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Detection of Bioorthogonal Groups by Correlative Light and Electron Microscopy allows Imaging of Degraded Bacteria in Phagocytes^{*}

Introduction

Phagocytic degradation is a question of great biological relevance, as it is one of the key mechanisms by which the immune system keeps pathogens at bay. As a consequence, subversion of the phagolysosomal pathway is a survival strategy employed by a wide range of parasites, which collectively are responsible for a great amount of human morbidity and mortality.¹

The interaction between immune cells and pathogenic bacteria is very difficult to study², as intracellular pathogens can be non-trivial to grow *ex vivo*³ and very difficult to genetically alter. Even in (rare) cases where these bacteria can be genetically modified,⁴ imaging their encounters with host phagocytes is limited to encounters where successful infection is established. Encounters whereby the pathogens are killed and degraded are difficult to image, as the proteolysis that is

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a hallmark of successful phagocytic maturation⁵ results in the degradation of reporter proteins and epitopes.⁶

Bioorthogonal chemistry is a powerful tool for labelling of (sub)-populations of biomolecules in complex biological systems⁷ and could be employed to circumvent these problems. The approach relies on the introduction of a small, physiologically inert chemical group into a biomolecule of interest that can subsequently be visualised using a selective reaction.⁸ The small size, biological stability of the chemical group, and the wide range of biomolecules that can be labelled with this approach makes this method a valuable part of the biochemist's toolkit.^{9, 10}

Bolstered by the recent successful imaging of a pathogen inside a host phagocyte through the use of a bioorthogonally-modified cell wall component, D-alanine,¹¹⁻¹³ it was envisaged that bioorthogonal bacteria could also be used to image degradation events in host phagocytes. Bioorthogonal non-canonical amino acid tagging (BONCAT)^{14, 15} for pan-proteomic incorporation of bioorthogonal groups^{16, 17} would allow the labelling of a wide range of bacterial species without the need for genetic modification.¹⁸ Furthermore, unlike reporter proteins, bioorthogonal groups, such as azides,^{19, 20} have been shown to be stable in the harsh chemical environments of the phagolysosomal system and should therefore be detectable even when extensive proteolysis has occurred.

Information about subcellular localisation is of key importance when studying parasite–phagocyte-interactions as movement between organelles may be essential to the life cycle of certain parasites.^{1, 21} Only transmission electron microscopy (TEM)-based techniques allows the study of these pathogens in their subcellular context, as it provides substructural information on the position of any label/antigen within the cell.²² However, in contrast to superresolution imaging,^{23, 24} no methods have been reported that allow the visualisation of bacterial degradation using bioorthogonal labelling in combination with EM imaging.²⁵

This chapter describes the application of a correlative light electron microscopy (CLEM)-based imaging approach for the visualisation of bioorthogonal groups, which allows the imaging of BONCAT-labelled bacteria inside phagocytes (Figure 1); even as they are being degraded. This approach combines the benefits of

confocal microscopy – which allows widefield navigation to areas of interest²⁶ – with those of EM – which provides narrow-field high-resolution information about the interior of the cell.²² All approaches described here on the model organism *Escherichia coli* (*E. coli*) are amenable to application to pathogens, which would open new avenues for studying the events leading to bacterial clearance and/or establishment of intracellular residence by intracellular pathogens.

