Supporting Information for: Bioorthogonal Correlative Light-Electron Microscopy of *Mycobacterium tuberculosis* in macrophages reveals the effect of anti-tuberculosis drugs on subcellular bacterial distribution

Thomas Bakkum¹, Matthias T. Heemskerk², Erik Bos³, G. J. Mirjam Groenewold¹, Nikolaos Oikonomeas-Koppasis¹, Kimberley V. Walburg², Suzanne van Veen², Martijn J.C. van der Lienden¹, Tyrza van Leeuwen¹, Marielle C. Haks², Tom H.M. Ottenhoff², Abraham J Koster³, Sander I. van Kasteren¹,*

Author address: ¹Leiden Institute of Chemistry and The Institute of Chemical Immunology, Leiden University, Einsteinweg 55, Leiden, The Netherlands; ²Department of Infectious Diseases, Leiden University Medical Center, Albinusdreef 2, 2333 ZC, Leiden, The Netherlands; ³Department of Cell and Chemical Biology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, The Netherlands

*Keywords: Mycobacterium tuberculosis, Mtb, Correlative Light-Electron Microscopy, CLEM, bioorthogonal chemistry, click reaction, metabolic labeling*

ABSTRACT: Bioorthogonal correlative light-electron microscopy (B-CLEM) can give a detailed overview of multi-component biological systems. It can provide information on the ultrastructural context of bioorthogonal handles and other fluorescent signals, as well as information about subcellular organization. We have here applied B-CLEM to the study of the intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*) by generating a triply labelled *Mtb* through combined metabolic labeling of the cell wall and the proteome of a DsRed-expressing *Mtb* strain. Study of this pathogen in a B-CLEM setting was used to provide information about the intracellular distribution of the pathogen, as well as its *in situ* response to various clinical antibiotics, supported by flow cytometric analysis of the bacteria, after recovery from the host cell (*ex cellula*). The RNA polymerase-targeting drug rifampicin displayed the most prominent effect on subcellular distribution, suggesting the most direct effect on pathogenicity and/or viability, while the cell wall synthesis-targeting drugs isoniazid and ethambutol effectively rescued bacterial division-induced loss of metabolic labels. The three drugs combined did not give a more pronounced effect but rather an intermediate response, whereas gentamicin displayed a surprisingly strong additive effect on subcellular distribution.

Pages: 22
Figures: 12
Tables: 3
**Methods.**

**Safety statement.** All biological experiments with *M. tuberculosis* described in this study must be performed under Bio Safety Level 3 conditions. Following fixation and disinfection of the tubes, further sample preparation for CLEM was performed under normal laboratory conditions. No unexpected or unusually high safety hazards were encountered.

**Reagents.** Difco Middlebrook 7H9 broth and ADC growth supplement were purchased from Becton Dickinson, Breda, The Netherlands. Hygromycin B, gentamicin and azide- or alkyne-modified Alexa Fluor dyes (AF488 and AF647) were purchased from Thermo Fisher Scientific, Bleiswijk, The Netherlands. Dulbecco’s modified Eagle medium (DMEM), GlutaMAX, Copper(II) sulfate pentahydrate, (+)-sodium L-ascorbate, tris(3-hydroxypropytriazolylmethyl)amine (THPTA), aminoguanidine hydrochloride, paraformaldehyde, glycine, gelatin type A bloom 300 (gelatin), cold water fish skin gelatin, Lysozyme from hen egg white, bovine serum albumin (BSA), IGEPAL CA-630, Triton-X100 and Tween-80 were purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands. D-propargylglycine (alkynyl-d-alanine; alkDala) was purchased from Combi-Blocks, San Diego, USA. EM-grade 8% paraformaldehyde and EM-grade 8% glutaraldehyde were purchased from Electron Microscopy Sciences, Hatfield, USA. Fetal calf serum (FCS) was purchased from VWR International, Amsterdam, The Netherlands. Penicillin G sodium and streptomycin sulphate were purchased from Duchefa, Haarlem, The Netherlands.

**Organic synthesis.**

**Synthesis of (S)-2-amino-4-azidobutanoic acid (L-azidohomoalanine; Aha).**

\[
\begin{align*}
\text{N}_3 & \quad \text{O} \\
\text{NH}_2 & \quad \text{OH}
\end{align*}
\]

Aha was synthesized according to a previously described procedure by Zhang et al., 2010. \(^1\) \(^{1}\text{H}-\text{NMR (D}_2\text{O), 400 MHz: } \delta [\text{ppm}] = 4.05 (\text{t, 1H, } \alpha-\text{CH}), 3.55 (\text{t, 2H, } \gamma-\text{CH}_2), 2.15 (\text{m, 2H, } \beta-\text{CH}_2).

**Synthesis of (S)-2-Aminohex-5-ynoic acid (L-homopropargylglycine; Hpg).**

\[
\begin{align*}
\text{O} & \quad \text{NH}_2 \\
\text{C} & \quad \text{OH}
\end{align*}
\]

Hpg was synthesized according to previously described procedure by Li et al.\(^2\), adjusted to obtain the chirally pure L-Hpg variant based on Chenault et al.\(^3\), Biagini et al.\(^4\) and Dong et al.\(^5\).
Chiral deprotection of N-acetyl-DL-homopropargylglycine (2-acetamidohex-5-ynoic acid). A solution of 303 mg (1.13 mmol, 1 eq.) N-acetyl-DL-homopropargylglycine in 20 mL H$_2$O and adjusted to pH 7.5 using 1M NH$_4$OH. 1 mg kidney acylase I (≥2000 units/mg) was added and the mixture was stirred for 16 h at 37 °C. The enzyme was recovered by centrifugation dialysis, using a 10kDa membrane at 4000 rpm for 35 min at 10 °C. Next, the solution was acidified to pH 3 with 2M HCl and extracted with 3 x 20 mL diethyl ether. The organic layers were concentrated to retrieve the N-acetyl-D-homopropargylglycine. The aqueous layer was loaded on a pre-washed and regenerated Dowex 50WX8 cation exchange resin (60 mL). The column was washed with 5 x bed volume of water, maintaining a pH of 5.5 at the exit and eluted with 200 mL 1.5M NH$_4$OH. Product was detected by TLC and the eluate was concentrated and lyophilized to yield chirally pure L-Hpg (68 mg, 0.535 mmol, 95%) as a white powder.

