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A bioorthogonal chemistry approach to the study of biomolecules in their ultrastructural cellular context

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Towards Ultrastructural Imaging of Enzyme Activity with Correlative Light and Electron Microscopy^{*}

Introduction

Enzymes are proteins that are involved in the metabolic pathways of living systems, and catalyse chemical reactions that modify, take apart and construct biomolecules. Their activity is regulated by post-translational modifications, protein-protein interactions and/or endogenous small-molecule inhibitors, and as a consequence their expression levels do not necessarily correlate with their activity.¹ Labelling methods such as genetically-encoded reporter proteins and/or antibodies can therefore not be used to obtain information on their active populations.² To this end chemical probes termed activity-based probes (ABPs) have been developed, which target the active site of an enzyme and only label its catalytically active form.³ ABPs consist of three essential structural elements; (1) a recognition element, (2) a reactive group or 'warhead' and (3) a detectable group. The recognition element is recognised as substrate by the enzyme of interest and

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ensures the selectivity of the ABP. The warhead ensures (covalent) binding of the ABP to the enzyme of interest upon reaction with the catalytically-active amino acid residue of the enzyme. The detectable group can either be a bioorthogonal ligation handle or a reporter (e.g. biotin or a fluorophore) (Figure 1A/B, respectively).² If the detectable group is a bioorthogonal ligation handle, the strategy is referred to as two-step ABP labelling and the modification of the ABP takes place after the ABP has reacted with its target enzyme (Figure 1A). Bioorthogonal ligation handles are mostly azide- or alkyne moieties and can be ligated to a fluorescent group or an affinity handle depending on the experimental setup. When the detectable group itself is a reporter moiety, the ABP labelling strategy is referred to as direct ABP labelling, as enzymes are directly labelled with the ABP and can be directly visualised or isolated (Figure 1B). The main advantage of this strategy is that no additional labelling steps are necessary; however, the direct attachment of reporter groups may result in a lower binding affinity and/or specificity of the ABP.^{4, 5}

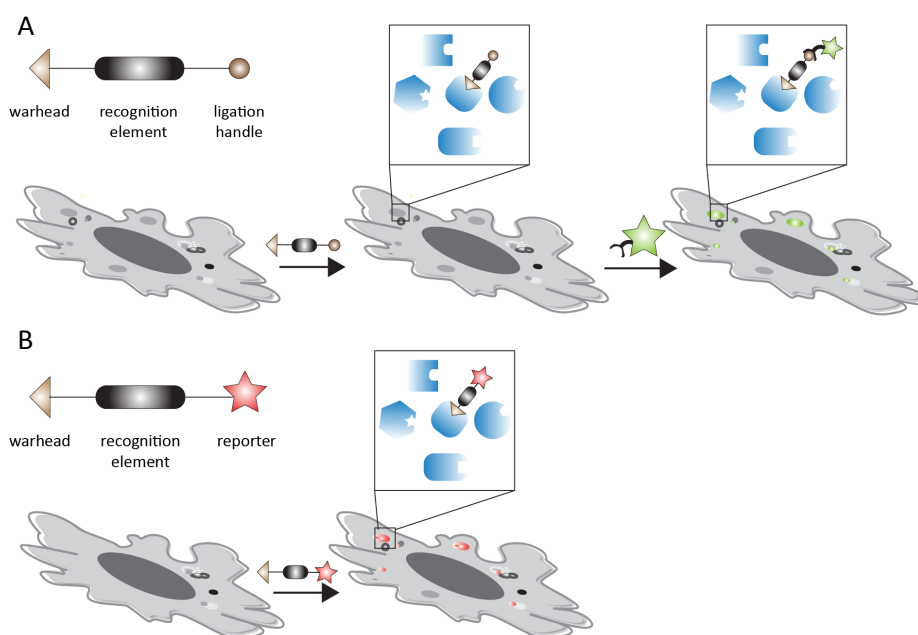


Figure 1: Two-step and direct labelling of enzymes with ABPs. A) The installation of a chemical reporter on active enzyme populations can be achieved upon use of two-step ABPs. Two-step ABPs consist of a warhead, recognition element and a chemical ligation handle. B) Direct ABPs consist of a warhead, recognition element and a detection group.

Over the past decades, a large number of ABPs have been developed that enabled the identification and monitoring of enzyme activity in various disease states.^{3, 6-8} Moreover, fluorescence imaging of enzyme activity with ABPs has yielded valuable information on subcellular enzyme localisation.^{9, 10} For example, imaging of cathepsin S activity has revealed the existence of distinct populations of cathepsin S-positive vesicles in bone marrow-derived immune cells.¹⁰ However, these imaging studies did not reveal any information on the cellular ultrastructures in which these active enzymes reside. To this end correlative light and electron microscopy (CLEM) imaging of active enzyme populations is explored in this chapter. It is demonstrated that CLEM imaging of both two-step and direct ABP labelling is feasible. With both strategies active populations of cysteine proteases could be identified in their ultrastructural cellular context. Moreover, upon combining direct ABP and CLEM the relative ultrastructural localisation of glucocerebrosidases and cathepsins could be revealed.

