Progress and prospects for small-molecule probes of bacterial imaging

Ozden Kocaoglu[1] and **Erin E Carlson**[1,2,3,*]

[1]Department of Molecular and Cellular Biochemistry, Indiana University, Bloomington, Indiana, USA

[2]Department of Chemistry, Indiana University, Bloomington, Indiana, USA

Abstract

Fluorescence microscopy is an essential tool for the exploration of cell growth, division, transcription and translation in eukaryotes and prokaryotes alike. Despite the rapid development of techniques to study bacteria, the size of these organisms (1–10 μm) and their robust and largely impenetrable cell envelope present major challenges in imaging experiments. Fusion-based strategies, such as attachment of the protein of interest to a fluorescent protein or epitope tag, are by far the most common means for examining protein localization and expression in prokaryotes. While valuable, the use of genetically encoded tags can result in mislocalization or altered activity of the desired protein, does not provide a readout of the catalytic state of enzymes and cannot enable visualization of many other important cellular components, such as peptidoglycan, lipids, nucleic acids or glycans. Here, we highlight the use of biomolecule-specific small-molecule probes for imaging in bacteria.

While bacteria do not have the distinct internal compartments seen in eukaryotes, these unicellular organisms possess highly organized cellular structures that are essential to orchestrating cell growth, division, motility, DNA transcription, protein translation and secretion in a well-defined synchrony[1]. Visualization of interactions between the constituents of this organization is critical to our understanding of bacterial growth and pathogenesis, and although great progress has been made in these areas in the past decade, many questions cannot be addressed using existing techniques. Imaging-based methods are and will continue to be essential for the study of the biomolecules involved in cellular and physiological processes in their native environment, with fluorescence microscopy being particularly valuable for examination of the subcellular structures of bacteria.

Until the application of fluorescent tagging techniques in bacteria, these cells were considered amorphous bags in which chromosomes and proteins were randomly distributed. However, work over the last several decades has revealed that bacteria are well-organized multicomponent systems and that individual proteins, such as the cytoskeletal proteins MreB

*carlsone@umn.edu.
[3]Current address: Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, USA.

and FtsZ and the chemoreceptors, localize to specific sites of the cell in a dynamic fashion[1,2]. Fluorescent protein fusion (for example, with green fluorescent protein, GFP; Fig. 1a)[3] and epitope tagging (for example, with FLAG or c-Myc; Fig. 1b)[4,5] are the most widely used strategies for monitoring protein expression and localization in bacteria[3,5,6]. Small protein and peptide tags have also been used to facilitate labeling, whereby a fluorescent tag is conjugated to the protein of interest by association with a tetracysteine motif (CCXXCC; FlAsH or ReAsH)[7,8] or through the action of enzymes such as biotin ligase or sortase[9]. Alternatively, the protein target is fused to an enzyme that can be specifically labeled with a small dye-containing substrate (such as HaloTag or SNAP-tag)[9].

Ideally, the addition of this appendage, which can be rather large (for example, 27 kDa for GFP), does not affect the functionality or localization of the targeted protein. However, recent work has demonstrated that the fusion component can dramatically affect protein localization[10,11]. For instance, the actin-like protein MreB in *Bacillus subtilis* and *Escherichia coli* was thought to be organized in a helical structure, on the basis of microscopy results obtained with fluorescent-protein-tagged constructs (GFP and YFP, respectively). However, it has subsequently been shown that MreB does not form a helical pattern during exponential growth and that this pattern was an artifact of the protein tag[12–14]. Although fluorescent protein tags have been undeniably valuable for protein localization studies, it is now clear that target proteins need to be stringently tested for functionality (such as native-like catalytic activity and growth phenotype) with various fluorescent tags.

Another critical barrier that must be overcome in the study of bacteria arises from the fact that they are so small (1–10 μm). The spatial resolution of conventional fluorescence microscopes is insufficient for this, being limited by the diffraction of light to 200–300 nm and 500–700 nm in the lateral and axial dimensions, respectively. Thus, to adequately visualize the subcellular structures of these organisms, the use of super-resolution (SR) microscopy has become essential (Box 1)[15,16]. SR imaging has already enabled elucidation of several bacterial processes that was not possible with conventional optical microscopy. For example, the actions of FtsZ, a tubulin-like GTPase involved in cell division that recruits other proteins to the division site for cytokinesis, have been mapped in multiple organisms. *In vitro* polymerization of FtsZ showed that this protein could assemble into short, single-stranded protofilaments[17]. These protofilaments were thought to form bundles to produce a ring, called the Z-ring, during division *in vivo,* but the optical resolution of conventional microscopes was insufficient to validate this hypothesis in live cells. When, more recently, PALM was used to image the Z-ring of *E. coli*, it showed that protofilaments make randomly overlapping bundles and form a Z-ring with a width of ~110 nm (FtsZ-GFP or FtsZ-mEos2 fusions; Fig. 1c)[18,19]. Similarly, this technique was used to examine the organization of FtsZ in live *Caulobacter crescentus*. FtsZ in this organism localizes mainly to the midcell as a patchy band because of the random distribution of protofilaments (FtsZ-Dendra2 fusion)[20]. Finally, 3D-SIM was used to examine FtsZ localization and Z-ring structure in *B. subtilis* and *Staphylococcus aureus*, showing that there is heterogeneous FtsZ distribution in the Z-ring and the ring has a bead-like discontinuous pattern (FtsZ-GFP)[21].