$^1$H NMR (400MHz, D$_2$O): δ [ppm] = 4.11 (t, J = 6.4 Hz, 1H), 2.42 – 2.36 (m, 2H), 2.36 (s, 1H), 2.19 – 2.10 (m, 1H), 2.07 – 1.98 (m, 1H); 13C NMR (101MHz, D$_2$O): δ 82.28, 71.16, 52.05, 28.44, 14.16; HRMS (ESI): C$_6$H$_9$NO$_2$ [M+H]$^+$ 128.06, found 128.07; [α]$_{20}$D: +32.4 (c = 1, 1 M HCl); Ref. 5: +28 (c = 1, 1 M HCl).

Biological experiments.

**Bacterial culture, metabolic labeling and viability assessment based on growth rate.** *Mycobacterium tuberculosis* (*Mtb*) strain H37Rv, expressing DsRed from a pSMT3[Phsp60/DsRed] plasmid$^6$, was cultured in Difco Middlebrook 7H9 broth with 10% ADC, 0.05% Tween-80, 0.2% glycerol and 50 µg/mL hygromycin B (Thermo Fisher Scientific) shaking at 37°C. Fresh cultures were inoculated from glycerol stocks every 2 months due to loss of DsRed expression over time. For metabolic labeling of *Mtb*, cultures were supplemented with 4 mM Hpg, 4 mM Aha, 5 mM alkDala or a combination of 4 mM Aha and 5 mM alkDala (‘dual’) and incubated under normal culturing conditions. After 0h, 8h, 24h, 48h, 72h and 144h, bacterial growth was assessed by OD$_{600}$ measurement and normalized on the first time point to determine the growth rate in the presence or absence of metabolic labels. These growth rates were plotted as percentages of the unlabeled *Mtb* control culture. After the last time point, the bacteria were collected by centrifugation (15 min at 3200 rcf) and resuspended in fresh 7H9 to assess the bacterial growth recovery after label incorporation, over another incubation period of 144h.

**Mammalian cell culture and infection experiments.** RAW 264.7 cells (ATCC TIB-71), a mouse monocyte/macrophage cell line, were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FCS, GlutaMAX, penicillin 100 I.U./mL and streptomycin 50 µg/mL and incubated at 37°C, 5% CO$_2$. For infection experiments, 10 million cells were seeded on a 10 cm dish for each condition in minimal medium (DMEM, 10% FCS, GlutaMAX), allowed to attach for 8 hours and pre-stimulated with 25 ng/mL LPS-B4 (Sigma-Aldrich) for an additional 18 hours. Cells were infected with triple label *Mtb* at an MOI of 25 for 1 hour, washed three times with minimal medium containing 30 µg/mL gentamicin and incubated for 24 hours, in the presence or absence of 5 µg/mL gentamicin. For intracellular treatment with antibiotics, 1
µg/mL (1.2 µM) rifampicin, 2 µg/mL (14.6 µM) isoniazid, 5 µg/mL (18.0 µM) ethambutol or a combination of the three (‘triple-antibiotics cocktail’), was added to the medium during the 24-hour incubation post-infection. For in vitro Mtb treatment with antibiotics, triple label Mtb were incubated for 24 hours in minimal cell medium containing the triple-antibiotics cocktail, washed once with fresh minimal cell medium and subsequently added to the cells for 1 hour to allow phagocytosis (infection).

Analysis of label incorporation by in-gel fluorescence. Mtb expressing DsRed were metabolically labeled as described above and samples of OD<sub>600</sub> ≈ 0.5 were collected after 8h, 24h and 48h to analyze the label incorporation levels into the bacterial proteome, by in-gel fluorescence. Bacterial samples were pelleted by centrifugation (10 min at 9000 rcf), washed once with PBS and resuspended in 100 µL lysis buffer. Lysis buffer and conditions were varied (Table S1) to allow for optimal killing and recovery of bacterial proteins, while maintaining compatibility with subsequent copper(I)-catalyzed Huisgen cycloaddition (ccHc or ‘click’) reaction. Optimal lysis and protein recovery was achieved with 1% SDS and immediate heat treatment (30 min at 80°C). Protein concentration of the lysates was determined using Qubit Protein Assay (Thermo Fisher Scientific) and 10 µg protein was diluted to 20 µL total volume. Lysates were reacted with AF647-azide (Hpg/alkDala) or alkyne (Aha/dual) by ccHc, through direct addition of 10 µL 3X concentrated ‘click cocktail’ (3 mM copper sulfate, 30 mM sodium ascorbate, 3 mM THPTA ligand, 30 mM aminoguanidine, 100 mM HEPES pH 8 and 15 µM fluorophore; mixed in this exact order) and incubated for 1 hour at room temperature. The reaction was quenched by adding 10 µL 4X concentrated Laemmli sample buffer and boiling for 5-10 min at 95°C, followed by brief cooling on ice. 10 µL of the fluorophore-labeled lysates was loaded on a 15-slot 12.5% gel and separated by SDS-PAGE. In-gel fluorescence was measured on a Bio-Rad Chemidoc MP Imaging System (Bio-Rad Laboratories), followed by Coomassie Brilliant Blue staining and imaging as a protein loading control. Gel images were analyzed with Bio-Rad Image Lab Software (Bio-Rad Laboratories).