Results

CLEM imaging of enzyme activity with two-step ABPs

To verify whether enzyme activity could be imaged with CLEM, a two-step ABP labelling sequence was implemented in the previously reported bioorthogonal on-section labelling strategy.¹¹ Mouse bone marrow-derived dendritic cells (BM-DCs), which express high levels of active cathepsins in their endolysosomal system, were incubated with the azide-functionalised ABP E-64 (Figure 2A). This irreversible cysteine protease inhibitor reacts selectively with cysteine proteases of the cathepsin family,¹² that localise inside lysosomes and have the ability to degrade internalised matrix proteins.¹³ After incubation with E-64-azide, cells were washed, fixed and subjected to Tokuyasu sample preparation and cryosectioning. After cryosectioning, the E-64-azide incubated cells were labelled with an AlexaFluor-488 alkyne fluorophore using an on-section copper-catalysed Huisgen cycloaddition (ccHc) reaction (Figure 2B).¹¹ This reaction ensured covalent attachment of the AlexaFluor-488 fluorophore to the E-64-azide probe (Figure 2C) and thus to the active population of cathepsins. Sections were subsequently imaged with confocal microscopy (Figure 2D, left upper panel), after which a methylcellulose/uranylacetate embedding step was performed. The sections were then EM imaged and correlation of the confocal and EM-images was performed using fluorescent electron-dense beads as reference markers (Figure 2D, lower left panel/right panel).

The results show that E-64-azide could be successfully imaged using CLEM after on-section bioorthogonal ligation with an AlexaFluor-488 alkyne fluorophore. CLEM images show that the cysteine proteases of the cathepsin family -labelled by E-64- solely localised in membrane-limited lysosome-like structures of around 50-250 nm in diameter (Figure 2D). The intensity of the fluorescent signal varied between these ultrastructures, which possibly reflects the variations in cysteine protease activity within lysosomes.

CLEM imaging of enzyme activity with a direct ABP

To determine whether direct ABP labelling was also applicable with the CLEM protocol, CLEM imaging of a BODIPY-TMR functionalised E-64 probe was performed (Figure 3A). With exception of the bioorthogonal ligation step, CLEM images of the E-64-BODIPY-TMR treated BM-DCs were obtained according to the exact same protocol as for E-64-azide. The CLEM results show that upon labelling with E-64-BODIPY-TMR membrane-limited lysosome-like structures of around 50-250 nm in diameter were marked cysteine protease-positive. E-64-BODIPY-TMR-labelled structures were detected with varying fluorescence intensities, again indicating differences in cysteine protease activity throughout these organelles. These results are in accordance with the results obtained with the two-step protocol and indicate that with both direct and two-step E-64 variants ultrastructures can be labelled and CLEM-imaged in a similar fashion.