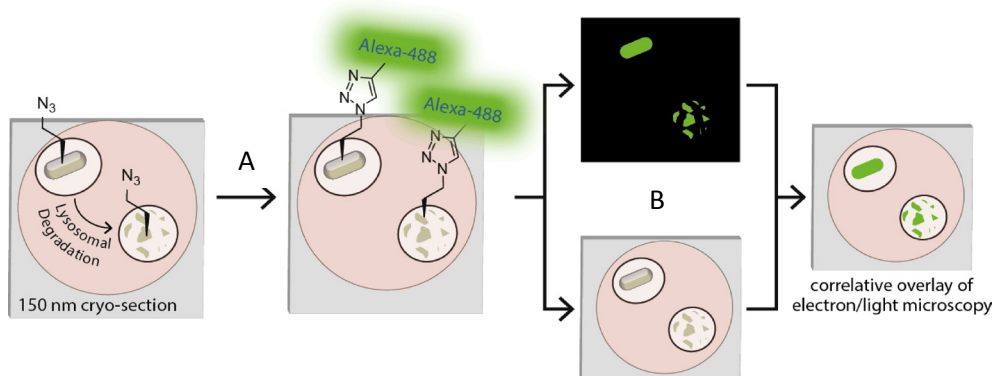


Figure 1: Overview: (A) phagocytosed azido-*E. coli* can be fluorescently visualised in an ultrathin cryosection using a copper-catalysed Huisgen cycloaddition reaction with a fluorophore; (B) overlay of this image on an electron micrograph provides an ultrastructural context for the signal with nanometer-scale resolution. As the bioorthogonal handle is stable to proteolysis, degraded bacteria can be visualised in this manner.

Results

Comparison of GFP-*E. coli* and azido-*E. coli* for imaging phagolysosomal degradation

Most CLEM studies employ the fusion of fluorescent proteins to a protein of interest or antibody-based approaches to allow their identification and localisation.²² These labelling approaches have shown to be of great value for the imaging of specific proteins in their cellular context, but only in the cases where genetic modification of the organism is possible and where the attachment of the fluorescent proteins does not affect protein function.²⁷ Immunofluorescence has also been used, but combined with CLEM it either compromises ultrastructure (by virtue of the need of fixation and permeabilisation prior to CLEM-sample preparation)²⁸, or suffers from a notoriously low success rate due to compromised epitope availability in samples prepared for TEM.²⁹

To determine whether the BONCAT-based labelling approach has advantages over genetic methods for the detection of phagocytosed bacteria during degradation, the fate of azido-*E. coli* was compared to that of GFP-expressing *E. coli* (Figure 2). Mouse bone marrow-derived dendritic cells (BM-DCs)^{30, 31} were incubated with azido-*E. coli* or GFP-*E. coli* for 45 minutes. After washing, the cells were chased for 1 h, 2 h or 3 h prior to fixation, bioorthogonal modification of the azides (where present), and confocal imaging (Figure 2) – time points in which maturation of a phagosome to a phagolysosome is known to take place in these cells.³²

To assess whether the fluorescent signal originated from intact or (partially) degraded bacteria, extranuclear DAPI staining was analysed: colocalisation of the fluorescent signal with the extra-nuclear DAPI indicates the intactness of the bacterial DNA, which in turn indicates the intactness of the bacterium.³³ Absence of this colocalisation (i.e. 488 nm single-positive foci) indicated the degradation of the bacterial genome and thus death. The azide-based signal persisted significantly more than the GFP-signal after killing of the bacterium; as indicated by the significantly larger number of DAPI-negative/azide-positive foci at all time points of the chase period compared to DAPI-negative/GFP-positive foci (Figure 3). Many of the azide-positive foci were smaller than intact DAPI/azide double positive foci, indicating these signals to originate from partially degraded bacteria.

CLEM imaging of azido-E. coli after uptake by BM-DCs.

We obtained ultrastructural information about the location of these smaller, DAPI-negative foci by performing CLEM analysis on azido-*E. coli*-treated BM-DCs samples at all four time points (Figure 4 and Figure 5). Co-staining with the lysosomal marker Lysosomal Associated Membrane Protein-1 (LAMP-1) revealed that these degraded fragments only partially resided in LAMP-1-positive late endosomes/lysosomes. This ties in with previous studies showing the existence of a second population of phagosomes in DCs, which do not acidify and never become LAMP-1 positive.³⁴ This set of phagosomes has been implicated in DC-specific functions, such as cross-presentation.^{35, 36} Morphological information obtained from TEM showed that the azide-positive/DAPI-positive foci were intact bacteria, whereas the DAPI-negative foci showed no identifiable bacterial morphology, indicating that this technique allows the imaging of partially degraded bacteria inside mammalian phagocytes (Figure 4 and Figure 5).