Analysis of label incorporation by flow cytometry. Mtb expressing DsRed were metabolically labeled as described above and samples of OD<sub>600</sub> ≈ 0.5 were collected after 8h, 24h and 48h to analyze the label incorporation levels per bacterium, by flow cytometry. Bacterial samples were pelleted by centrifugation (10 min at 9000 rcf), washed once with PBS and resuspended in 100 µL fixative. Fixation and permeabilization conditions were varied (Table S2) to allow for optimal penetration of click fluorophores into the fixed bacteria, to reach the targeted bioorthogonal labels. Washing conditions were varied (Table S3) to allow for optimal resuspension and recovery of fixed bacteria, using an anti-clumping additive. Optimal fluorophore penetration and recovery of fixed bacteria were achieved by pre-permeabilizing the bacteria with 1% SDS in PBS for 15 min at room temperature, followed by fixation with 4% paraformaldehyde in PBS for 24 hours at room temperature, after collecting the bacteria by centrifugation (10 min at 9000 rcf). Fixed bacteria were collected by low-speed centrifugation (10 min at 2000 rcf) and stored in 0.5% paraformaldehyde in PBS at 4°C until all time points were collected. Low-speed centrifugation was used to collect the fixed bacteria in between all further steps, to avoid damage due to
excessive G-forces. Bacteria were washed once with PBS to remove residual paraformaldehyde and once with FACS buffer (0.1% BSA, 20 mM glycine in PBS) to quench free aldehydes. The first ccHc reaction, with AF647-azide, was performed, by resuspending the bacteria in ‘click cocktail’ (1 mM copper sulfate, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM aminoguanidine, 100 mM HEPES pH 8 and 5 µM fluorophore; mixed in this exact order), and incubating for 1 hour at room temperature. After a single wash with FACS buffer, the second ccHc reaction, with AF488-alkyne, was performed in the same manner. After another wash with FACS buffer, the bacteria were resuspended in blocking buffer (1% BSA, 10 mM EDTA in PBS) and incubated for 30 min at room temperature, to assist the removal of non-specifically bound fluorophores. After a final wash with FACS buffer, the bacteria were resuspended in FACS buffer and measured on a BD FACSLyric Flow Cytometer (BD Biosciences). The UV-autofluorescence of Mtb was detected in the V450 channel, Aha-AF488 was detected in the FITC channel, DsRed was detected in the PE channel and alkDala-AF647 was detected in the APC channel. Automatic compensation was provided by the integrated BD FACSuite software (BD Biosciences) and all subsequent analysis was performed with FlowJo V10 (FlowJo software). The measured events were gated on size, shape and fluorescence to accurately select single bacteria (Fig. 2A). Quantification of label incorporation was achieved by selecting the median fluorescence intensity (MFI) of the major [Aha+/alkDala+] population for dually-labeled Mtb or the major [Aha-/alkDala-] population for unlabeled Mtb (Fig. 2A, Fig. S2).

**Preparation of single-bacteria samples for CLEM.** Mtb expressing DsRed were metabolically labeled as described above and samples of 50 mL were collected after 1h, 24h and 48h to analyze the label incorporation levels into the bacterial proteome and cell wall, by on-section ccHc reaction. Unlabeled control bacteria were included as a control for background fluorescence of on-section ccHc reaction. Bacteria were collected by centrifugation (15 min 3000 rcf), washed once with PBS and resuspended in a fixation solution optimized for transmission electron microscopy (TEM) (2% EM-grade paraformaldehyde, 0.2% EM-grade glutaraldehyde in 0.1M phosphate buffer pH 7.2) and rotated for 2 hours at room temperature. The fixed bacteria were collected by centrifugation and stored in storage buffer (0.5% EM-grade paraformaldehyde in 0.1M phosphate buffer pH 7.2) at 4°C until all time points were collected. The fixed bacteria were washed with PBS and aldehyde residues were quenched with 20 mM glycine in PBS. Next, the bacteria were collected by centrifugation, resuspended in warm 1% gelatin in PBS, transferred to a 1.5 mL Eppendorf tube, resuspended in warm 12% gelatin in PBS and pelleted by centrifugation (3 min at 5000 rcf). After jellification on ice, the sample pellet was cut off from the tube and cut in half with a razor knife. Sample cubes of approx. 1 mm² were prepared and rotated in a 2.3 M sucrose solution for 18 hours to allow for sucrose infiltration, as a cryo-protectant, followed by plunge-freezing the cubes on metal support pins.

**Preparation of Mtb-infected macrophage samples for CLEM.** Following infection and post-infection incubation of triple labeled Mtb in RAW 264.7 cells, in the presence or absence of antibiotics, the cells were washed three times with minimal cell medium containing 30 µg/mL gentamicin and once with PBS.
The cells were fixed with TEM-optimized fixation solution (2% EM-grade paraformaldehyde, 0.2% EM-grade glutaraldehyde in 0.1M phosphate buffer pH 7.2) for 2 hours at room temperature, rinsed with PBS and stored in storage buffer (0.5% EM-grade paraformaldehyde in 0.1M phosphate buffer pH 7.2) at 4°C until all time points were collected. Fixed cells were rinsed with PBS, harvested in warm 1% gelatin in PBS with cell scrapers and transferred to a 15 mL Falcon tube. The cells were collected by centrifugation, resuspended in warm 1% gelatin in PBS, transferred to a 1.5 mL Eppendorf tube, resuspended in warm 12% gelatin in PBS and pelleted by centrifugation (3 min at 800 rcf). Samples were further processed for plunge-freezing as described for the bacteria-only samples above.