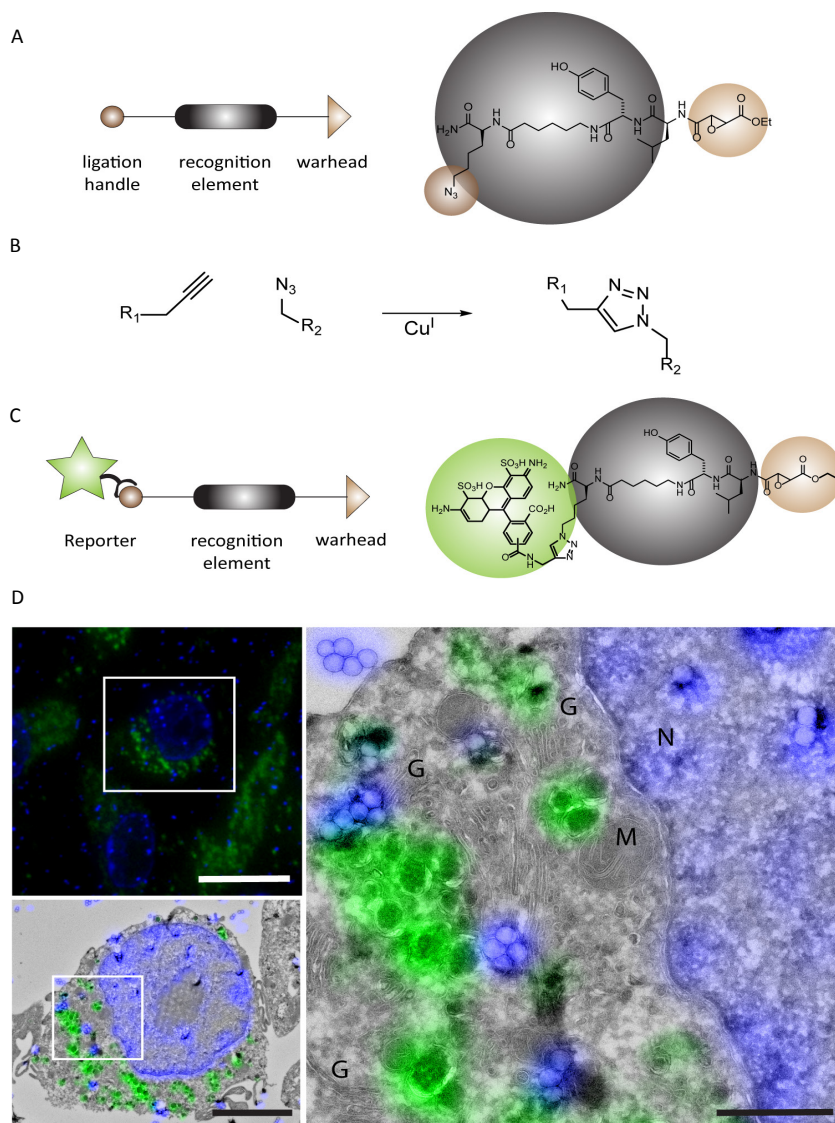


Figure 2: CLEM imaging of active populations of cysteine proteases in BM-DCs using a two-step ABP. A) Azide functionalised E-64 probe. The recognition element of the E-64 probe contains a leucine side chain that ensures its selectivity to cysteine proteases. The warhead is an epoxide moiety that forms a stable covalent bond between enzyme and probe. The ligation handle is represented by an azide moiety that -after covalent interaction of the warhead with the enzyme- can be ligated to a detection group of interest upon a bioorthogonal ligation reaction. B) Bioorthogonal ligation strategy for the covalent attachment of a detection moiety on the azide functionality of the E-64 probe. C) Product formed after ligation of the E-64-azide with a AlexaFluor-488 fluorophore. D) BM-DCs were incubated for 2 h with 10 μ M E-64-azide. Cells were fixed in 2% PFA, subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were reacted with

AlexaFluor-488 alkyne (green) using cHc-conditions and DAPI (blue). DAPI staining and blue fiducials were used for correlation purposes. D) (Upper left) Confocal microscopy image of sample section; scale bar 5 μm . D) (Lower left) CLEM image obtained from overlay of confocal and EM pictures; scale bar 2 μm . D) (Right) High magnification detail showing the following structures: M, mitochondria; N, nucleus; G, Golgi. Scale bar 500 nm.