Box 1

Super-resolution microscopy

Several platforms for super-resolution (SR) microscopy have been devised. Importantly, SR microscopy techniques often require a limited set of fluorophores, with each technique necessitating fluorescent molecules with different optical properties.

Structured-illumination microscopy (SIM) and stimulated emission depletion (STED) achieve improved resolution by reducing the size of the effective point spread function (PSF), the diffraction pattern produced by an infinitely small point source[94–96]. SIM stands out among the SR microscopy methods in that it is not dependent upon specialized fluorescent molecules. It can be used with the same variety of fluorescent protein tags and commercially available fluorophores as wide-field fluorescence microscopes, making this method perhaps the most amenable to the utilization of small-molecule probes[15,97]. SIM enables 100- to 130-nm lateral and 250- to 340-nm axial resolution. Because STED microscopy requires fluorophores with high-intensity brightness and photostability, ATTO dyes have been widely used for this technique, and resolutions of ~50 nm lateral and ~100 nm axial are possible with this combination.

Methods that are based on the sequential detection of discrete fluorophores, such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), enable single-molecule imaging as they can achieve resolutions of ~20 nm lateral and ~50 nm axial[98–100]. These techniques necessitate the use of specialized fluorophores, with PALM requiring photoactivatable fluorescent protein tags, such as Dendra, Dronpa, Eos and mCherry, and STORM using photoswitchable organic fluorophores including Cy3, Cy5 and Alexa Fluor 647.

The vast majority of studies to examine bacterial substructures have used fluorescent protein fusions and epitope tags to shed light on protein expression and localization. While important, genetically encoded tags are not applicable to the visualization of cellular components such as peptidoglycan (PG), lipids, nucleic acids, metabolites or glycans[22]. Nucleic-acid-based probes have been used with much success and have been described elsewhere[23–27]. Here, we focus on small-molecule probes that target the cell envelope components and/or provide a readout of enzymatic activity. These compounds are much less likely to perturb the native system than encoded tags[28]. However, a major obstacle in their application is the largely impenetrable cell envelope of these organisms. Because of that, the majority of studies using these compounds to date have focused on visualization of the extracellular components (Table 1).

Antibiotic-inspired chemical probes

PG, also called murein, is a major component of the bacterial cell wall, which maintains the organism's shape and protects it from turgor pressure. PG is a polymeric structure composed of alternating β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic (MurNAc) acid glycan chains that are crosslinked by pentapeptides (Fig. 2)[29]. Although most bacteria have PG, the length of the glycan chains and the amino acid composition of

the stem peptides vary between organisms[30,31]. This structure is unique to bacteria, and inhibition of its biosynthesis by antibacterial agents leads to cell lysis[32]. A particularly fruitful strategy for probe design has been to harness the power of known antibiotic scaffolds that interfere with cell wall construction.

The PG precursors (glycan chains) are polymerized and crosslinked by membrane-anchored proteins called the penicillin-binding proteins (PBPs)[33,34]. Although their catalytic functions are conserved, most organisms possess a suite of PBPs that are postulated to be critical in different stages of cellular growth and division. The peptide crosslink formed by the PBPs is required for to cellular integrity, and inhibition of PBP transpeptidation activity is the basis for the antibacterial activity of penicillin and other β-lactams. The β-lactam ring is a structural mimetic of the terminal portion of the PG stem peptide substrate, D-alanyl-D-alanine (D-Ala-D-Ala), and covalently labels the PBPs in an activity-dependent fashion[35,36]. Accordingly, the β-lactam scaffold has served as inspiration for a number of probes devised to examine bacterial protein function.