**Cryo-sectioning and on-section ccHc reaction.** Ultrathin (75 nm) cryo-sections were prepared according to the Tokuyasu technique \(^7,8\), using a cryo-ultramicrotome (Leica) and diamond knife (Diatome). Sections were thawed on a droplet of pickup fluid (1.15 M sucrose, 1% methylcellulose) and transferred to a Formvar/carbon-coated titanium TEM grid, pre-coated with blue 0.2 µm FluoSpheres (Thermo Fisher) as fiducial markers. Thawed cryo-sections attached to the TEM grid were incubated on 1% gelatin in PBS for 30 min at 37°C, followed by several washing steps with 20 mM glycine in PBS and subsequent ccHc reaction with AF647-azide (or AF647-alkyne for analysis of label incorporation) on a droplet of ‘click cocktail’ (1 mM copper sulfate, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM aminoguanidine, 100 mM HEPES pH 8 and 5 µM fluorophore; mixed in this exact order), for 1 hour at room temperature. After several washes with PBS, the second ccHc reaction with AF488-alkyne was performed, followed by several washed with PBS. The sections were then incubated on droplets of 1% BSA in PBS for 3 x 10 min at room temperature, to assist the removal of non-specifically bound fluorophores. Nuclear staining of the host cells was achieved by incubating the sections on a droplet of 0.2 µg/mL DAPI for 5 min.

**Confocal microscopy.** After several washes with PBS, the fluorescently-labeled sections attached to TEM grids were mounted in water containing 30% glycerol between a microscopy slide and a coverslip, and imaged on an Andor Dragonfly 505 Spinning Disk Confocal (Oxford Instruments), containing an 8-line integrated laser engine, on a Leica DMi8 inverted microscope equipped with a 100X/1.47 HC PL APO TIRF-corrected oil objective. FluoSpheres were excited with the 405 line and collected with the 450/50 BP emission filter, AF488 was excited with the 488 line and collected with the 525/50 BP emission filter, DsRed was excited with the 561 line and collected with the 620/60 BP emission filter and AF647 was excited with the 637 line and collected with the 700/75 BP emission filter. Images were acquired with the Zyla 2048x2048 sCMOS camera and 2x2 camera binning controlled with the integrated Fusion software. Z-series optical sections were collected with a system-optimized step-size of 0.13 microns and deconvolved using the integrated ClearView-GPUTM deconvolution software. Z-series are displayed as maximum z-projections, and gamma, brightness and contrast were carefully adjusted (identically for compared image sets) using FIJI.\(^9\) Note that fluorescence images with insufficient brightness/contrast will result in a poor quality fluorescence image after correlation.
TEM imaging and correlation. After acquiring the fluorescence microscopy (FM) images, the TEM grids containing the sections were recovered from the microscopy slides, rinsed in distilled water and incubated for 5 min on droplets of uranyl acetate/methylcellulose. The negatively-stained sections were then imaged on a FEI Tecnai 12 BioTwin TEM System (FEI Technologies) at 120 kV acceleration voltage. Correlation of FM and TEM images was performed in Adobe Photoshop CC 2020 (Adobe Systems). The separate fluorescence channels were imported as layers, set to overlay mode ‘Lighten’, then grouped and set to overlay mode ‘Color’, placed on top of the TEM image. Transformation of the FM image to match the TEM image was achieved by isotropic scaling with interpolation setting ‘bicubic smoother’, translation and rotation. Alignment at low magnification was guided by the grid bars and sample shape for single bacteria or nuclei for Mtb-infected cells. Alignment at high magnification was guided by the fiducial beads and the position of the bacteria. Minor manual corrections for chromatic aberration were applied where required, guided by the obvious bacterial shapes, showing a clear internal [DsRed+/Aha+] part and a ring-like [alkDala+] cell wall.

Quantification of intracellular Mtb localization and shape in situ by CLEM-based data analysis. The intracellular localization and shape of triple label Mtb, in the presence or absence of antibiotics, was quantified by manual counting of bacteria in the large CLEM image, using Adobe Photoshop CC 2020 (Adobe Systems). Fluorescence was used to guide the detection of bacteria, and the geometrical shapes tool (‘rectangle’, ‘rounded rectangle’, ‘ellipse’, ‘polygon’ and ‘custom shape’) was used to classify bacteria and host cells based on their appearance and localization, which allowed for facile automatic counting for each of the categories (geometric shapes) by Photoshop. The complete large field of view CLEM image was used for quantification, which allowed for a large data set of over 500 bacteria per condition. The distribution of intracellular bacteria was classified as either ‘no vacuole’, ‘small/tight vacuole’ or ‘large/spacious vacuole’ to allow unbiased counting, and plotted as percentage relative to the total number of intracellular bacteria counted. Extracellular bacteria were counted separately and plotted as percentage relative to the total number of intracellular and extracellular bacteria counted. The appearance (bacterial profile) of intracellular bacteria was classified as either ‘regular shape’ or ‘irregular shape’ to allow unbiased counting, and plotted as percentage relative to the total number of bacteria counted. Host cell death was quantified by counting disintegrated cell profiles, still containing a DAPI-positive compartment to allow unbiased counting, and plotted as percentage relative to the total number of nucleus-containing cell profiles counted. Each of the conditions was tested at least twice but the quantified data were obtained from a single experiment in which all conditions were tested simultaneously in parallel, to avoid technical variations.

