Detection of Protein-Synthesizing Microorganisms in the Environment via Bioorthogonal Noncanonical Amino Acid Tagging (BONCAT)

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Abstract

Bioorthogonal noncanonical amino acid tagging (BONCAT) is a recently developed method for studying microbial in situ activity. This technique is based on the in vivo incorporation of artificial amino acids that carry modifiable chemical tags into newly synthesized proteins. BONCAT has been demonstrated to be effective in labeling the proteomes of a wide range of taxonomically and physiologically distinct Archaea and bacteria without resulting in preferential synthesis or degradation of proteins. After chemical fixation of cells, surrogate-containing proteins can be detected by whole-cell fluorescence staining using azide-alkyne click chemistry. When used in conjunction with rRNA-targeted fluorescence in situ hybridization (FISH), BONCAT allows the simultaneous taxonomic identification of a microbial cell and its translational activity. Rather than studying the bulk proteome, BONCAT is able to specifically target proteins that have been expressed in reaction to an experimental condition. BONCAT-FISH thus provides researchers with a selective, sensitive, fast, and inexpensive fluorescence microscopy technique for studying microbial in situ activity on an individual cell level.

This protocol provides a detailed description of how to design and perform BONCAT experiments using two different bioorthogonal amino acids, *L*-azidohomoalanine (AHA) and *L*-homopropargylglycine (HPG), which are both surrogates of *L*-methionine. It illustrates how incorporation of these noncanonical amino acids into new proteins can be detected via copper-catalyzed or strain-promoted azide-alkyne click chemistry and outlines how the visualization of translational activity can be combined with the taxonomic identification of cells via FISH. Last, the protocol discusses potential problems that might be encountered during BONCAT studies and how they can be overcome.

Keywords: AHA, Anabolic activity, Bioorthogonal chemistry, Click chemistry, Ecophysiology, FISH, HPG, Protein synthesis, Single cell, Translation

1 Introduction

If the physiology and in situ activity of uncultured cells is to be determined, approaches that target the individual cell level are essential in order to link particular taxonomies to specific functions. While observations on the rRNA and mRNA level have provided us with important insights into the functioning of microbes in the

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environment, protein synthesis is generally considered to be a more reliable marker for cellular activity [1–8]. However, until recently the visualization of the level and location of proteins within uncultured cells was limited to a single technique, immunohistochemistry. This approach, however, requires prior knowledge of the target protein and cannot provide information of the timing of protein synthesis. This is problematic when very slow-growing microorganisms, such as those that inhabit subsurface environments, or proteins with unknown lifetimes are to be studied.

A solution to this problem was recently presented by the adaptation of bioorthogonal noncanonical amino acid tagging (BONCAT) to environmental systems [9]. Developed in the early 2000s for the study of neuron cells [10–12], BONCAT has since then been applied to a range of eukaryotic systems (e.g., [13–17]) and more recently has been used to study a few select microbial pathogens [18–21]. The technique is based on the in vivo incorporation of synthetic (i.e., noncanonical, not biologically produced) amino acids that exploit the substrate promiscuity of the translational machinery, while not negatively interfering with processes within the cell [12]. In a BONCAT experiment, an artificial amino acid that carries a chemically modifiable tag (an azide or alkyne group), such as L-azidohomoalanine (AHA) or L-homopropargylglycine (HPG), which are both surrogates of L-methionine (Fig. 1a) [12], is added to a sample. If the artificial amino acid is taken up by a cell (the exact process of how this happens is currently unknown), it is incorporated into new proteins if the cell is anabolically active. This is possible due to the low specificity of the methionyl-tRNA synthetase, the enzyme that catalyzes the esterification of Met with its tRNA, which to some extent misrecognizes Met for its surrogates [12]. After incorporation, the artificial amino acids can be fluorescently detected via azide-alkyne click chemistry (Fig. 1b–f), a highly selective and biocompatible labeling reaction (for recent reviews, *see* [22–25]).

There are two variants of azide-alkyne click reactions: (1) a Cu (I)-catalyzed cycloaddition reaction [26–28] (Fig. 1b) and (2) a strain-promoted version that exploits the high reactivity of a cyclooctyne system that allows the click reaction to take place in the absence of a catalyst [29, 30]. Both labeling reactions are simple (only a small number of inexpensive chemicals are involved) and fast to perform (1–2 h) and are not influenced by the presence of complex organic (e.g., an extracellular matrix) or inorganic (e.g., minerals or sediment particles) substances. This results in a low level of background noise when applied to environmental samples. While copper-catalyzed click chemistry can be employed to detect both AHA- and HPG-tagged proteins, the strain-promoted click reaction is restricted to the visualization of AHA uptake (because HPG does not contain an azide group).