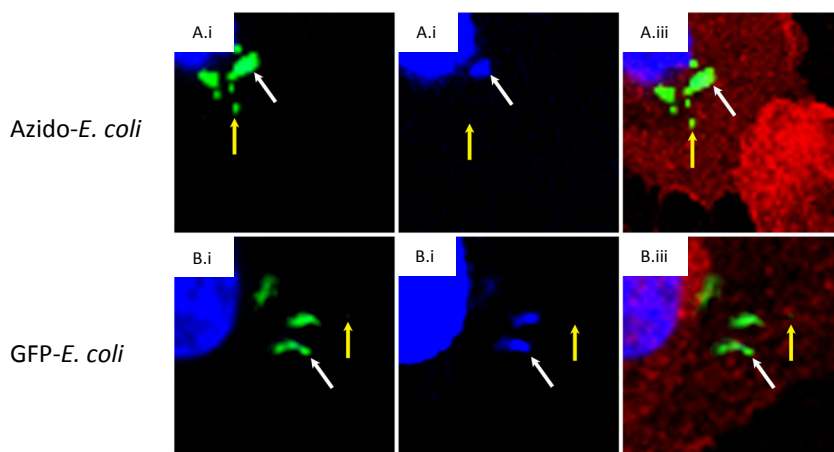


Figure 2: Confocal microscopy of (A) azido-*E. coli* or (B) GFP-*E. coli* after phagocytosis. BM-DCs were pulsed with either azido-*E. coli* or GFP-*E. coli* (45 minutes pulse). Cells were fixed after a 2 h chase and stained with DAPI (blue), anti-actin (red) and, in case of azido-*E. coli*, AlexaFluor-488 alkyne (green = either GFP or AlexaFluor-488). (i) DAPI/488 nm overlay; (ii) DAPI only; (iii) all fluorescent channels overlay. Yellow arrows indicate a 488-single positive focus, white arrows a DAPI/488 nm double positive focus.

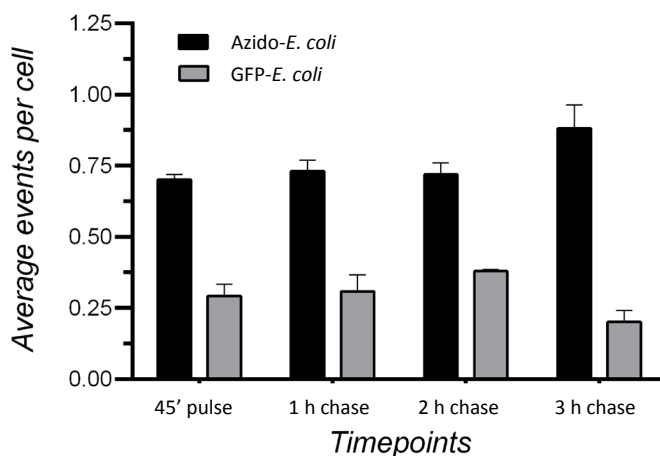


Figure 3: Comparison of GFP-*E. coli* and azido-*E. coli* for imaging phagolysosomal degradation in BM-DCs. BM-DCs were incubated with either GFP-*E. coli* or azido-*E. coli* cells for 45 minutes. Cells were washed with PBS to remove unbound/non-internalised *E. coli*. After a 45 minutes pulse, 1 h chase, 2 h chase or 3 h chase cells were fixed with 4% PFA for 15 minutes. Azido-*E. coli* containing cells were labelled with AlexaFluor-488 alkyne using copper-catalysed Huisgen cycloaddition-conditions. From each condition confocal microscopy pictures were made. Based on these pictures the average number of green foci, that represented degraded bacteria per cell, was determined. Only the foci that were within the focus plane of the cell (determined by the actin staining) were counted and had no overlap with extra-nuclear DAPI stain. Per condition 50 cells were counted. N=2.