Quantification of intracellular Mtb size and label retention in situ by CLEM-based data analysis. Label retention of intracellular triple label Mtb, in the presence or absence of antibiotics, was quantified by semi-automatic analysis of the fluorescence (DsRed, Aha-AF488, alkDala-AF647). To this end, a JavaScript was created using the Adobe Photoshop CC Script Listener Plug-in for Windows
Quantification of Mtb label retention by flow cytometry after recovery from host cell (ex cellula). Following infection and post-infection incubation of triple labeled Mtb in RAW 264.7 cells, in the presence or absence of antibiotics (identical to CLEM experiment), the cells were washed three times with minimal cell medium containing 30 µg/mL gentamicin and once with PBS. Cells were lysed with lysis buffer (0.1% Triton-X100, 150 mM NaCl, 20 mM Tris-HCl) for 15 min at room temperature and the cell debris was separated from the bacteria by centrifugation (5 min at 600 rcf) and pellet discarded. The bacteria were collected by centrifugation (15 min at 3000 rcf), pre-permeabilized with 1% SDS in PBS and fixed with 4% paraformaldehyde in PBS for 24 hours at room temperature. Fixed bacteria were collected by low-speed centrifugation (10 min at 2000 rcf) and stored in 0.5% paraformaldehyde in PBS at 4°C until all time points were collected. The isolated bacteria were further processed and measured by flow cytometry, as described for the freshly labeled Mtb above. The measured events were gated on size, shape and fluorescence to accurately select single bacteria (Fig. 2A). Quantification of label retention was achieved by selecting the MFI of the major [Aha+/alkDala+] population and corresponding [autofluorescence+/DsRed+] population (Fig. 6B, Fig. S12). Each of the conditions was tested at least twice but the quantified data were obtained from a single experiment in which all conditions were tested simultaneously in parallel, to avoid technical variations.

Statistics. The unpaired two-tailed Mann-Whitney U test and Chi-Square ($\chi^2$) test were performed using GraphPad Prism 8 (GraphPad Software). The Benjamini-Hochberg procedure for multiple testing was performed in Microsoft Excel 2016 (Microsoft), with a False Discovery Rate (FDR) of 0.1.
Supplemental tables.

Table S1. Optimization of lysis conditions for analysis of label incorporation by in-gel fluorescence.

| Lysis conditions | Fluorescence | Protein recovery |
|------------------|--------------|------------------|
| 1% IGEPAL*¹      | +/-          | -²               |
| 1% SDS¹          | +/-          | -²               |
| 5% SDS¹          | +/-          | -²               |
| Lysozyme³; 1% IGEPAL*¹ | +    | +/-²            |
| Lysozyme³; 10% IGEPAL*¹ | -    | -²              |
| Lysozyme³; 1% IGEPAL* + 1% SDS¹ | +       | ++/-            |
| Lysozyme³; heat⁴ | -            | -                |
| Lysozyme³; 4% IGEPAL* + heat⁴ | -      | -                |
| Lysozyme³; 4% SDS + heat⁴ | +      | +/-             |
| Lysozyme³; 4% CHAPS + heat⁴ | -      | -                |
| Lysozyme³; 6M urea + heat⁴ | +/-    | -                |
| Lysozyme³; 1M NaCl + heat⁴ | -      | -                |
| Lysozyme³; 4% CHAPS + 6M urea + 1M NaCl + heat⁴ | +/-   | -                |
| Lysozyme³; 4% CHAPS + 4% IGEPAL* + 4% SDS + heat⁴ | +/-   | +/-             |
| 1% SDS + heat⁴   | +            | ++               |
| 1% SDS 30 min at 4°C; heat⁴ | +/-   | ++               |
| 1% SDS 18 hours at -30°C; heat⁴ | +/-   | ++               |
| 1% SDS added to growth culture + heat⁴ | +/-   | +++⁵             |

* = IGEPAL CA-630; ¹ = in 150 mM NaCl, 50 mM HEPES pH 8, O/N at 4°C; ² = after 0.2 µm filtration to remove live/intact bacteria, due to insufficient killing of Mtb (!); ³ = 2 mg/mL lysozyme for 1 hour at 37°C; ⁴ = 30 min at 80°C, to guarantee sufficient killing of Mtb (!); ⁵ = may include both interesting secreted proteins and potentially unwanted proteins from growth culture (dismissed).

Table S2. Optimization of fixation and permeabilization conditions for analysis of label incorporation by flow cytometry.

| Fixation conditions | Permeabilization conditions | Aha-click positive |
|---------------------|-----------------------------|--------------------|
| 2% PFA¹             | Lysozyme²; 0.1% IGEPAL*³    | 7%⁴                |
| 2% PFA¹             | Lysozyme²; 1% SDS³          | 18%⁴               |
| 2% PFA¹             | Lysozyme²; 0.1% IGEPAL* + 1% SDS³ | 12%⁴  |
| 2% PFA¹             | Lysozyme²; 1% IGEPAL* + 1% SDS³ | 9%⁴              |
| 2% PFA¹             | Lysozyme²; 1% SDS³          | 14%⁴               |
| 2% PFA¹             | Lysozyme²; 2% SDS³          | 22%⁴               |
| 2% PFA¹             | Lysozyme²; 4% SDS³          | 21%⁴               |
| 2% PFA¹             | Lysozyme²; 1% SDS + 5 mM EDTA³ | 19%⁴               |
| 2% PFA¹             | Lysozyme²; 2% SDS + 5 mM EDTA³ | 26%⁴              |
| 2% PFA¹             | Lysozyme²; 1% SDS + 1% EtOH³ | 16%⁴               |
| 2% PFA¹             | Lysozyme²; 2% SDS + 1% EtOH³ | 18%⁴               |
| Anti-clumping additive | Effect on aggregation | Effect on yield |
|------------------------|-----------------------|-----------------|
| 0.1% Tween\# in PBS    | ++                    | +/- 1           |
| 0.1% SDS in PBS        | +                     | - 2             |
| 0.1% gelatin\† in PBS | +                     | +/-             |
| **0.1% BSA in PBS**    | +                     | +               |