Fluorescent β-lactams such as BODIPY-conjugated penicillin V analogs, BOCILLIN FL (**1**; Fig. 3a) and BOCILLIN 650/665 (derived from BODIPY FL and BODIPY 650/665, respectively)[37,38] have been used in gel-based studies to examine global PBP activity in both membrane preparations and live cells, a strategy also known as activity-based protein profiling[39,40]. Additionally, a set of β-lactam probes based on both natural and synthetic compounds appended with an alkyne tag have been reported. Following labeling of target proteins with these molecules, the bacteria are lysed and a fluorophore or biotin conjugated to an azide handle is attached using the Hüisgen 1,3-dipolar cycload-dition reaction ('click chemistry') for in-gel fluorescence detection or mass spectrometry (MS) analysis, respectively. Natural β-lactam analogs label PBPs, whereas synthetic monocyclic β-lactam probes have affinity for virulence-associated enzymes[41]. Similarly, a library of β-lactone probes has been synthesized and applied to several Gram-positive and -negative bacteria. Identification of the labeled proteins by MS revealed that most of the probe-labeled proteins are involved in primary and secondary metabolism, virulence, antibiotic resistance and detoxification[42–44].

To enable visualization of the actions of discrete PBPs, we reported the design and synthesis of cephalosporin-based probes that target a PBP subset, the high-molecular-weight homologs. We synthesized cephalosporin C–based probes (Ceph C–carboxyte-tramethylrhodamine (Ceph C-T), **2**; Fig. 3b) and demonstrated that they selectively label a small subset of PBPs in *B. subtilis* and *Streptococcus pneumoniae*. Fluorescence imaging with 3D-SIM indicated that tagged PBPs localized at the division site in both organisms[45,46]. Dual labeling experiments were performed by incubating whole cells with Ceph C-T to label Ceph C–sensitive PBPs, and then BOCILLIN FL was applied to saturate the remainder of the PBPs, enabling simultaneous analysis of two PBP subsets. Tagged PBPs were localized at the division site but were not mixed; instead, they displayed distinct localization patterns (Fig. 3c). Such details were not observed with wide-field fluorescence microscopes before our study. Recently, we reported PBP selectivity profiling of 20 β-lactam antibiotics in *E. coli* and *S. pneumoniae* that will serve as a foundation for future probe generation[47,48].

Vancomycin is a clinically important antibiotic that targets PG biosynthesis by binding to the D-Ala-D-Ala motif of the stem peptide. Vancomycin binds to nascent PG, and it was postulated that a fluorescent version of this antibiotic (Van-FL, **3**) could be used as a tool to visualize PG biosynthesis in live cells. Exponentially growing *B. subtilis* cells treated with **3** show the brightest staining at the division site where nascent PG synthesis was predicted to occur, along with less apparent sidewall staining in a helical pattern (Fig. 3d,e)[49]. Van-FL has been applied in other Gram-positive bacteria such as *S. pneumoniae*, *Streptomyces coelicolor* and *Corynebacterium glutamicum* and now serves as an important marker for active PG biosynthesis. Another antibiotic, ramoplanin, targets cell wall biosynthesis by sequestering lipid II. Fluorescein-conjugated ramoplanin was used as a chemical probe on *B. subtilis* cells and displayed dose-dependent labeling. At low probe concentration, staining at the divisional septa and the cell poles, as well as helix-like sidewall patterns, were observed[50]. The fluorescent variants of both vancomycin and ramoplanin have been valuable tools for understanding PG biosynthesis, but their use is limited to Gram-positive bacteria because the outer membrane of Gram-negative bacteria acts as a barrier to these large molecules.

Peptidoglycan-inspired chemical probes

Imaging of the bacterial cell wall has also been accomplished through the incorporation of unnatural biosynthetic precursors or substrate mimics[51]. For example, fluorophore-tagged UDP-MurNAc pentapeptide was synthesized and tested for incorporation into bacterial cells[52]. Fluorescence microscopy images show that this UDP-MurNAc analog is readily incorporated by Gram-positive bacteria. Pretreatment of Gram-negative bacteria with EDTA weakened the thick liposaccharide layer and enabled incorporation of the probe in these cells[53]. In another study, a derivative of the PG tripeptide, L-alanyl-γ-D-glutamyl-L-lysine, was generated with *N*-7-nitro-2,1,3-benzoxadiazol-4-yl in the lysine position for *in situ* detection of nascent PG synthesis in *E. coli*[54].