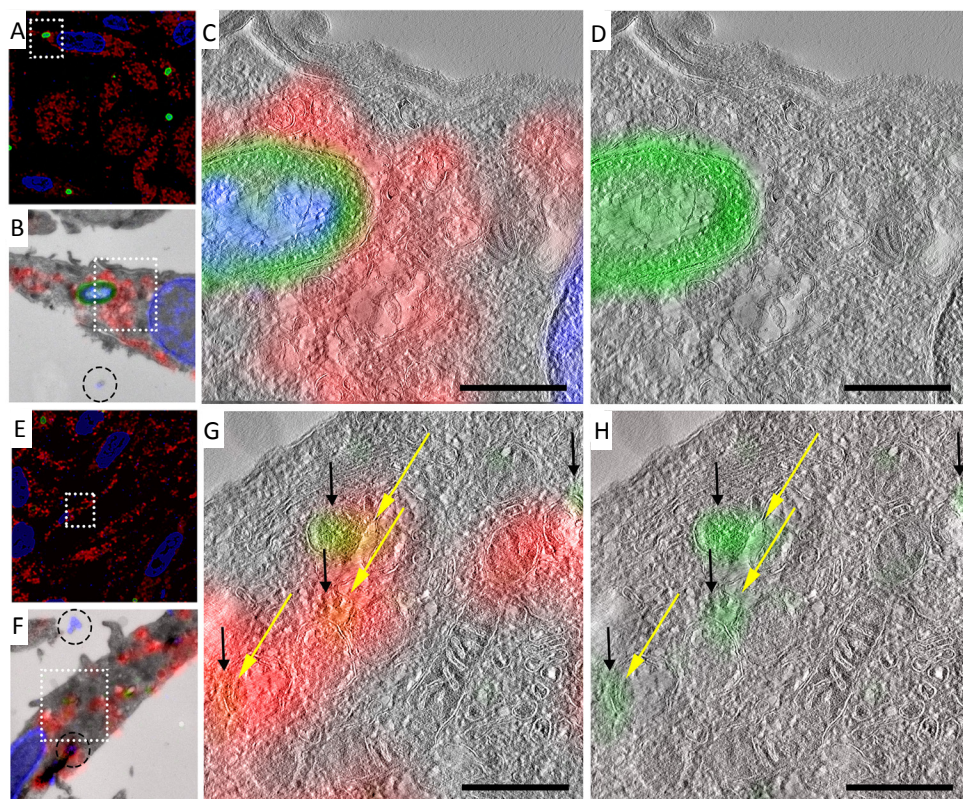


Figure 4: CLEM imaging of phagocytosed azido-*E. coli*: BM-DCs were pulsed with azido-*E. coli* (45 minutes pulse). Cells were washed with PBS to remove unbound/non-internalised *E. coli*. Samples were fixed immediately after pulsing (A–D) or after a 3 h chase (E–H). Cells were subjected to Tokuyasu sample preparation and cryosectioned into 150 nm sections. Sections were reacted with AlexaFluor-488 alkyne using ccHc-conditions (green), anti-LAMP-1 (red) and DAPI (blue). DAPI staining and blue fiducials (indicated with circles in B and F) were used for correlation purposes. (A/E) Confocal microscopy images; (B/F) CLEM image obtained from overlay LM and EM pictures; (C, D, G and H) CLEM details from (B/F), showing LAMP-1 and 488 nm channels (C/G) or 488 nm alone (D/H). Green = AlexaFluor-488, Red = LAMP-1, Blue = nuclear DAPI stain and fiducial beads. Scale bar 500 nm.