\* = IGEPA LA CA-630; \# = Tween-80; † = Triton-X100; \(1\) = in PBS for \(\geq 18\) h at 4°C, unless otherwise specified; 
\(2\) = 2 mg/mL lysozyme for 60 min at 37°C, unless otherwise specified; 
\(3\) = in PBS; 
\(4\) = no anti-clumping agent used, resulting in a very low yield after staining; 
\(5\) = 0.1% SDS used as anti-clumping agent during all washing steps; 
\(6\) = 0.1% Tween-80 used as anti-clumping agent during all washing steps; 
\(7\) = 0.1% BSA used as anti-clumping agent during all washing steps; 
\(8\) = higher signal observed for alkDala, compared to other conditions.

Table S3. Anti-clumping additives to reduce hydrophobic aggregation of fixed *Mtb* during washing steps, during sample preparation for flow cytometry.

---

---

S10
**Supplemental figures.**

**Figure S1.** Growth recovery of *Mtb* after 144h label incorporation relative to control. DsRed-expressing *Mtb* H37Rv were first incubated with 4 mM Hpg, 4 mM Aha, 5 mM alkDala or a combination of 4 mM Aha and 5 mM alkDala (dual), for 144h in Middlebrook 7H9 broth (shown in Fig. 1A). The medium was then replaced by fresh 7H9 broth without metabolic labels and the bacterial growth recovery after labeling was assessed for another 144h by OD\textsubscript{600} measurements, normalized on the first time point. The number of biological replicates for each OD\textsubscript{600} measurement is indicated above the bar.
Figure S2. Controls of label incorporation over time by flow cytometry. DsRed-expressing *Mtb* H37Rv were incubated with a combination of 4 mM Aha and 5 mM alkDala or without labels, for the indicated time in Middlebrook 7H9 broth. Bacterial samples of 0.5 OD were collected, pre-permeabilized with 1% SDS and fixed in 4% paraformaldehyde. Fixed and permeabilized bacteria were reacted with AF647-azide (alkDala) and AF488-alkyne (Aha) by two sequential ccHc reactions. The effect of label incorporation on DsRed fluorescence was assessed after 8 h (A), 24 h (B) and 48 h (C) incubation and compared to incubation without metabolic labels after 8 h (D), 24 h (E) and 48 h (F). The background fluorescence of the ccHc (click) reaction was analyzed after incubation without metabolic labels for 8 h (G), 24 h (H) and 48 h (I). J: The effect of label incorporation on DsRed fluorescence over time is shown as the median fluorescence intensity (MFI), on the same scale as used in Fig. 2A for comparison. K: The relative change in label incorporation over time and the resulting effect on DsRed fluorescence are shown as normalized MFI values, corresponding to the MFI values presented in Fig. 2A and panel J of this figure.
Figure S3. Controls of label incorporation over time by flow cytometry in presence of increasing concentrations of Rifampicin. DsRed-expressing *Mtb* H37Rv were cultured and analyzed as for Figure S2 after pretreatment with for either 1h (A-E) or 24h (F-J) with rifampicin in increasing concentrations.
Figure S4. Controls of label incorporation over time by flow cytometry in presence of increasing concentrations of D-cycloserine. DsRed-expressing Mtb H37Rv were cultured and analyzed as for Figure S2 after pretreatment with for either 1h (A-E) or 24h (F-J) with D-cycloserine in increasing concentrations.
Figure S5. Controls of label incorporation by flow cytometry after heat-killing or PFA-fixation. A) live cell positive control; B) no-label negative control; C/D) Heat-killed bacteria (30 min at 80 °C) with (C), or without (D) the dual label; E/F) after 30 min in 4% PFA.
**Figure S6.** Signal and background fluorescence of on-section click reactions. Triple label *Mtb* and DsRed-only *Mtb* were prepared, followed by cryo-sectioning and dual on-section ccHc reaction with AF647-azide (alkDala) and AF488-alkyne (Aha), sequentially. The resulting confocal microscopy images are shown as separated fluorescence channels for clarity. All scale bars represent 5 µm.

**Figure S7.** Large area CLEM image of *Mtb* *in vitro*, corresponding to the details presented in Fig. 3. Triple label *Mtb* and DsRed-only *Mtb* were prepared and subsequently prepared for cryo-sectioning, followed by cryo-sectioning and dual on-section ccHc reaction with AF647-azide (alkDala) and AF488-alkyne (Aha), sequentially. The sections were imaged by confocal microscopy, then stained with uranyl acetate and imaged by TEM. The
resulting fluorescence and TEM images were correlated in Photoshop to obtain the CLEM image, shown in the bottom panel. The top panel shows the corresponding fluorescence channels separately for clarity. All scale bars represent 5 µm.