The use of fluorophore-conjugated D-amino acids that can be incorporated into the PG stem peptide has been reported by several research groups (fluorescent D-amino acid (FDAA) labeling; Fig. 4a). D-Alanine and D-lysine analogs containing small fluorophores that enable detection of newly synthesized PG in live cells by fluorescence microscopy have been developed (hydroxycoumarin-carbonyl-amino-D-alanine, HADA; **4**)[55]. These probes were initially tested on various Gram-positive and -negative bacteria such as *B. subtilis*, *E. coli*, *S. aureus*, *S. pneumoniae*, *Agrobacterium tumefaciens* and *C. crescentus*. A clickable alkyne-containing D-alanine analog ((*R*)-propargylglycine; **5**) has also been reported and used to label nascent PG of *Listeria monocytogenes* during macrophage infection (Fig. 4b)[56]. More recently, cyclooctyne D-amino acids were combined with near-infrared fluorogenic azides to facilitate visualization of PG without the need for washes to remove unreacted probe (Fig. 4c,d)[57]. Intriguingly, several studies have demonstrated that the efficiency of incorporation of a number of DAA derivatives is organism specific[58,59], information that is useful for probe design.

Since their introduction, FDAAs have been used in a number of investigations[60,61], including elegant studies to determine if Chlamydiae produce PG, a question that has been

investigated for decades using indirect techniques[62,63]. Probes based on D-alanine and D-alanyl-D-alanine were used in *Protochlamydia amoebophila* and *Chlamydia trachomatis*, respectively (Fig. 4e). Fluorescence microscopy imaging demonstrates conclusively that these Chlamydia species do in fact synthesize PG[64,65]. PG assembly in ovoid-shaped bacteria (ovococci) has also been examined with FDAAs in combination with other tools, including Van-FL. These experiments revealed that ovococcus cells have a unique PG structure that is dissimilar to those of other Gram-positive bacteria (Fig. 4f)[66,67]. None of these studies would have been possible without the generation of applicable small-molecule probes as they required direct visualization of the PG biopolymer.

Carbohydrate-derived chemical probes

Bacterial cells are surrounded by glycan structures that have been shown to have important functions in pathogenesis. For example, Gram-positive bacteria have teichoic acids on their cell surface, and organisms lacking these sugar moieties exhibit an attenuated ability to cause infection[68]. Mycobacterial species have a thick PG layer, similar to that of Gram-positive bacteria, and an unique outer membrane called the mycomembrane, which is composed of a layer of arabinogalactan and mycolic acids that is coated with the non-mammalian disaccharide trehalose[69]. Absence of trehalose causes proliferation defects[70]. As a specific and sensitive strategy to detect mycobacteria such as *Mycobacterium tuberculosis*, a fluorescein- conjugated trehalose analog (**7**) has been developed and used for visualization of *M. tuberculosis* within infected macrophages (Fig. 5a,b)[71]. Azide-functionalized trehalose analogs have been generated as bioorthogonal chemical reporters for imaging[72,73]. This strategy is suitable for studying mycobacterial glycolipid function and dynamics in living cells.

Gram-negative bacteria have an outer membrane that is associated with a dense lipopolysaccharide (LPS), which distinguishes them from Gram-positive bacteria. Modification of the LPS layer enables the host immune system to more readily combat these organisms. A clickable version of 3-deoxy-D-mannooctulosonic acid (KDO), thought to be an essential component of LPS, has been reported. Following incorporation, azido-KDO was conjugated to a fluorophore, and the resulting labeling pattern demonstrated that KDO localized at the inner core of LPS in Gram-negative bacteria (*E. coli*, *Salmonella typhimurium*, *Legionella pneumophila*). No labeling was observed in Gram-positive organisms (*Shawanella oneidensis*, *B. subtilis*, *S. aureus*), demonstrating that the LPS can be selectively targeted and used for bacterial imaging[74].

Several additional strategies have been reported for the visualization of extracellular components in bacteria, including global conjugation of cell surface amines to a fluorophore followed by SR microscopy[75–77] and utilization of the bacterial enzyme sortase to incorporate non-native small molecules into the cell wall for fluorescence microscopy, flow cytometry, electron microscopy and MS applications[78].

Additional small-molecule probes

Replacement of an amino acid with an unnatural analog can yield a chemical handle for subsequent detection of a protein of interest[9,79,80]. For example, co-translational labeling of proteins with noncanonical amino acids that contain an alkyne provides a site for attachment of a fluorogenic dye appended to an azide group. This strategy has been used to visualize newly synthesized proteins in *E. coli*[81] and environmental microbes (L-azidohomoalanine)[82]. Recently, tagging with azidonorleucine enabled detection of secreted bacterial proteins in host cells[83], and the outer-membrane proteins of *E. coli* have been visualized by STORM following incorporation of an unnatural amino acid (homopropargylglycine) and attachment of a dye by 'click chemistry'[84].