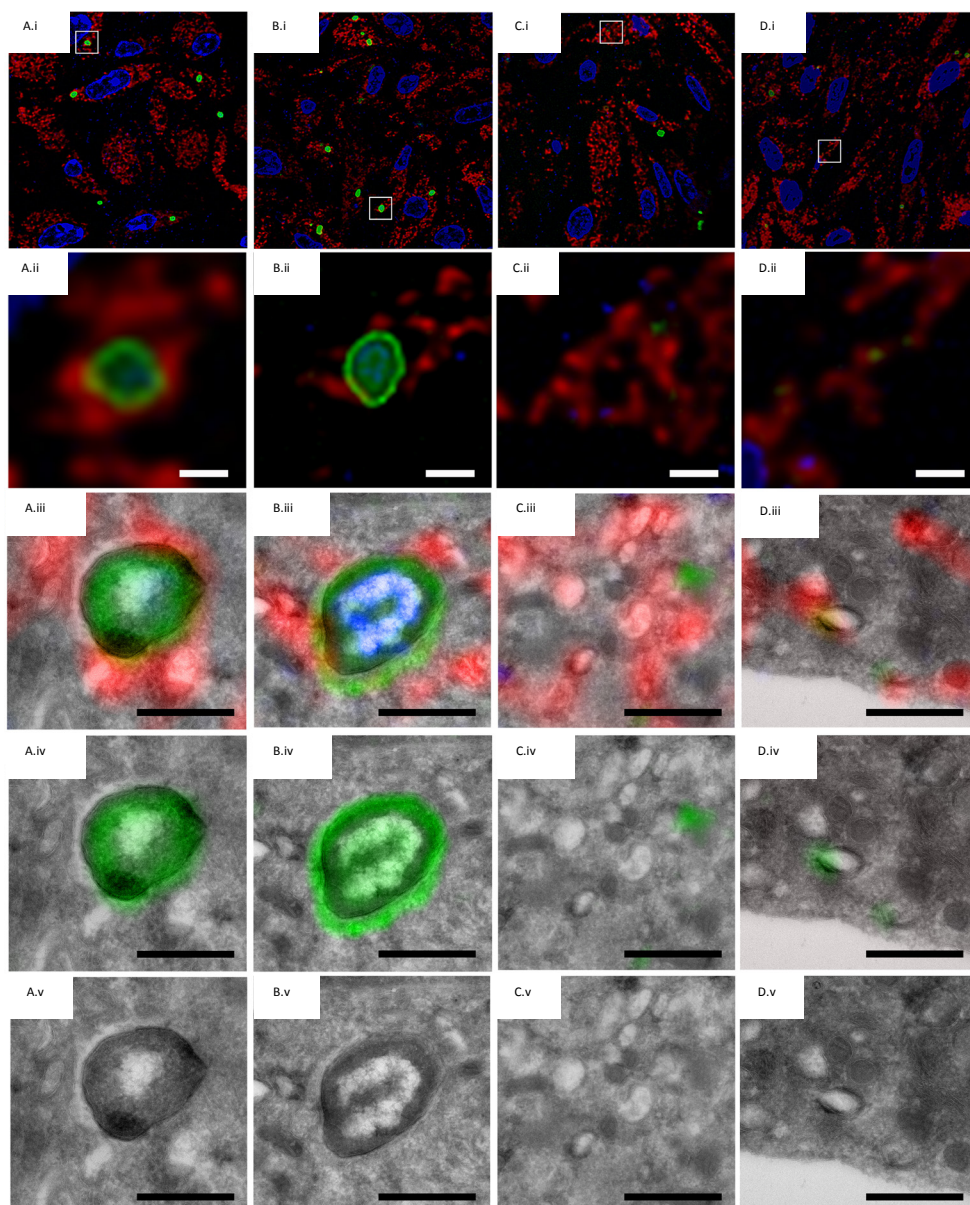


Figure 5: CLEM imaging of mouse BM-DCs infected with azido-*E. coli*. BM-DCs were incubated with *E. coli* cells for 45 minutes. Cells were washed with PBS to remove unbound/non-internalised *E. coli*. After a 45 minutes pulse (A.i-v), 1 h chase (B.i-v), 2 h chase (C.i-v) or 3 h chase (D.i-v) cells were fixed in 2% PFA, subjected to Tokuyasu sample preparation (including gelatin embedding and sucrose infiltration) and crysectioned into 150 nm sections. Sections were reacted with AlexaFluor-488 (green) alkyne using copper-catalysed Huisgen cycloaddition reaction, anti-LAMP-1 (red) and DAPI (blue). DAPI staining and blue fiducials were used for correlation purposes. (A.i-D.i) Confocal microscopy image of bioorthogonally tagged *E. coli* B834 incubated with mouse BM-DCs (A.i) 45

minutes pulse (B.i) 1 h chase (C.i) 2 h chase (D.i) 3 h chase. Green = AlexaFluor-488, Red = LAMP-1, Blue = nuclear DAPI stain and fiducial beads. (ii) Detail from i. (iii) CLEM image obtained from overlay EM picture and figure ii, using blue fiducials and DAPI stain for correlation. (iv) Similar detail from a.iii-d.iii without red and blue signals. (v) Similar detail from a.iii-d.iii EM image only. Scale bar 500 nm.

Conclusion

By combining BONCAT with CLEM-imaging, we have established a new approach that allowed us to visualise bioorthogonally modified bacteria in an ultrastructural cellular context, even during late stages of bacterial degradation. With this approach degradation events in a cell can be identified at low magnification with confocal microscopy using bioorthogonal labelling, after which ultrastructural information about their subcellular location and context can be obtained with EM. This is of great interest for the study of obligate intracellular parasites that are very hard to study by any other means.