Figure S8. Stepwise zooming in from an ultra large field of view CLEM image, corresponding to the details presented in Fig. 4. RAW 264.7 macrophages were infected with triple label *Mtb*, incubated for 24 hours without antibiotics, and subsequently prepared for cryo-sectioning, followed by dual on-section ccHc reaction with AF647-azide and AF488-alkyne. The resulting confocal fluorescence image was correlated onto the corresponding large field of view (stitched) TEM image, using Photoshop. A: An entire window in the TEM grid could be partially correlated with the available fluorescence image, depending on the orientation of the grid.
Figure S9. *Mtb* displays a heterogeneous intracellular distribution in host cells. RAW 264.7 macrophages were infected with triple label *Mtb* and incubated with or without antibiotics, and subsequently prepared for cryosectioning, followed by dual on-section ccHc reaction with AF647-azide and AF488-alkyne. The resulting confocal fluorescence image was correlated onto the corresponding large field of view (stitched) TEM image, using Photoshop. A small percentage of bacteria appears to have escaped into the cytosol, as no vacuole can be observed (A). These bacteria may fuse again with a vacuole (ii), divide cytosolically (iii) or potentially be re-compartmentalized into a double membrane autophagosome (iv). An intermediate percentage of bacteria resides in a small vacuole (B), that can appear empty (i) or filled with smaller vacuoles/granules (ii). Bacteria may divide within small vacuoles (iii) and the vacuole may wrap tightly around the bacterium (iv). A large percentage of bacteria resides in large vacuoles (C) that may appear spacious (i) or filled with smaller vesicles/granules (ii). Vacuoles containing a cluster of bacteria were classified as large, regardless of being smaller (iii) or larger (iv). A dotted line indicates the apparent vacuole where relevant. All scale bars represent 500 nm.
Figure S10. *Mtb* is released from host cell and subsequently re-internalized by surrounding macrophages. RAW 264.7 macrophages were infected with triple label *Mtb* and incubated with or without antibiotics. The resulting confocal fluorescence image was correlated onto the corresponding large field of view (stitched) TEM image, using Photoshop. Some *Mtb*-containing macrophages are disintegrated (A), probably as a result of the *Mtb* infection. The escaped bacteria reside extracellularly (B), surrounded by cell debris. Extracellular bacteria appear to be re-internalized by neighboring cells (C), through phagocytosis of separate bacteria (i) or internalization of the entire necrotic cell (ii), which can result in large vacuoles that contain both bacteria and cell debris (iii). A dotted line indicates the apparent cell outline or vacuole where relevant. All scale bars represent 1 µm.
Figure S11. The occurrence of extracellular Mtb and apparent host cell death changes upon treatment with antibiotics. RAW 264.7 macrophages were infected with triple label Mtb for 1 hour and immediately analyzed (0h untreated) or further incubated for 24 hours with rifampicin (RIF), isoniazid (INH), ethambutol (EMB), a combination of the three (triple) or no antibiotics (24h control) and compared to Mtb pre-treated with triple antibiotics before infection (0h pre-treated). An additional control with heat-killed Mtb (prior to infection) was included as a negative control for pathogenicity. (A) Mtb in the absence of host cell or inside a disintegrating host cell were classified as extracellular Mtb. Shown as percentage relative to total number of Mtb counted in the analyzed region; n>500 bacteria counted. (B) Disintegrating and/or phagocytosed macrophages were classified as host cell death. Shown as percentage relative to total number of macrophages counted in the analyzed region; n>80 cells counted.

Figure S12. Mtb resides primarily in large vacuoles directly after infection. RAW 264.7 macrophages were infected with triple label Mtb for 1 hour with viable Mtb (0h control) or Mtb pre-treated with rifampicin, isoniazid and ethambutol (0h pre-treated), and analyzed immediately after infection. The resulting confocal fluorescence image was correlated onto the corresponding large field of view (stitched) TEM image, using Photoshop. A: Intracellular distribution of Mtb was manually classified as no vacuole/cytosolic (none), small/tight vacuole (small) or large/spacious vacuole (large). Shown as percentage relative to total number of intracellular Mtb counted in the analyzed region; n>200 bacteria counted. B: Most untreated bacteria reside in large clusters of smaller vacuoles (i), that were considered large vacuoles for unbiased counting, while most of the pre-treated bacteria reside in large (ii) and spacious (iii) vacuoles. This distinction was not considered in the quantification in order to avoid interpretation bias. A dotted line indicates the apparent vacuole where relevant. All scale bars represent 1 µm.
Intracellular Mtb displays bacterial profiles in various shapes. RAW 264.7 macrophages were infected with triple label Mtb for 1 hour (0h untreated) and incubated for 24 hours without antibiotics (24h control) or with rifampicin, isoniazid and ethambutol (24h triple antibiotics) and compared to Mtb pre-treated with triple antibiotics before infection (0h pre-treated). The variety of shapes, observed for the bacterial profiles, were classified into ovaloid (A) or irregular (B). In some cases, clear fluorescence was observed without any recognizable bacterial structure (C), containing either alkDala (i), Aha (ii), DsRed (iii) or all three combined (iv). Relevant structures are indicated with an asterisk (*). A dotted line indicates the apparent vacuole where...
relevant. All scale bars represent 1 µm. D: The intracellular appearance of *Mtb* was manually classified as ovaloid or irregular, shown as percentage relative to the total number of *Mtb* counted in the analyzed region; *n>*500 bacteria counted. E: The additional effect of gentamicin (GEN) on the intracellular appearance of *Mtb* was assessed after 24 hours of incubation with triple antibiotic cocktail (triple +/-GEN) or without antibiotics (control +/-GEN); *n>*500 bacteria counted. F: The early intracellular appearance of *Mtb* was assessed directly after infection with *Mtb* pre-treated with triple antibiotics cocktail or with untreated control bacteria; *n>*200 bacteria counted. Raw distributions were pairwise compared using the chi-square test and corrected for multiple testing using the Benjamini-Hochberg procedure, with a false discovery rate (FDR) of 0.1 (****: *p*<0.0001, ***:** *p*<0.001, **:** *p*<0.01, *:** *p*<0.05, ns: not significant).