Although probe development for the exploration of bacterial proteins has been an active area of study for many years, relatively few of these compounds have been applied in microscopy-based experiments, a lack that is likely due in part to the difficulty of generating compounds that can penetrate the cell envelope of bacteria. Activity-based probes have been designed to detect a number of bacterial proteins, such as sulfatases[85,86], serine proteases[87], the cell wall biosynthetic protein MurA[88], glycoside hydrolases[89], redox-sensitive proteins[90,91] and histidine kinases[92]; however, most of these probes have been used only in gel-based analyses. One additional strategy that has been applied to bacterial imaging is the use of a probe that becomes fluorescent upon turnover by nitroreductase. In this way, the probe (nitro-aryl fluorogen **8**, Fig. 5c,d) acts as a readout of enzyme activity in live cells, enabling visualization of the location of nitroreductase in an approach called enzymatic-turnover-activated localization microscopy (ETALM)[93].

Summary and future outlook

In this Review, we have provided a survey of small-molecule probes that have been used to explore proteins, carbohydrates and PG in bacterial cells. To date, relatively few of these compounds have been applied in imaging experiments, and only a handful have been utilized with SR imaging technologies. Looking forward, the use of biomolecule-selective small molecules carries both great potential and significant challenges. Perhaps one of the most difficult obstacles will be the development of probes and bright, photostable fluorophores that can readily penetrate the cell envelopes of bacteria, which will be necessary to examine many targets in living cells. To date, most success in the use of small-molecule probes has been in the examination of extracellular components, making the majority of this work complementary to the use of genetically encoded tags. A true challenge will be to devise strategies to utilize target-selective molecules inside live bacteria. Continued improvements in SR microscopy image acquisition and data analysis processes will also be necessary to increase temporal resolution, which is critical for the visualization of the many events that occur on a biological timescale. These challenges aside, it is clear that much can be learned from biomolecule-specific fluorescent probes and that combination of these molecules with the improved spatial and temporal resolution achievable with SR microscopy will continue to shed light on growth, division, pathogenesis and virulence processes in bacteria. Creative solutions will be required to move the field of probe-promoted bacterial imaging beyond the

investigation of 'readily accessible' components so as to provide a comprehensive toolkit for visualization of all bacterial biomolecules.

Acknowledgments

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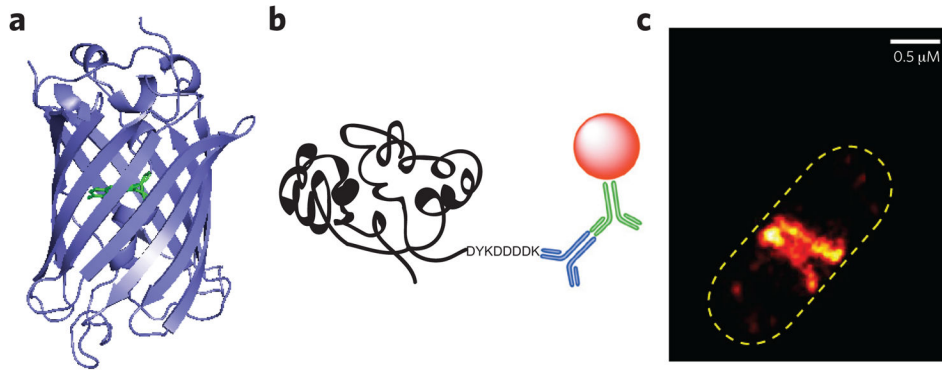
Figure 1. Genetically encoded tags for bacterial imaging