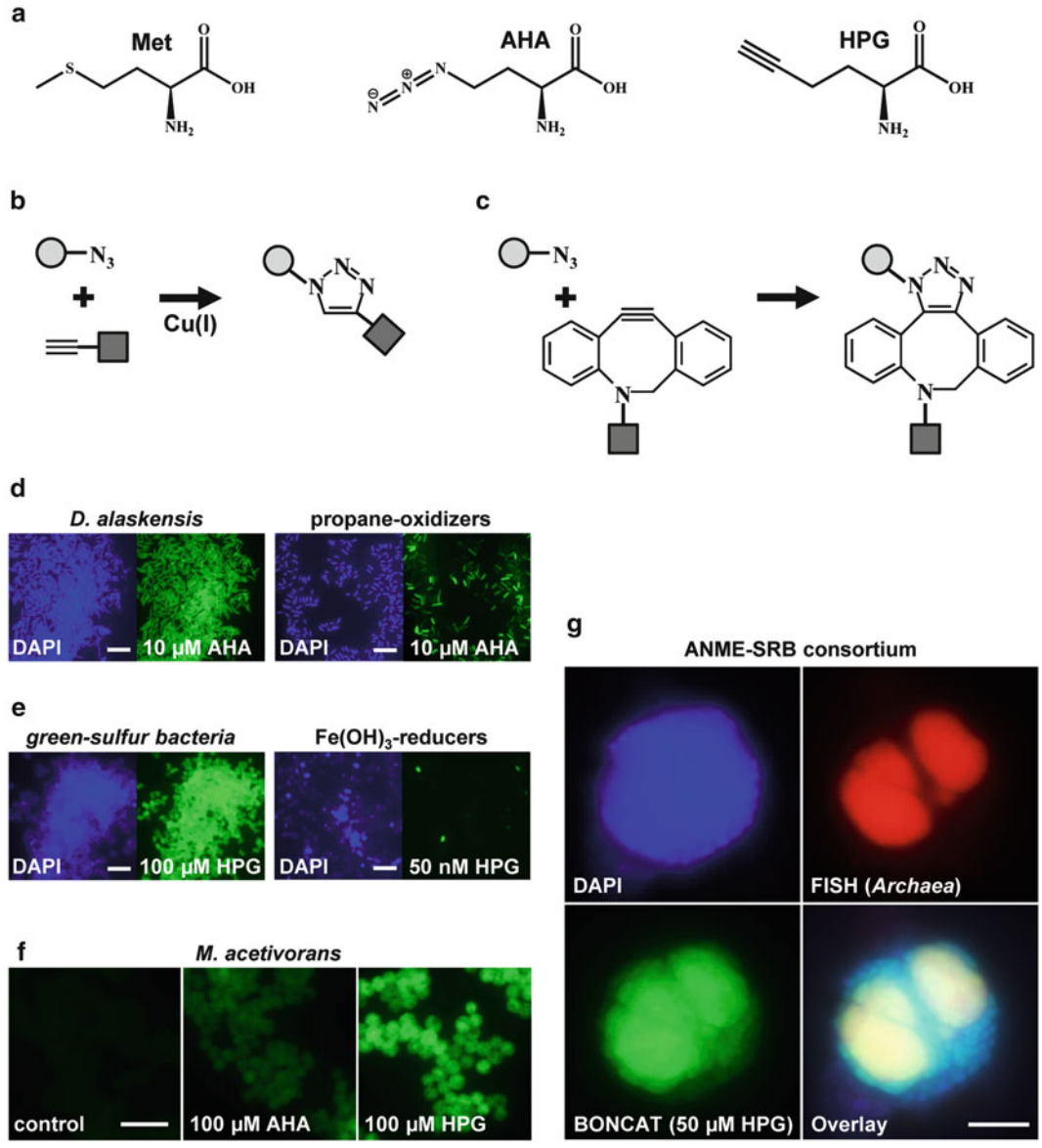

Fig. 1 Overview of BONCAT for visualizing newly made proteins. (**a**) Structures of L-methionine (Met) and its surrogates L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG), which compete with Met during translation. (**b**) In Cu(I)-catalyzed click chemistry, an azide group (N_3) is linked to a terminal alkyne residue, yielding a triazole conjugate. (**c**) Strain-promoted click chemistry allows the copper-less conjugation of an azide group (N_3) with a cyclooctyne-carrying molecule, yielding a triazole conjugate. (**d**) Cultures of the sulfate reducer *Desulfovibrio alaskensis* and an aerobic propane-oxidizing enrichment were incubated for ~1 generation in the presence of AHA. After cell fixation, AHA incorporation was visualized via strain-promoted click chemistry. Scale bars equal 10 μm. (**e**) Enrichment cultures of green sulfur bacteria and ferrihydrite reducers were incubated for ~20 h in the presence of HPG, which was then detected via Cu(I)-catalyzed click chemistry. Scale bars equal 10 μm. (**f**) Demonstration of the low labeling efficiency of AHA as compared to HPG under conditions of both elevated pH (here, pH 7.5) and sulfide (~2 mM), which in combination lead to the

It was recently demonstrated that BONCAT is able to label the proteomes of a range of phylogenetically and metabolically diverse pure and enrichment cultures (e.g., *see* Fig. 1d–g) without resulting in changes to protein expression or degradation [9, 31]. It was shown that the translational activity, as exemplified by AHA labeling, correlates with cellular ^{15}N-ammonia uptake, an established proxy for microbial growth [9]. Most importantly, a protocol was established that combines BONCAT with rRNA-targeted fluorescence in situ hybridization (FISH), allowing one to directly link the identity of a cell with its translational activity in the environment (Fig. 1g; [9]) using only fluorescence microscopy, a standard technique in molecular biology.