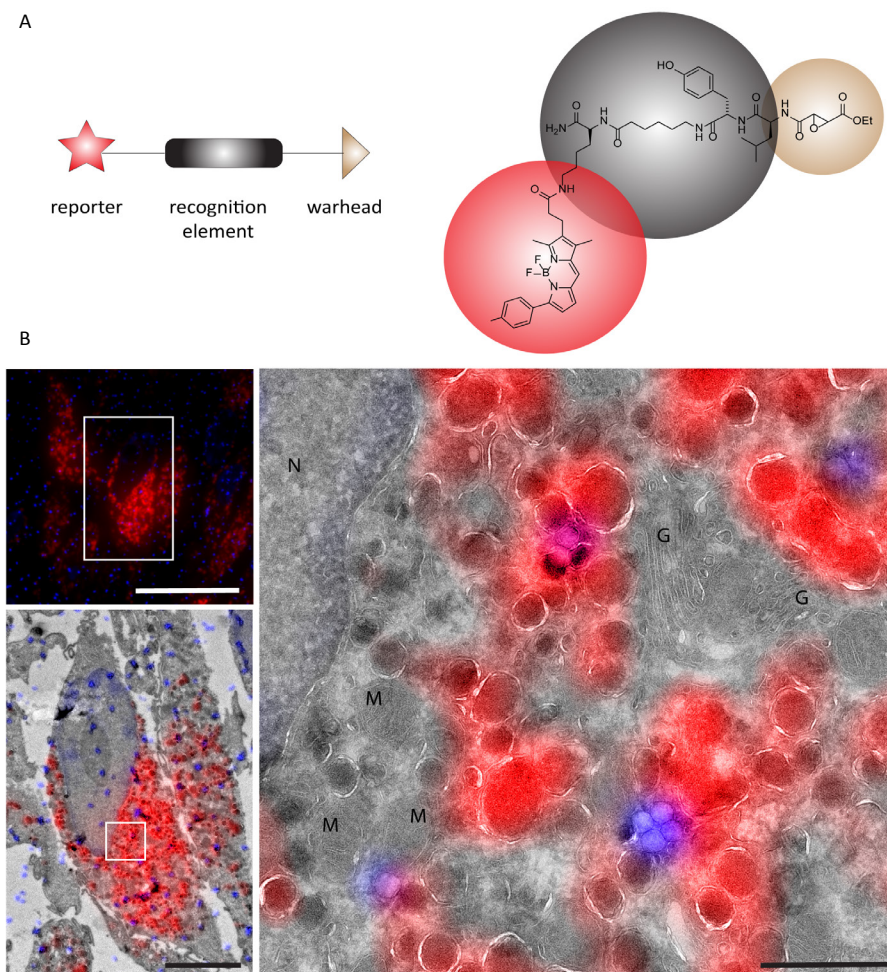


Figure 3: CLEM imaging of active populations of cysteine proteases in BM-DCs using a direct ABP. A) BODIPY-TMR-functionalised E-64 probe. B) BM-DCs were incubated for 2 h with 10 μM E-64-BODIPY-TMR (red). Cells were fixed in 2% PFA, subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were additionally stained with DAPI (blue). DAPI staining and blue fiducials were used for correlation purposes. B) (Upper left) Confocal microscopy image of sample section; scale bar 10 μm . B) (Lower left) CLEM image obtained from overlay of confocal and EM pictures; scale bar 5 μm . B) (Right) High magnification detail showing the following structures; M, mitochondria; N, nucleus; G, Golgi. Scale bar 500 nm.

Comparison of direct and two-step ABP labelling for the CLEM imaging

To further analyse whether CLEM-sample preparation and CLEM-imaging influences the labelling efficiencies of the direct and two-step ABP E-64-BODIPY-TMR was modified with an additional azide moiety (Figure 4).¹⁴ After ABP incubation and CLEM sample preparation the azide was functionalised with an AlexaFluor-488 fluorophore, using the exact same protocol as for the on-section labelling of the E-64-azide. In this manner, fluorescence signals of the direct and two-step ABP labelling strategies could be compared in one single experiment. The results show that both fluorescent signals label the exact same membrane-limited lysosome-like structures (Figure 5). Colocalisation analysis of two signals resulted in a Pearson's R value of 0.82. Most probably this slightly lowered colocalisation is due to the high magnification of the confocal image, whereby chromatic aberration distortions of the fluorescent signals become visible. Overall these results corroborated that CLEM imaging of direct ABP labelling results in similar labelling efficiencies as CLEM imaging of two-step ABPs.

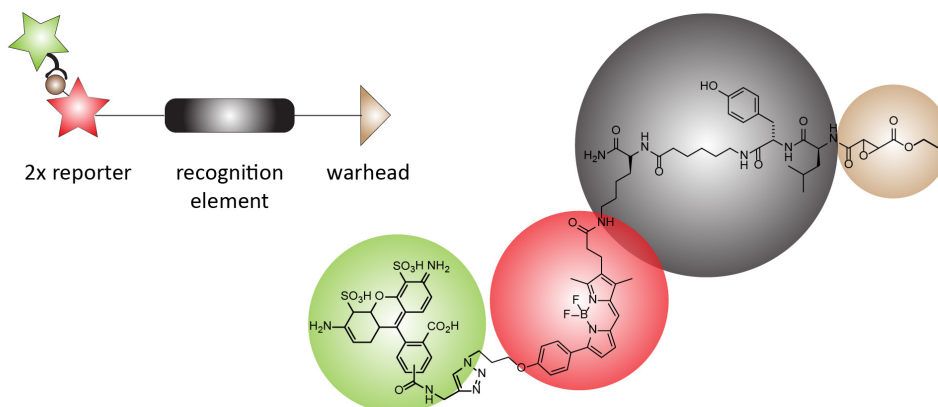


Figure 4: Azide-functionalised E-64-BODIPY-TMR ABP¹⁴ conjugated to AlexaFluor-488 alkyne upon a ccHc reaction.