(**a**) Crystal structure of green fluorescent protein (GFP) (PDB 1GFL). Ser65-Tyr66-Gly67 residues that form the chromophore (*p*-hydroxybenzylideneimidazolinone) are highlighted in green. (**b**) Schematic representation of FLAG epitope tag. The FLAG tag is an octapeptide with a sequence of DYKDDDDK. Cartoon representation shows detection of the FLAG sequence by an anti-FLAG primary antibody (blue), followed by the introduction of secondary antibody (green) carrying a fluorophore (red sphere). (**c**) *E. coli* expressing FtsZ-mEos2 imaged with PALM. The number of molecules used to construct the PALM image was 2,843. Image in **c** reproduced from ref. 18.
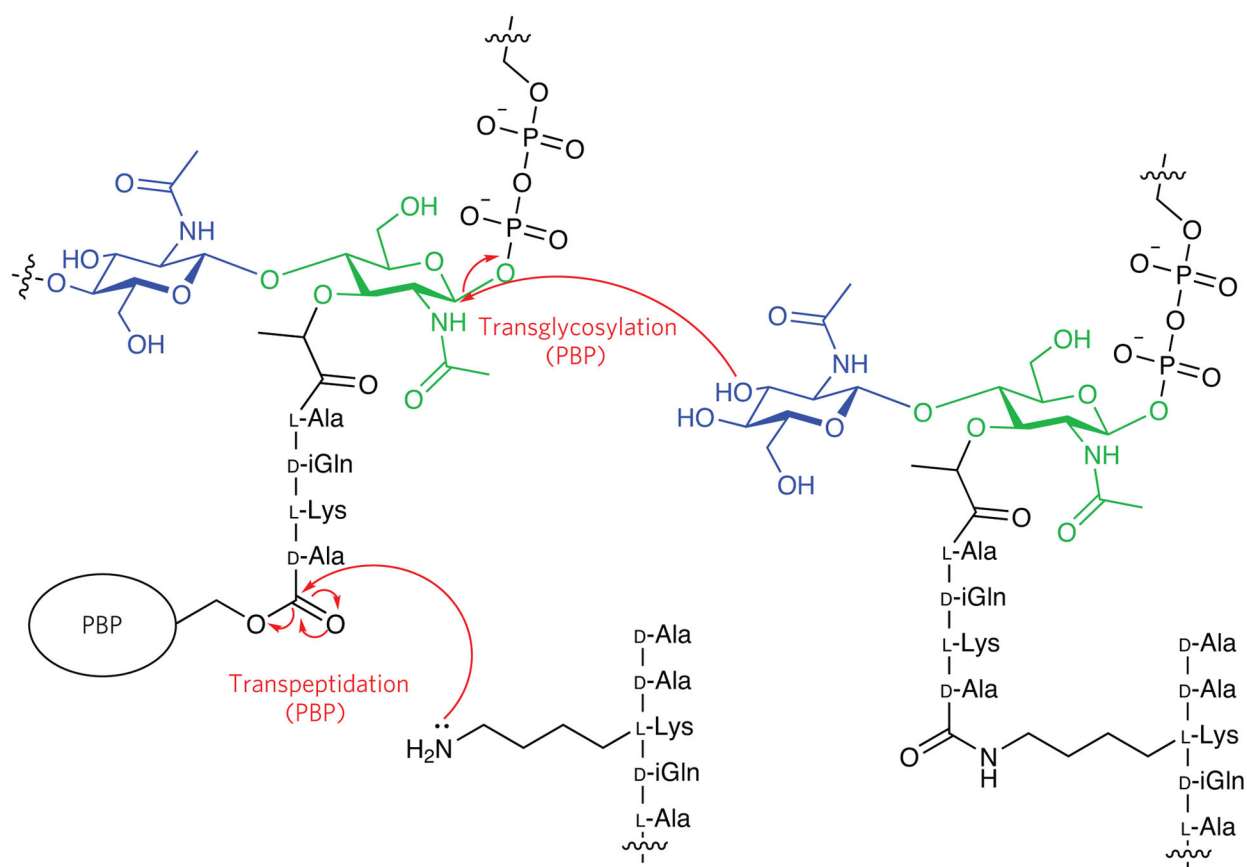
Figure 2. Schematic representation of bacterial peptidoglycan (PG)
PG is composed of alternating β-(1,4)-linked *N*-acetylglusosamine (blue) and *N*-acetylmuramic acid (green) glycan chains that are crosslinked by peptides. Polymerization (transglycosylation) and crosslinking (transpeptidation) between glycan chains are catalyzed by the PBPs. The peptides shown are those from *S. pneumoniae*.