In addition to these AHA-centered approaches, HPG has recently been used to visualize protein synthesis in cultured and environmental microbes (examples shown in Fig. 1e–g) ([32]; Hatzenpichler et al., unpublished), and promises to be a valuable alternative to AHA. However, it should be noted that HPG has only been employed in a limited number of studies [15, 32, 33] and that it is more discriminated against by the cellular machinery than AHA [12], which might result in lower labeling efficiency for some microorganisms. AHA, on the other hand, suffers from the problem that long-term incubations of samples characterized by high concentrations of sulfide in combination with alkaline pH are prohibited due to the reduction of the azide group under such conditions (Fig. 1f; [9]). For the study of alkaline (>pH 7.5), highly sulfidic (>1 mM HS^-) habitats, such as marine sediments (Fig. 1g), the use of HPG is recommended.

In this chapter we both describe click chemistry-mediated approaches for detecting newly made proteins via incorporation of AHA or HPG and discuss considerations to be taken when applying this technique to environmental systems.

2 Materials

If not indicated otherwise, all reagents can be stored at room temperature (RT).

Fig. 1 (continued) reduction of the azide group of AHA [9]. Cultures of *Methanosarcina acetivorans* were incubated in the presence of either 100 μM AHA or 100 μM HPG for ~1 generation. Then, Cu(I)-catalyzed click chemistry was performed and microscopic images taken at identical settings. Scale bar equals 5 μm. (**g**) Visualization of newly made proteins in a consortium of anaerobic methanotrophic Archaea (ANME) and sulfate-reducing bacteria (SRB) via a combination of BONCAT (*green*) with 16S rRNA-targeted FISH (*red*). Marine methane seep sediment was incubated in the presence of 50 μM HPG for 5 weeks before Cu(I)-catalyzed click chemistry was performed, which was followed by FISH. DAPI staining in blue. Scale bar equals 5 μm

2.1 Bioorthogonal Amino Acids

1. L-2-amino-4-azidobutanoic acid (L-azidohomoalanine, AHA), Click Chemistry Tools, www.clickchemistrytools.com. Dissolve in nano-pure water, adjust to pH 7.0, filter sterilize (0.2 μm), and store in the dark at 4°C. Prepare stock solutions of 1–100 mM (*see* **Notes 1** and **2**).
2. L-2-amino-5-hexynoic acid (L-homopropargylglycine, HPG), Click Chemistry Tools, www.clickchemistrytools.com. Dissolve in nano-pure water, adjust to pH 7.0, filter sterilize (0.2 μm), and store in the dark at 4°C. Prepare stock solutions of 1–100 mM (*see* **Note 2**).