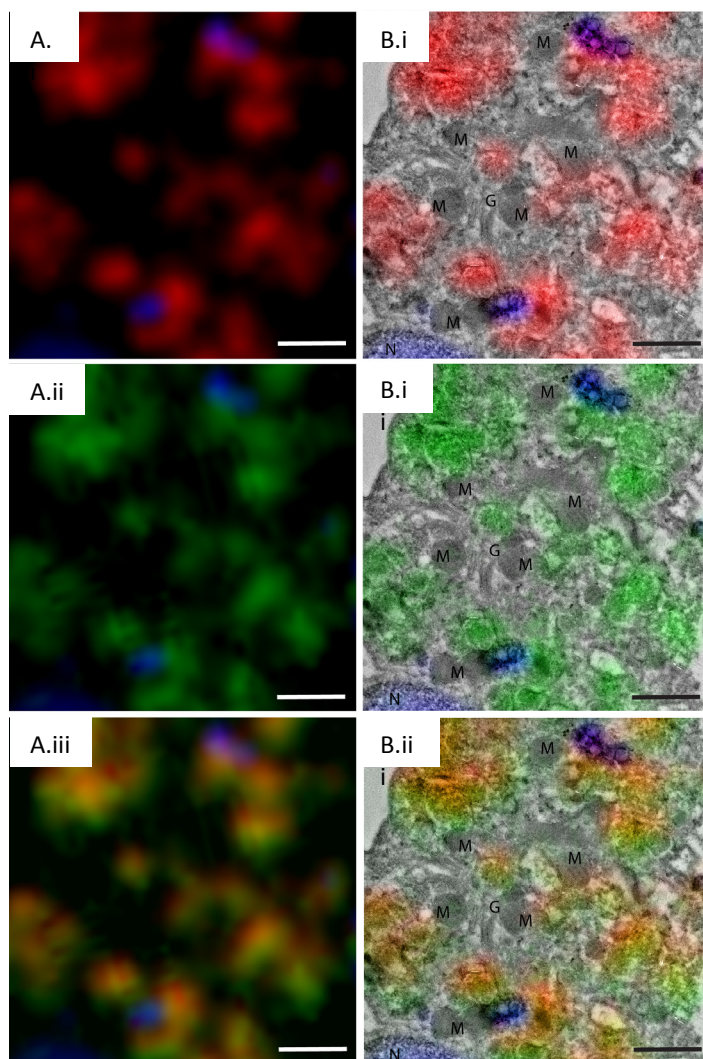


Figure 5: CLEM imaging of active populations of cysteine proteases in BM-DCs using direct and two-step ABPs in a single experiment. BM-DCs were incubated for 2 h with 10 μ M E-64-BODIPY-TMR-azide (red). Cells were fixed in 2% PFA, subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were labelled with DAPI (blue) and AlexaFluor-488 alkyne (green) using ccHc conditions. DAPI staining and blue fiducials were used for correlation purposes. (A.i) Confocal microscopy of direct ABP labelling of cysteine proteases (red); scale bar 500 nm. (B.i) CLEM image of direct ABP labelling of cysteine proteases (red); scale bar 500 nm. (A.ii) Confocal microscopy of two-step ABP labelling of cysteine proteases (green), scale bar 500 nm. (B.ii) CLEM image of two-step ABP labelling of cysteine proteases (green), scale bar 500 nm. (A.iii) Confocal microscopy of simultaneous direct and two-step cysteine protease labelling, scale bar 500 nm. (B.iii) CLEM image of direct and two-step cysteine protease labelling, scale bar 500 nm. M, mitochondria; N, nucleus; G, golgi.

Simultaneous CLEM imaging of glucocerebrosidase and cathepsins using ABP-CLEM

Glucocerebrosidase (GBA) is a lysosomal hydrolase that hydrolyses glucocerebrosides. Mutations in the GBA gene result in Gaucher disease, a lysosomal storage disease that is characterised by buildup of glucocerebrosides in lysosomes. GBA is a relatively short-lived enzyme in lysosomes¹⁵ and is subject to intra-lysosomal proteolytic degradation by cathepsins.^{16, 17} In case of the very common N370S GBA mutation in Caucasian Gaucher disease patients, resistance against intra-lysosomal proteolytic degradation is reduced. Inhibition of intra-lysosomal turnover of GBA through cathepsin inhibition is therefore considered a therapeutic option for some Gaucher disease patients. Insight in the actual (co-)localisation of reactive cathepsins and active GBA is therefore of great interest.

Monitoring of GBA activity using ABPs has been described using a fluorophore-functionalised cyclophellitol ABP (INHIBODY) that targets active variants of GBA (Figure 6).¹⁸ In a preliminary experiment to determine whether GBA and cathepsin co-localisation can be monitored using ABP-CLEM, HeLa and MelJuSo cells were labelled with both INHIBODY and E-64 probes. These cells were then subjected to the same imaging strategy described above for the CLEM imaging of E-64-BODIPY-TMR-treated BM-DCs. CLEM images of labelled HeLa cells show that cysteine proteases and GBA co-localised in membrane-limited lysosome-like structures (Figure 7). This colocalisation of GBA and cysteine proteases in HeLa cells was confirmed with whole-cell confocal microscopy (Figure 8). In contrast to HeLa cells, conventional confocal microscopy and CLEM of dual-labelled MelJuSo cells showed only a partial colocalisation of cysteine proteases and GBA in lysosome-like structures (Figure 9 and 10). Labelling of cysteine proteases was primarily associated with lysosome structures, indicated with solid arrows (Figure 10A/B). However, in case of GBA labelling, GBA was shown to be present in both lysosome-like cysteine protease-positive structures and cysteine protease-negative ultrastructures. These cysteine protease-negative structures were found to be in very close proximity to the cysteine protease-positive structures, but had a distinct morphology. These results show that with direct ABP-CLEM multiple enzyme populations can be simultaneously imaged and reveal differences in the relative ultrastructural localisation of enzyme populations.