**Figure S14.** Semi-automatic CLEM-based quantification of label retention in intracellular *Mtb*. RAW 264.7 macrophages were infected with triple label *Mtb* for 1 hour and incubated for 24 hours with rifampicin, isoniazid and ethambutol (24h triple antibiotics) or no antibiotics (24h untreated control). The resulting confocal
fluorescence image was correlated onto the corresponding large field of view (stitched) TEM image, using Photoshop. Bacterial outline was drawn manually in Photoshop, using the 'lasso tool', followed by automatic cropping and separation of channels, using a JavaScript. Raw fluorescence images (brightness/contrast unchanged) were subsequently analyzed based on the average fluorescence intensity of the non-white area (masked), using an ImageJ macro. A: Two examples of untreated control bacteria, incubated for 24 hours intracellularly without antibiotics, showing the color-merged CLEM image (i,v), isolated alkDala signal (ii,vi), isolated Aha signal (iii,vii) and isolated DsRed signal (iv,viii). B: Two examples of triple antibiotics treated bacteria, incubated for 24 hours intracellularly with rifampicin, isoniazid and ethambutol, showing the color-merged CLEM image (i,v), isolated alkDala signal (ii,vi), isolated Aha signal (iii,vii) and isolated DsRed signal (iv,viii). All scale bars represent 500 nm.
Figure S15. Quantification of label retention by flow cytometry. RAW 264.7 macrophages were infected with triple label Mtb for 1 hour (0h control; A) and further incubated for 24 hours without antibiotics (24h control; B) or with rifampicin (RIF; C), isoniazid (INH; D), ethambutol (EMB; E) or a combination of the three (triple; F). Bacteria were recovered by selective lysis of the host cell, fixed and processed for flow cytometry. The average label retention was quantified by selecting the Aha+/alkDala+ quadrant or the autofluorescence+/DsRed+ quadrant. (G) The median fluorescence intensity (MFI) was used for the quantification of all signals, as it reflects
the average signal intensity for a local population most accurately. Corresponding normalized MFI values are presented in Fig. 6B.
Figure S16. Additional controls for bacterial recovery from host cells. RAW 264.7 macrophages were infected with triple label Mtb for 1 hour (0h control; A) and further incubated for 24 hours without antibiotics (24h control; B). Additional controls include the use of rifampicin (RIF) at 0.1 μg/mL (C) and 1 μg/mL (D) and heat-killed Mtb (E). In vitro controls were performed at 0h (F) and 24h (G) for comparison. Figure (I) illustrates the effect of different concentrations of RIF on label retention, while figures (J) and (K) depict dead Mtb control and label loss in vitro, respectively. The data shows normalized MFI for each condition, highlighting the impact of RIF concentrations and the survival status of Mtb on label retention and MFI.
B) or with increasing concentrations of rifampicin (RIF; C, D, E) or heat-killed (30 min at 80°C) Mtb (F). Bacteria were recovered by selective lysis of the host cell, fixed and processed for flow cytometry. Additional in vitro controls were included, in which the Mtb were incubated in cell medium for 0h (G) or 24h (H). The average label retention was quantified by selecting the Aha+/alkDala+ quadrant or the autofluorescence+/DsRed+ quadrant. (I-K) MFI was used for the quantification of all signals and subsequently normalized to the 0h time point.

References

(1) Zhang, M. M.; Tsou, L. K.; Charron, G.; Raghavan, A. S.; Hang, H. C. Tandem Fluorescence Imaging of Dynamic S-Acylation and Protein Turnover. Proc. Natl. Acad. Sci. U. S. A. 2010, 107 (19), 8627–8632.

(2) Li, N.; Lim, R. K. V; Edwardraja, S.; Lin, Q. Copper-Free Sonogashira Cross-Coupling for Functionalization of Alkyne-Encoded Proteins in Aqueous Medium and in Bacterial Cells. J. Am. Chem. Soc. 2011, 133 (39), 15316–15319.

(3) Chenault, H. K.; Dahmer, J.; Whitesides, G. M. Kinetic Resolution of Unnatural and Rarely Occurring Amino Acids: Enantioselective Hydrolysis of N-Acyl Amino Acids Catalyzed by Acylase I. J. Am. Chem. Soc. 1989, 111 (16), 6354–6364.

(4) Biagini, S. C. G.; Gibson, S. E.; Keen, S. P. Cross-Metathesis of Unsaturated Alpha-Amino Acid Derivatives. J. Chem. Soc. Perkin Trans. 1 1998, 1, 2485–2500.

(5) Dong, S.; Merkel, L.; Moroder, L.; Budisa, N. Convenient Syntheses of Homopropargylglycine. J. Pept. Sci. 2008, 14 (10), 1148–1150.

(6) Korbee, C. J.; Heemskerk, M. T.; Kocev, D.; Van Strijen, E.; Rabiee, O.; Franken, K. L. M. C.; Wilson, L.; Savage, N. D. L.; Džeroski, S.; Haks, M. C.; Ottenhoff, T. H. M. Combined Chemical Genetics and Data-Driven Bioinformatics Approach Identifies Receptor Tyrosine Kinase Inhibitors as Host-Directed Antimicrobials. Nat. Commun. 2018, 9 (1).

(7) Tokuyasu, K. T. A Technique for Ultracryotomy of Cell Suspensions and Tissues. J. Cell Biol. 1973, 57 (2), 551–565.

(8) Peters, P. J.; Bos, E.; Griezspoor, A. Cryo-Immunogold Electron Microscopy. Curr. Protoc. Cell Biol. 2006, 30 (1), 4.7.1-4.7.19.

(9) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J. Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A. Fiji: An Open-Source Platform for Biological-Image Analysis. Nat. Methods 2012, 9 (7), 676–682.