2.2 Cell Fixation

1. Phosphate buffer: 20:80 (v/v) mix of 200 mM NaH_2PO_4 and 200 mM Na_2HPO_4 in nano-pure water, pH 7.4.
2. Phosphate-buffered saline (PBS): 130 mM NaCl, 5% (v/v) phosphate buffer in nano-pure water, adjust to pH 7.4, and sterilize filter (0.2 μm).
3. Paraformaldehyde 20%, Electron Microscopy Sciences, www.emsdiasum.com. Prepare a solution of 3% paraformaldehyde in PBS for chemical fixation.
4. 1:1 mix of PBS and absolute ethanol (EtOH) for cell storage (1:1 PBS:EtOH).

2.3 Reagents for Cu (I)-Catalyzed Click Chemistry

1. Copper sulfate ($CuSO_4 \times 5\ H_2O$), 20 mM in nano-pure water, sterilize filter (0.2 μm), and store at room temperature or 4°C.
2. Tris[(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (THPTA), Click Chemistry Tools, www.clickchemistrytools.com. 50 mM in nano-pure water, sterilize filter (0.2 μm), and store in aliquots at −20°C.
3. Sodium ascorbate, Sigma-Aldrich, www.sigmaaldrich.com. 100 mM in PBS, make fresh!
4. Aminoguanidine hydrochloride, Sigma-Aldrich, www.sigmaaldrich.com. 100 mM in PBS, make fresh!
5. PBS, pH 7.4 (see above).
6. 50, 80, and 96% ethanol in nano-pure water (for ethanol dehydration series), 50 mL each.

2.4 Reagents for Strain-Promoted Click Chemistry

1. 2-chloroacetamide, 100 mM in PBS (pH 7.4), make fresh!
2. PBS, pH 7.4 (see above).
3. 50, 80, and 96% ethanol in nano-pure water (for ethanol dehydration series), 50 mL each.

2.5 Clickable Dyes

A range of clickable fluorophores is available, including standard dyes such as Cy3, Cy5, Cy5.5, Cy7, TAMRA (similar spectral characteristics to Cy3), and carboxyrhodamine 110 (similar to

Fluos/FITC/FAM) at Click Chemistry Tools, www.clickchemistrytools.com. Oregon Green and Alexa dyes are available at Invitrogen, www.invitrogen.com. See Hatzenpichler et al. (2014) [9] for a comparative analysis of some of these dyes.

1. Alkyne dyes
   For the detection of AHA, dyes featuring either a terminal alkyne (for Cu(I)-catalyzed click chemistry) or a cyclooctyne group (for strain-promoted click chemistry) may be used. Prepare 1–10 mM stock solutions in either dimethylsulfoxide (DMSO) or dimethylformamide (DMF). Make sure to check provider instructions.
2. Azide dyes
   For the detection of HPG, azide-conjugated fluorophores are used. Prepare 1–10 mM stock solutions in either dimethylsulfoxide (DMSO) or dimethylformamide (DMF). Make sure to check provider instructions.

2.6 Carrier Materials

In addition to performing click chemistry in solution, slides (we recommend to use Teflon-coated slides, such as the ones available at Tekdon Inc., www.tekdon.com) or filters (e.g., Millipore, www.millipore.com) can be used, without any detectable differences in signal-to-noise ratio.

2.7 Fluorescence In Situ Hybridization (FISH)

After BONCAT, perform FISH according to standard protocols [9, 34].

2.8 Microscopy and Image Analysis

1. Citifluor AF-1 anti-fading solution, Electron Microscopy Sciences, www.emsdiasum.com.
2. 4,6-diamidino-2-phenylindole (DAPI). Dissolve 1 mg mL^{-1} in Citifluor and store in the dark at 4°C.
3. For image analysis we use a combination of the photo acquisition software provided by the manufacturer of our microscopes (e.g., cellSense Dimension for our Olympus microscope) and the free image analysis and visualization interfaces imageJ (available at http://imagej.nih.gov/ij/) and daime (www.microbial-ecology.net/daime) [35].