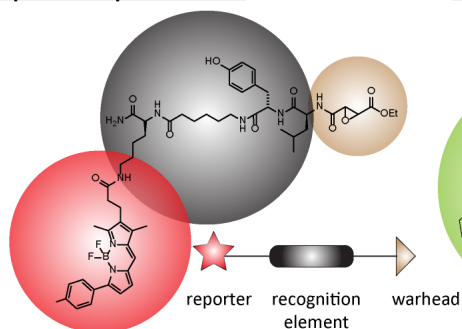
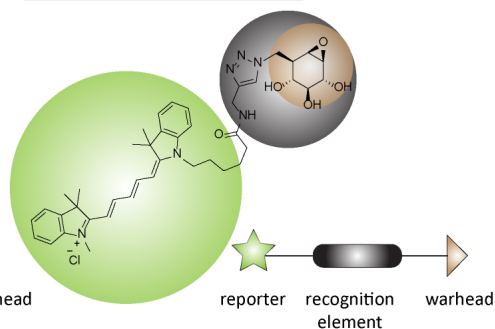
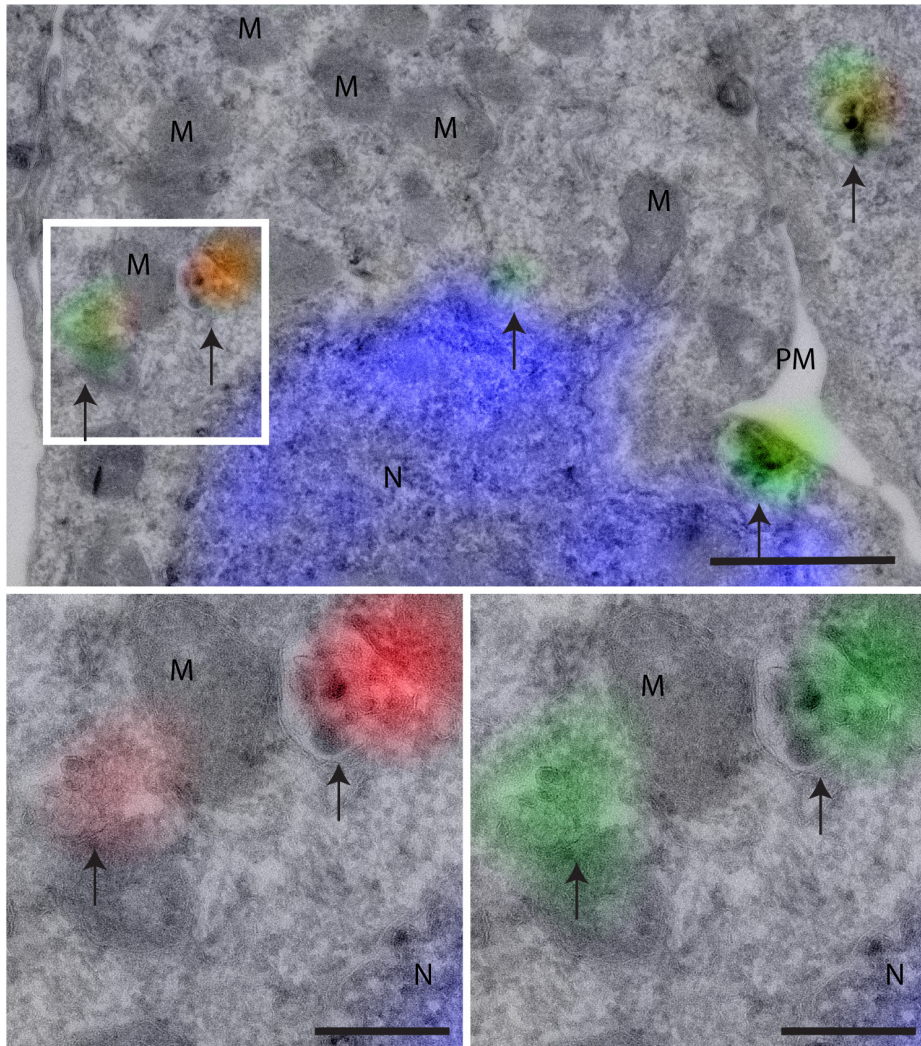
Cysteine proteasesGlucocerebrosidases

Figure 6: E-64-BODIPY-TMR ABP and INHIBODY-Cy5 ABP. E-64-BODIPY-TMR: The recognition element of the E-64 probe contains a leucine side chain that ensures its selectivity to cysteine proteases. The warhead is an epoxide moiety that forms a stable covalent bond between enzyme and probe. The reporter is a BODIPY-TMR fluorophore. INHIBODY-Cy5: the warhead is a cyclophellitol moiety that forms a stable covalent bond between the enzyme and the probe upon interaction with the nucleophile of glucocerebrosidases. The reporter functionality is a Cy5 fluorophore.