As the application of bioorthogonal chemistry is ever expanding, the CLEM-imaging method of bioorthogonal groups described here could also be of great benefit to the study of labelled biomolecules in other fields in which bioorthogonal imaging has proven its value.²⁵ Application of this approach to other bioorthogonal assays (for instance, lipid imaging³⁷ and the imaging of newly synthesised proteins³⁸), and perhaps in combination with some of the more recently developed bioorthogonal chemistries³⁹ will allow the provision of additional structural information to the current imaging methods available for these types of biomolecules.

Experimental

E. coli culturing conditions and growth measurements

E. coli B834(DE3) bacteria were grown overnight at 37°C in Lysogeny Broth (LB) medium. The following day cultures were diluted 1:50 in LB medium and grown at 37°C till an OD₆₀₀ between 0.3-0.5. Subsequently cells were collected and resuspended in Selenomet medium (Molecular Dimensions) and supplemented with either 4 mM Azidohomoalanine (Aha) (Bachem) or 4 mM Methionine (Met) (Sigma-Aldrich). After 1 h OD₆₀₀ were measured and cells were collected by centrifugation for BM-DC infection experiments.

E. coli B834(DE3) GFP_{A206K} was grown overnight at 37°C in LB medium. The following day cultures were diluted 1:50 in LB medium and grown at 37 °C till an OD₆₀₀ between 0.3-0.5. Throughout culturing, cultures were supplemented with 100 µg/ml Ampicillin. The vector pRD35 for the constitutive expression of GFP_{A206K}, was constructed by cloning GFP into pUC21 using NsiI and MluI restriction sites. An A206K mutation was introduced by site-directed mutagenesis PCR to prohibit dimerisation of GFP.⁴⁰ The constitutive *hns* promoter and ribosomal binding site (the 258 bases upstream of the *E. coli hns* gene), were amplified by PCR, using *E. coli* K12 as a template, and positioned upstream of GFP by way of XhoI and NsiI. Used primers are indicated in Table 1.