3 Methods

3.1 Incubation with Bioorthogonal Amino Acid

Directly add AHA or HPG using a sterile-filtered (0.2 μM), pH-adjusted (pH 7.0) stock solution yielding a final concentration of 1 nM to 1 mM. Always perform replicate experiments and include replicated incubations without AHA/HPG. These controls serve three roles: (1) to determine the background fluorescence from

naturally occurring azides [36] or alkynes [37] in the sample (however, we never observed any reaction of click dyes with natural compounds that is due to their clickable group); (2) to correct for the autofluorescence of cells and certain abiotic materials; and (3) to test for community shifts due to the addition of the bioorthogonal amino acid (e.g., via 16S rRNA gene libraries, tag sequencing, or FISH). This is of particular importance when long-term incubations are performed or high concentrations of AHA/HPG are used (*see* **Notes 3–5**).

3.2 Cell Fixation

Fix cells according to standard protocols [34] immediately after sampling either by (1) fixation in 3% formaldehyde (PFA) in PBS or (2) by resuspending pelleted biomass in a 1:1 mix of PBS:EtOH. For fixation with PFA, pellet the biomass, remove the supernatant (SN), and resuspend cells in 3% PFA in PBS. For aqueous samples, directly add PFA to reach a final concentration of 3% PFA. Fix for either 3 h on ice or 1 h at RT. Afterwards, pellet biomass by centrifugation or filter onto 0.2 μm filters. Wash with PBS to remove remaining PFA, before resuspending biomass in 1:1 PBS:EtOH. Store at −20°C. Make sure to deposit PFA in the chemical waste. For EtOH-fixation, pellet biomass, remove SN, resuspend in 1:1 PBS:EtOH, and store at −20°C.

3.3 Preparing the Sample for Click Labeling

3.3.1 Immobilized Samples

Immobilize biomass either on glass slides or filters. Dry at 46°C or, if not available, at 37°C or RT. Dehydrate and permeabilize cells by sequentially placing slides or filters for 3 min into 50 mL tubes that contain 50, 80, and 96% ethanol. Dry biomass using pressurized air.

3.3.2 Samples in Solution

Pellet sample via centrifugation (16,100*g* or max. setting for 5 min at RT) and resuspend in 250 μL 80% EtOH. Mix by vortex and incubate for 3 min at RT. Add 1.5 mL 96% EtOH, mix by vortex, and incubate for 3 min at RT. Afterwards, pellet sample via centrifugation and resuspend in 221 μL PBS. Removing small volumes of leftover EtOH is not necessary as it does not interfere with the click reaction.

3.4 Fluorescence Labeling of Newly Made Proteins

3.4.1 Cu(I)-Catalyzed Click Chemistry

Cu(I) click chemistry can be used to detect either AHA or HPG, with the only difference being the functional group linked to the fluorescent dye (an alkyne side group for the detection of AHA; a terminal azide if HPG is to be visualized). The copper catalyst is prepared with a chelating ligand, typically THPTA [38, 39], to keep the metal in its Cu(I) oxidation state. Because of the instability of Cu(I) under standard conditions, it is added in large excess (100 μM) and in the presence of the reductant sodium ascorbate. To prevent protein cross-linking and precipitation, aminoguanidine is added. We recommend to perform Cu(I)-catalyzed click

chemistry at a dye concentration of 1–5 μM (final concentration) to guarantee for best signal-to-noise ratios, but substantially lower or higher concentrations can be used, if necessary. We successfully tested concentrations as low as 10 nM and as high as 50 μM.

If using immobilized biomass, after dehydration of the sample, prepare the dye premix by mixing 1.25 μL of 20 mM $CuSO_4$ solution with 2.50 μL of 50 mM THPTA and 0.30 μL of alkyne dye. Allow to react for 3 min at RT in the dark. In the meantime, add 12.5 μL of each 100 mM sodium ascorbate and 100 mM aminoguanidine hydrochloride to 221 μL PBS. Then, add the dye premix and invert the tube once (do not mix by vortex to maintain reducing conditions). Cover the sample with 20 μL of the click solution, transfer the slide into a humid chamber (water on tissue paper), and incubate in the dark at RT for 30 min. Increasing the incubation time is possible, but typically does not increase fluorescence signal. Afterwards, wash the slide or filter three times for 3 min each in PBS-filled 50 mL tubes, before dehydrating it by incubating it for 3 min in 50% EtOH at RT (*see* **Notes 6** and 7).

