Step 5E, virtual time-lapse using short pulses of multiple FDAAs

i–iv: 10 min

vi–vii: 10 min

viii–x: 10 min

xi–xv: 10 min

xvi: 20 min

Step 5F, labeling environmental samples: 30 min–4 h

Step 6, washing and resuspending cells, 10 min

Step 7A, preparation of imaging agar pads: 10 min

Step 7B, preparation of nutrient agar pads for time-lapse microscopy of live cells prepared in Step 5B: 10 min

Steps 8–10, imaging and evaluation, 1 h 30 min

ANTICIPATED RESULTS

Analytical data

HADA: pale yellow solid, yield after reverse-phase HPLC (rpHPLC) purification: 58%

^{1}H NMR (400 MHz, DMSO-*d6*): δ = 2.46 (s, 1H), 3.69–3.77 (m, 1H), 3.79–3.87 (m, 1H), 4.07 (t, *J* = 5.6 Hz, 1H), 6.85 (d, *J* = 2.0 Hz, 1H), 6.89 (dd, *J* = 2.0, 8.4 Hz, 1H), 7.89 (d, *J* = 8.8 Hz, 1H), 8.43 (br s, 3H), 8.76 (s, 1H), 8.86 (t, *J* = 6.2 Hz, 1H), 11.34 (br s, 1H).

^{13}C NMR (100 MHz, DMSO-*d6*): δ = 52.1, 102.3, 111.3, 113.5, 115.0, 132.5, 148.8, 156.8, 161.2, 163.1, 164.6, 169.7; the signal for one carbon was overlapping with the solvent peak.

$[\alpha]^{20}_D$ -21.8 (c 2.2, DMSO-*d6*).

High-resolution MS–electrospray ionization–time of flight (HRMS-ESI-TOF) *m/z* calcd. for $C_{13}H_{12}O_6N_2$ ($[M+H]^+$): 293.0774, found 293.0774.

HPLC: t_R = 5.96 min (10–90% acetonitrile/H_2O over 10 min).

NADA: orange solid, yield on a 100-mg scale after rpHPLC purification: 71%, larger scale (≥500 mg) reactions gave erratic and low-yielding results.

$[\alpha]^{20}_D$ -32.0° (c 1.1, DMSO-*d6*).

^{1}H NMR (400 MHz, DMSO-*d6*): δ = 4.06 (m, 2H), 4.29 (m, 1H), 6.61 (d, *J* = 8.0 Hz, 1H), 8.56 (d, *J* = 8.0 Hz, 1H), 8.66 (br s, 3H), 9.32 (br s, 1H).

^{13}C NMR (100 MHz, DMSO-*d6*): δ = 43.4, 51.5, 100.5, 122.5, 138.1, 144.4, 144.9, 145.2, 169.2.

HRMS-ESI-TOF *m/z* calcd. for $C_9H_9O_5N_5$ ($[M+H]^+$): 268.0682, found 268.0680.

HPLC: t_R = 5.02 min (20–90% acetonitrile/H_2O over 10 min).

FDL: yellow solid, yield after rpHPLC purification: 72%.

$[\alpha]^{20}_D$ -7.1° (c 0.72, MeOH-*d4*).

^{1}H NMR (400 MHz, MeOD-*d4*): δ = 1.50–1.63 (m, 2H), 1.78 (quintet, *J* = 7.0 Hz, 2H), 1.90–1.99 (m, 1H), 2.00–2.10 (m, 1H), 3.66 (br s, 2H), 4.00 (t, *J* = 6.3 Hz, 1H), 6.59 (dd, *J* = 2.0, 8.8 Hz, 2H), 6.73(s, 2H), 6.74 (d, *J* = 8.90 Hz, 2H), 7.18 (d, *J* = 8.2 Hz, 1H), 7.76 (d, *J* = 8.2 Hz, 1H), 8.17 (s, 1H).