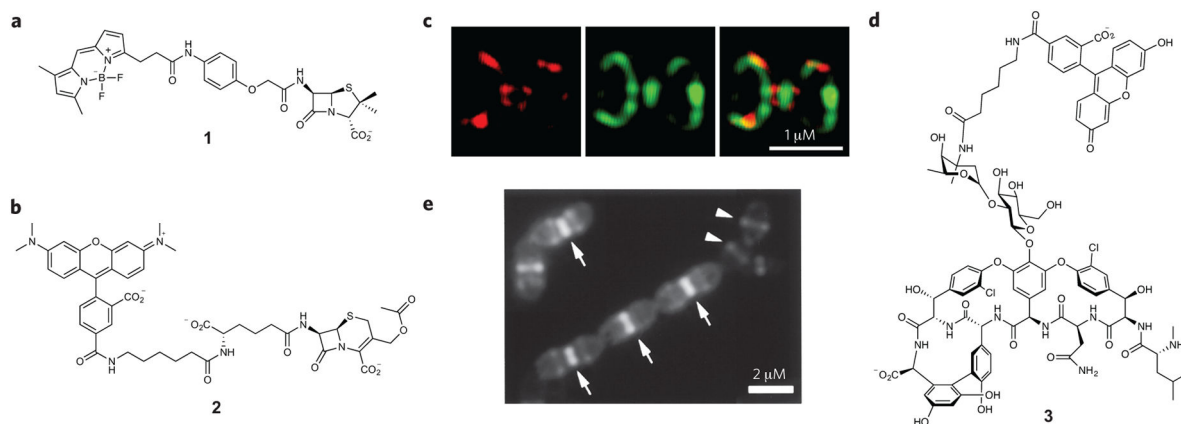
Figure 3. Antibiotic-inspired probes and images obtained with these compounds
(**a**,**b**) Structures of PBP imaging probes. Probe **1** (**a**) labels all PBPs that are present in
bacteria, whereas molecule **2** (**b**) selectively targets only a subset of PBPs in *B. subtilis* and
S. pneumoniae. (**c**) 3D-SIM images of PBPs in *S. pneumoniae* after dual labeling with **2**
(red) and **1** (green). (**d**) Vancomycin-fluorescein (**3**) enables visualization of nascent PG
synthesis. (**e**) Nascent PG in *S. pneumoniae* cells is labeled with molecule **3**. Arrows
indicate heavily stained division septum. Arrowheads indicate lightly stained equators of the
daughter cells that will become division sites. Figures reproduced with permission from: **c**,
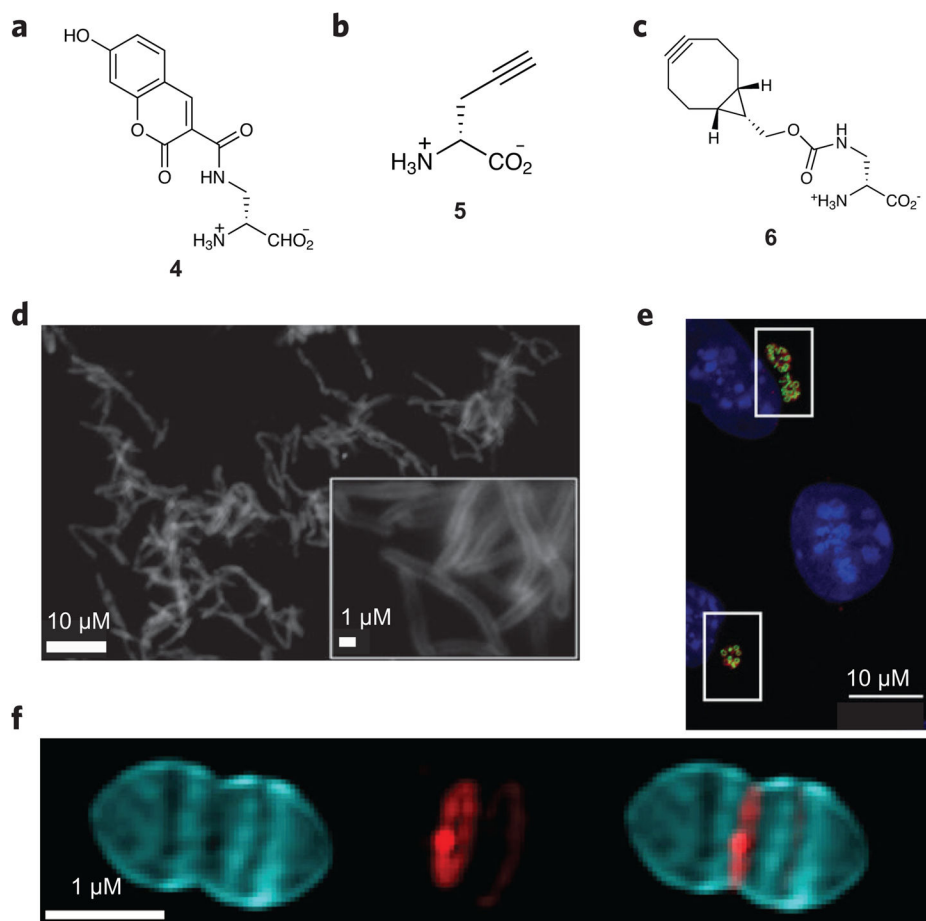ref. 45; **e**, ref. 49.