If the biomass is in solution, all reagents (sodium ascorbate and aminoguanidine, followed after 3 min by the dye premix, final concentrations as described above) are added directly to the sample. Invert tubes once and incubate in the dark at RT for 30 min. Afterwards, wash samples three times with PBS and then one time in 50% EtOH (RT). Between washing steps, pellet samples via centrifugation for 5 min at 16,100*g* (or highest setting) at RT. Finally, resuspend biomass in a 1:1 mix of PBS:EtOH, transfer onto a glass slide, and air-dry (*see* **Notes 6** and 7).

3.4.2 Strain-Promoted (Cu-Free) Click Chemistry

In strain-promoted click chemistry, the reaction rate is increased by using strained dibenzocyclooctyne (DBCO)-conjugated dyes [33, 40]. Strain-promoted click can only be used to detect azide-containing compounds, such as AHA. HPG features an alkyne group and thus can only be detected via Cu(I) click chemistry-enabled conjugation to an azide dye.

If using immobilized biomass, after dehydration of the sample, incubate the slide for 1 h in 100 mM 2-chloroacetamide in PBS at 46°C (or, if not available, 37°C or RT) in the dark to block free thiols (for easy handling, use a 50 mL tube). Then, remove the slide, add DBCO dye reaching a final concentration of 0.1–1 μM, mix by vortex, and incubate the slide in this solution for 30 min at 46°C (or 37°C, RT) in the dark. We successfully tested concentrations as low as 5 nM and as high as 10 μM, but found 100 nM to work best for nearly all samples. Afterwards, wash the slide or filter three times for 3 min each in PBS at RT, before dehydrating it by incubating it for 3 min in 50% EtOH at RT. If high amounts of dye remain, resulting in a high level of background fluorescence, either increase the number, duration, or temperature of PBS-washing steps or incubate for 15 min in a 1:1 mix of PBS:DMSO prior to PBS washing (*see* **Notes 6** and 7).

For strain-promoted click chemistry in the solution, resuspend pelleted biomass in 100 mM 2-chloroacetamide in PBS and incubate for 1 h at 46°C (or, if not available, 37°C or RT) in the dark. After blocking, add DBCO dye to reach a final concentration of 0.1–1 μM and incubate for 30 min at 46°C (or, if not available, 37°C or RT) in the dark. Afterwards wash samples three times with PBS and then one time in 50% EtOH at RT. If high background levels (due to nonremoved dye) are encountered, either increase the number, duration, or temperature of PBS washing or incubate for 15 min in a 1:1 mix of PBS:DMSO prior to the PBS-washing steps. Between washings, pellet samples via centrifugation for 5 min at 16,100*g* (or highest setting) at RT. Finally, resuspend biomass in a 1:1 mix of PBS:EtOH, transfer onto a glass slide, and air-dry (*see* **Notes 6** and **7**).

3.5 Fluorescence In Situ Hybridization

After successfully detecting newly made proteins, perform rRNA-targeted FISH according to standard protocols [9, 34]. If clicked samples had been stored in PBS or 1:1 PBS:EtOH, start with a complete ethanol series (3 min each in 50, 80, and 96% ethanol). If FISH is performed immediately after BONCAT, a single dehydration step for 1 min in 96% ethanol suffices. In order to guarantee specific detection of target species, perform FISH after BONCAT (*see* **Note 8**).

3.6 Microscopy

Dry slides with pressurized air, mount them with DAPI/Citifluor, and analyze samples via fluorescence microscopy. Adjust image acquisition settings in the microscope software according to the level of background fluorescence in the negative control (sample without bioorthogonal amino acid on which click chemistry has been performed). This background is a combination of unspecific autofluorescence of cellular material and some abiotic substances, such as minerals or salts, and fluorescence of nonremoved dye.

4 Notes

1. Vendors
   In the list of Materials, we provide the names of vendors from which we currently purchase reagents. We do not by any means endorse these particular vendors.
2. Click-it®
   Recently, Invitrogen started to offer "Click-It®" kits for the click chemistry-mediated detection of protein synthesis via AHA or HPG incorporation. While we acknowledge that this is a comfortable tool for initially testing the applicability of BONCAT to a new system, we stress that this kit currently is >20× over-priced as compared to when the individual

components (i.e., AHA or HPG, plus THPTA, $CuSO_4$, sodium ascorbate, aminoguanidine, and a clickable dye) are purchased separately.