^{13}C NMR (100 MHz, MeOD-*d4*): δ = 23.3, 29.5, 31.3, 45.0, 53.9, 103.5, 111.9, 114.1, 120.5, 126.1, 129.2, 130.5, 131.8, 142.5, 154.6, 162.1, 171.0, 171.9, 182.9.

HRMS-ESI-TOF *m/z* calcd. for $C_{27}H_{26}N_3O_7S$ ($[M+H]^+$): 536.1492, Found 536.1470.

HPLC: t_R =5.08 min (30–45% acetonitrile/H_2O over 10 min).

TDL: red solid, yield after rpHPLC purification: 61%.

Optical rotation is not reproducible owing to low energy on the polarimeter.

^{1}H NMR (400 MHz, MeOD-*d4*): δ = 1.25–1.35 (m, 2H), 1.46–1.62 (m, 2H), 1.68 (quintet, *J* = 7.2 Hz, 2H), 1.75–2.05 (m, 2H), 3.41 (t, *J* = 7 Hz, 1H), 3.92 (t, *J* = Hz, 1H), 7.01 (d, *J* = 2 Hz, 2H), 7.05 (dd, *J* = 2.0, 9.4 Hz, 2H), 7.13 (d, *J* = 9.4 Hz, 2H), 7.81 (d, *J* = 1.5 Hz, 1H), 8.19 (dd, *J* = 1.5, 8.6 Hz, 1H), 8.39 (d, *J* = 8.6 Hz, 1H);

HRMS-ESI-TOF *m/z* calcd. for $C_{31}H_{35}N_4O_6$ (M^+): 599.2557, Found 599.2559.

HPLC: t_R = 7.86 and 9.06 min (2 isomers isolated results from mixed isomer starting material) (20–40% acetonitrile/H_2O over 10 min).

Determination of species-specific optimum FDAA concentration with a long pulse (Step 5A)

For most species, we anticipate a DMSO concentration of 1% (vol/vol) and an FDAA concentration of 500 μM to represent near-optimal conditions that give a good signal without perturbing morphology and growth. Regardless, the user is advised to determine the optimal DMSO and FDAA concentrations under desired growth conditions empirically. This is especially true if the labeling is being performed in an untested bacterial species.

Long pulses for endpoint or time-lapse microscopy experiments (Step 5B)

A maximum steady-state labeling with a chosen FDAA concentration is reached after one or two generations of growth with the dye. Cells that are exposed to FDAAs for such long durations are expected to be labeled throughout the cell body. In *E. coli* and many Gram-negative species, the labeling is uniform, to an extent, where the signal from the septum is equal to the side-wall labeling. In *B. subtilis* and in many Gram-positive species, the side wall shows a uniform peripheral distribution of the signal, and the signal from the septum is usually ~2–6 times higher than the side wall.

Short pulses for detecting the site of active growth (Step 5C,D)

The short pulses are highly useful for showing sites of active cell wall synthesis. In a typical predivisional or dividing cell, the septum is expected to show the predominant signal.

Virtual time-lapse using short pulses of multiple FDAAs (Step 5E)

As discussed further in the introduction, the virtual time-lapse allows two unique applications: first, the sequential labeling with different colors can be used as a visual record for the growth and PG synthesis history of the cell, which can easily indicate the growth modes of the labeled species and provide insights about the dynamics of different morphological events at a single-cell level. For example, the virtual time-lapse of the polarly growing *S. venezuelae* shows a rainbow-like pattern at the growing tips of the cells, with the signal from the more recent FDAA pulses being closer to the tip (**Fig. 2b**). Second, the virtual time-lapse experiment can also be combined with a test condition such as a change in temperature or the addition of an antibiotic.

Labeling environmental samples (Step 5F)

Natural populations of bacteria are expected to show different modes of growth, including polar, septal or diffuse growth. Therefore, a short pulse labeling with environmental samples will likely give a collection of different labeling patterns (**Fig. 2a**).

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