GFP fw NsiI	ACA-ATG-CAT-AGT-AAA-GGA-GAA-GAA-CTT-TTC-ACT-GGA-GTT-G
A206K fw	CCT-GTC-CAC-ACA-ATC-TAA-ACT-TTC-GAA-AGA-TCC-C
A206K rev	GGG-ATC-TTT-CGA-AAG-TTT-AGA-TTG-TGT-GGA-CAG-G
GFP R	CAC-ACG-CGT-TTA-TTT-GTA-TAG—TTC-ATC-CAT-GCC-ATG-TGT-AAT-CC
HNS-Chrom-Fw-XhoI	GAA-CTC-GAG-GGT-CGT-CAG-CCT-ACG-ATA-ATC-TCC-CC
HNS-Chrom-Rev-NsiI	ACT-ATG-CAT-TCT-AGT-AAT-CTC-AAA-CTT-ATA-TTG-GGG-TGG-TTT-G

Table 1: Primers used for GFP-plasmid construction.

Mammalian cell culture conditions

Mouse bone marrow derived dendritic cells (BM-DCs) were generated from B57BL/6 mice bone marrow essentially as described³⁰ with some modifications. Briefly, bone marrow was flushed from femurs and tibia and cells were cultured in IMDM (Sigma Aldrich) supplemented with 8% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 μ M 2-Mercaptoethanol (Life Technologies), penicillin 100 I.U./mL and streptomycin 50 μ g/mL in the presence of 20 ng/mL GM-CSF (ImmunoTools). Medium was replaced on day 3 and 7 of culture and the cells were used between days 10 and 13.

E. coli B834(DE3) cells were added to the BM-DCs as suspensions in PBS in a ratio of approximately 25:1, respectively. After 45 minutes of incubation unbound/non-internalised *E. coli* cells were washed off (2x PBS) and medium was replaced. At the indicated time points cells were subjected to confocal microscopy or Tokuyasu sample preparation.

Whole cell confocal microscopy

BM-DCs were seeded (7×10^4) on a 12-well removable chamber slide (Ibidi) and left to grow O/N. The following day *E. coli* B834 cells harboring either GFP/Aha/Met were added to the BM-DCs as suspensions in PBS in a ratio of approximately 25:1, respectively. After 45 minutes of incubation unbound/non-internalised *E. coli* cells were washed off (2x PBS) and medium was replaced. At the indicated time points cells were fixed in 4% PFA for 15 minutes. Until further analysis cells were kept in PBS at 4°C. When all slides were collected, fixed cells were incubated for 30 minutes with blocking buffer (1% BSA, 1% gelatin cold water fish skin), for 1 h with click cocktail (0.1 M HEPES pH 7.3, 1 mM CuSO₄, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM amino-guanidine, 5 μ M AlexaFluor-488 Alkyne (Invitrogen)), O/N with anti-actin antibody (abcam), 1 h with goat anti-rabbit AlexaFluor-568 (Invitrogen) and DAPI (1 μ g/ml). After the staining procedures chambers were removed and cells were covered with a small drop of 50% glycerol, after which a coverslip was mounted over the grid. Coverslips were fixed using Scotch Pressure-Sensitive Tape. Samples were imaged with a Leica TCS SP8 confocal microscope (63x oil lens, N.A.=1.4).

Bioorthogonal labelling on cryosections

Samples were prepared for cryosectioning as described elsewhere.⁴¹ Briefly, BM-DCs were fixed for 24 h in freshly prepared 2% PFA in 0.1 M phosphate buffer. Fixed cells were embedded in 12% gelatin (type A, bloom 300, Sigma) and cut with a razor blade into 0.5 mm³ cubes. The sample blocks were infiltrated in phosphate buffer containing 2.3 M sucrose for 3 h. Sucrose-infiltrated sample blocks were mounted on aluminum pins and plunged in liquid nitrogen. The frozen samples were stored under liquid nitrogen.