3. Stability of AHA
   High concentrations (>1 mM) of sulfide in alkaline (pH >7) growth media (e.g., for methanogens) or environments (e.g., marine sediments) will lead to the reduction of the azide group of AHA (Fig. 1f) [9]. Under such conditions, HPG should be used instead of AHA. It should be pointed out that neither alkaline pH nor a high concentration of sulfide alone will negatively affect the stability of AHA [9, 41]. Furthermore, other reductants used in the preparation of anoxic media, such as cysteine or dithionite, have no measurable effect on the stability of AHA (Hatzenpichler and Scheller, unpublished). Likewise, we did not find any detrimental effect of elevated temperature (80 ± 3°C; AHA in water at pH 5.0 and 7.1) to the stability of AHA [9].
4. Methionine-free samples
   AHA and HPG compete with the intracellular Met pool for incorporation into newly made proteins. Thus, BONCAT is not applicable to pure cultures that depend on undefined media (such as Luria Broth) or any other samples with free Met.
5. Incubation time vs. concentration
   The two most important factors that dictate sensitivity of BONCAT are incubation time and concentration of bioorthogonal amino acid. The lowest concentration we have used in our experiments was 50 nM (Hatzenpichler, unpublished), but lower concentrations (8–20 nM HPG) were recently successfully tested in oligotrophic ocean water [32]. In general, incubation time and AHA/HPG concentration depend on the particular system and hypothesis to be tested. For high turnover, nutrient-replete systems, a low concentration of bioorthogonal amino acid is recommended to not interfere with the cellular machinery by overly substituting proteins with amino acid surrogate. However, if employed in a pulse-labeling setup with very short incubations times (minutes to a few hours), much higher concentrations (we successfully tested up to 1 mM) can be used [9]. On the other hand, if the long-term performance of a system is to be studied, regular supply ("re-feeding") of low amounts of substrate is the method of choice.
6. Dealing with background fluorescence
   Despite the fact that we regularly apply BONCAT to a wide range of environmental samples, we only rarely deal with problems of high background fluorescence due to nonremovable click dye. In case you are facing this problem, approach it in the following order: (a) decrease the dye concentration; we

successfully used dye concentrations as low as 10 nM, but best results are usually achieved at 1–5 μM and 0.1–1 μM for Cu(I)-catalyzed and strain-promoted click, respectively; (b) increase the number of washing steps, possibly including DMSO- and ethanol-washing steps (described in the Methods section); and (c) switch to another dye with different spectral characteristics and/or better solubility.

7. Quantifying protein synthesis rate?

   Recently, it was proposed that the fluorescence intensity of click-stained cells can be used to quantify the amount of proteins that have been newly synthesized in situ [32]. We acknowledge that semiquantitative comparisons of cells of a given type (a given species or genus), if coupled to its identification via FISH, are possible. We, however, doubt that absolute quantification (in fg new protein per cell) across many uncultured and unidentified species can be achieved. Most importantly, distinct proteins contribute differently to fluorescence due to contrasting contents of Met, the amino acid replaced by AHA or HPG. In an environmental sample, the average Met content, the individual abundance of Met-rich and Met-poor proteins, as well as the rate by which substitution with the bioorthogonal amino acid occurs are unknown for any particular cell type. The ratio of how much individual proteins (and how many copies thereof) are contributing to total fluorescence thus cannot be known (a single protein with ten Met residues contributes the same to overall fluorescence than ten copies of a single Met protein). Furthermore, the rate at which bioorthogonal amino acids are incorporated into proteins depends on several factors, most importantly the ability to take up the surrogate amino acid (unknown for both AHA and HPG) and load it onto the respective tRNA, a reaction that is achieved by methionyl-tRNA synthase. The activation rate of this enzyme currently is only known for *E. coli* [12] and might differ substantially in physiologically and taxonomically distinct microbes. Together, these limitations prohibit the absolute quantification of newly made protein within individual, uncultured cells.

8. FISH-BONCAT vs. BONCAT-FISH

   If your experimental setup demands that FISH is performed before detecting newly made proteins via click chemistry, adjust the salt concentration of the click buffer and all washing buffers to provide stringent conditions throughout the click protocol. When doing so, consider that every probe has different stringency conditions and that FISH usually is performed at higher temperatures (46 and 48°C for hybridization and washing, respectively) than BONCAT (i.e., consider concentrations of both salt and formamide as well as temperature).

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