Figure 4. Peptidoglycan-inspired probes and images obtained with these compounds
(**a,b**) Fluorescent D-amino acids hydroxycoumarin-carbonyl-amino-D-alanine (**4; a**) and
(*R*)-propargylglycine (**5; b**) for tracking PG production. (**c**) The cyclooctyne D-alanine
analog endobcnDala (**6**) used for no-wash PG labeling. (**d**) Fluorescence image of *M.
smegmatis* treated with **6** and labeled with bis-sulfated glycol-substituted azido silicon
rhodamine. (**e**) Fluorescence image of mouse fibroblast cells (L2 cells) infected with *C.
trachomatis* and labeled with the dipeptide ethynyl-D-alanyl-D-alanine followed by an
azide-modified Alexa Fluor 488 (green). An antibody for chlamydial major outer membrane
protein (red) was used to label chlamydial elementary bodies and reticulate bodies, and 4′,6-
diamidino-2-phenylindole (DAPI; blue) was used for nuclear staining. (**f**) 3D-SIM images of
S. pneumoniae PG pulse-chase labeled with **4** (blue) and tetramethylrhodamine 3-amino-D-
alanine (red). Using this strategy, older PG (blue) can be differentiated from areas of new PG
synthesis (red). Figures reproduced with permission from: **d**, ref. 57; **e**, ref. 65; **f**, ref. 67.
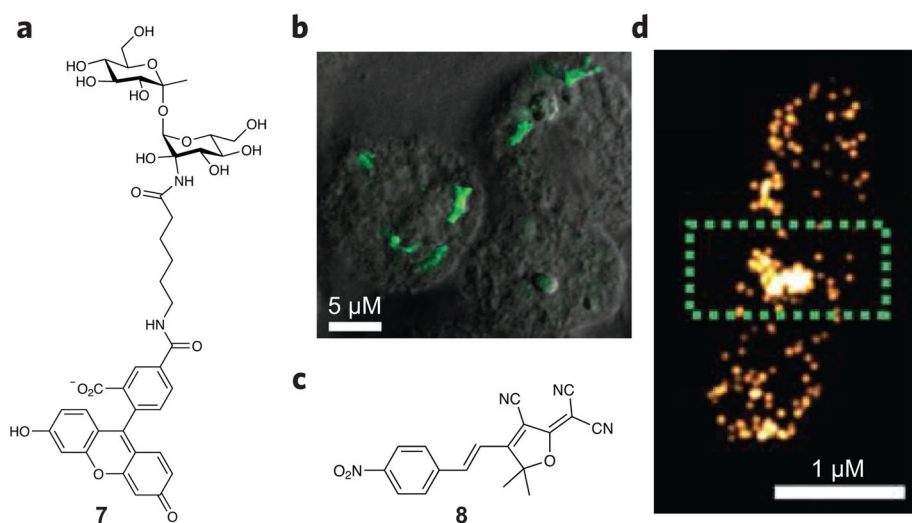
Figure 5. Carbohydrate-inspired and turnover-dependent probe designs
(**a**) Fluorescein-conjugated trehalose (**7**) for selective detection of *M. tuberculosis*. (**b**)
Fluorescence image of *M. tuberculosis*–infected macrophages labeled with fluorescein-trehalose. (**c**) Nitroreductases converts a nitro-aryl fluorogen (**8**) into a bright fluorophore.
(**d**) Enzymatic-turnover-activated localization microscopy (ETALM) images of
nitroreductase localization in *B. subtilis*. Figures reproduced with permission from: **b**, ref.
71; **d**, ref. 93.

Table 1

Highlighted probes and their cellular targets

Probe	Target of probe	Cell type and labeling conditions
BOCILLIN-FL	PBP activity	Live Gram positive and Gram negative
Ceph C-TAMRA	PBP activity	Live Gram positive
Van-FL	PG stem peptide	Live Gram positive
Ramoplanin-FL	PG stem peptide	Live Gram positive
UDP-MurNAc pentapeptide-FL	Nascent PG	Live Gram positive, permeabilized Gram negative
L-Alanyl-γ-D-glutamyl-L-lysine analog	PG stem peptide	Live Gram negative
FDAA	PG stem peptide	Live and permeabilized Gram positive and Gram negative
Trehalose analogs	Mycomembrane	Live mycobacterium
KDO	LPS	Live Gram negative (as no LPS in Gram positive)
Rhodamine spirolactam, Alexa Fluor 488 succinimidyl ester, Cy3-Cy5-succinimidyl ester	Cell surface amines	Live Gram positive and Gram negative
Noncanonical amino acids	Newly synthesized and secreted proteins	Live or permeabilized Gram negative
Nitro-aryl fluorogen	Nitroreductase activity	Live Gram positive and Gram negative