Ultrathin cell sections were obtained as described elsewhere. Briefly, the frozen sample was mounted in a cryo-ultramicrotome (Leica). The sample was trimmed to yield a squared block with a front face of about 300 x 250 µm (Diatome trimming tool). Using a diamond knife (Diatome) and antistatic device (Leica) a ribbon of 150 nm thick sections was produced that was retrieved from the cryochamber with a droplet of 1.15 M sucrose containing 1% methylcellulose. Obtained sections were transferred to a specimen grid previously coated with formvar and carbon. Grids were additionally coated as indicated with either 100 nm TetraSpeck beads or 100 nm FluoroSpheres (blue) carboxylate-modified (350/440) (Life Technologies).

To further improve the structural integrity of the ultrathin cryosections a novel micromanipulator (Manip, Diatome) was used that was mounted on the cryochamber of the ultramicrotome.⁴² This device facilitated section retrieval from the cryochamber and resulted in less overstretching of the sections during thawing.⁴³

Sections were labelled as follows; thawed cryosections on an EM grid were left for 30 minutes on the surface of 2% gelatin in phosphate buffer at 37°C. Subsequently grids were incubated on drops of PBS/glycine and PBS/glycine containing 1% BSA. Grids were then incubated on top of the ccHc-cocktail (0.1 M HEPES pH 7.3, 1 mM CuSO₄, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM amino-guanidine, 5 µM AlexaFluor-488 alkyne (Invitrogen) for 1 h and washed 6 times with PBS. Sections containing BM-DCs and Jurkat cells were then labelled with DAPI (2 µg/ml), and additionally washed with PBS and aquadest.

In case of additional immune-labelling against LAMP-1 grids were subjected to the following steps directly after the ccHc reaction. Grids were washed 5 times with PBS/glycine and blocked again with PBS/glycine containing 1% BSA after which the grids were incubated for 1 h with PBS/glycine 1% BSA supplemented with a rat anti-mouse LAMP-1 (BioLegend). Sections were subsequently washed 6 times with PBS, labelled with DAPI (2 µg/ml) and finally washed again with PBS and aquadest.

Microscopy and correlation

The CLEM approach used was adapted from Vicidomini et al.⁴⁴ Grids containing the sample sections were washed with 50% glycerol and placed on glass slides (pre- cleaned with 100% ethanol). Grids were then covered with a small drop of 50% glycerol, after which a coverslip was mounted over the grid. Coverslips were fixed using Scotch Pressure-Sensitive Tape. Samples were imaged with a Leica TCS SP8 confocal microscope (63x oil lens, N.A.=1.4). Confocal microscopy was used as it allowed to make image stacks from the sections at different focus planes; this was convenient as the sections were found to be in different focus planes whilst placed between the glass slides and coverslip. Confocal stacks were deconvolved with theoretical point spread functions using Huygens Essential deconvolution software (SVI, Hilversum, Netherlands). After fluorescence microscopy the EM grid with sections was removed from the glass slide, rinsed in distilled water and incubated for 5 minutes on droplets of unranylacetate/methylcellulose.

Excess of unranylacetate/methylcellulose was blotted away and grids were air-dried. EM imaging was performed with a Tecnai 12 Biotwin transmission electron microscope (FEI) at 120 kV acceleration voltage. Tilt series for electron tomography were collected using Xplore3D (FEI Company) software. The angular tilt range was set from -60° to 60° with 2° increments, and an objective lens defocus of -2 µm at a magnification of 20 K (pixel size is 1 nm). Alignments of the tilt series and weighted-back projection reconstructions for tomography were performed using IMOD software.⁴⁵

Correlation of confocal and EM images was performed in Adobe Photoshop CS6. In Adobe Photoshop, the LM image was copied as a layer into the EM image and made 50% transparent. Transformation of the LM image was necessary to

match it to the larger scale of the EM image. This was performed via isotropic scaling and rotation. Interpolation settings; bicubic smoother. Alignment at low magnification was carried out with the aid of nuclear DAPI staining in combination with the shape of the cells, at high magnification alignment was performed using fiducial beads.⁴⁶

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