HeLa



Cysteine proteases

Glucocerebrosidases

Figure 7: CLEM imaging of cysteine proteases and glucocerebrosidases (GBAs) in HeLa cells using direct ABPs. HeLa cells were incubated for 2 h with 10 μ M E-64-BODIPY-TMR (red) and 100 nM INHIBODY-Cy5 (green). Cells were fixed in 2% PFA, subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were additionally stained with DAPI (blue). (Upper) CLEM image of cysteine proteases (red) and GBA (green) in HeLa cells; arrows indicate locations of cysteine proteases and GBA labelling. Scale bar 500 nm. Lower left) High magnification detail of upper image showing ultrastructural context of E-64-BODIPY-TMR (red) labelling of cysteine proteases (arrows). M, mitochondria; N, nucleus, scale bar, 200 nm. Lower right) High magnification detail of upper image showing GBA labelling (arrows). Scale bar 200 nm. M, mitochondria; N, nucleus; PM, plasma membrane.

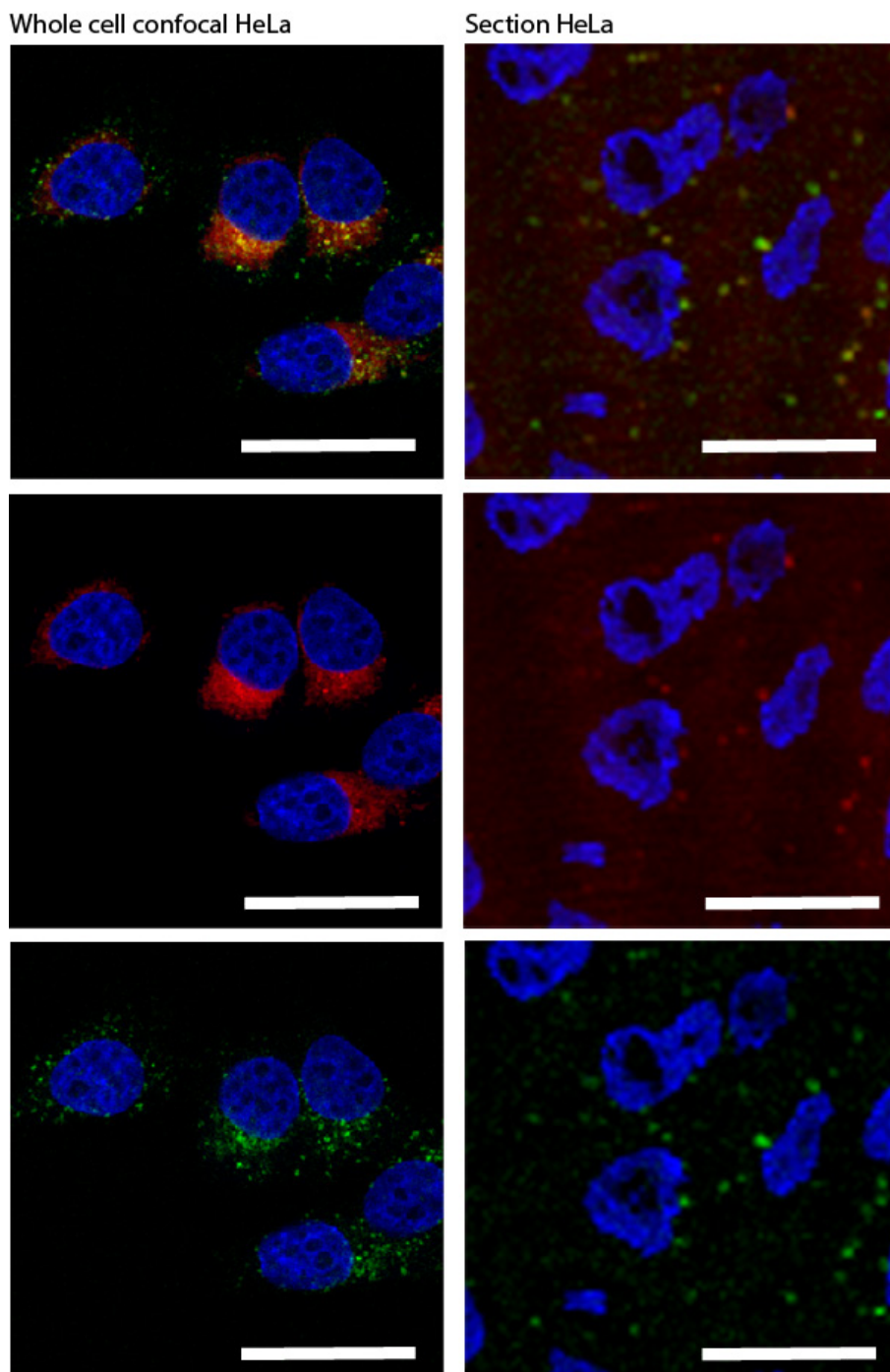


Figure 8: Whole cell and on-section confocal microscopy of HeLa cells incubated for 2 h with 10 μM E-64-BODIPY-TMR (red) and 100 nM INHIBODY-Cy5 (green). Cells were additionally stained with DAPI (blue). Scale bars, 25 μm .

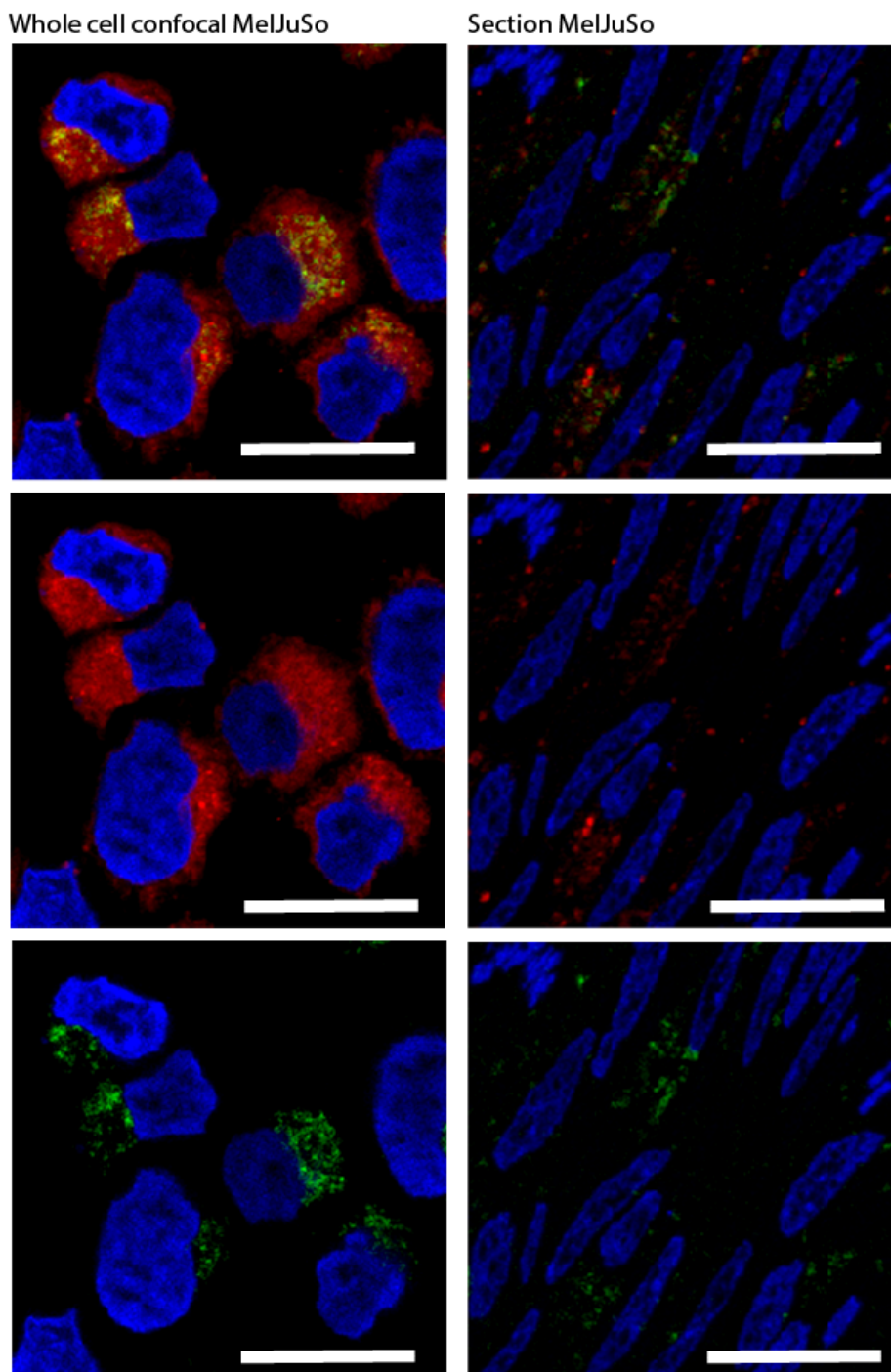
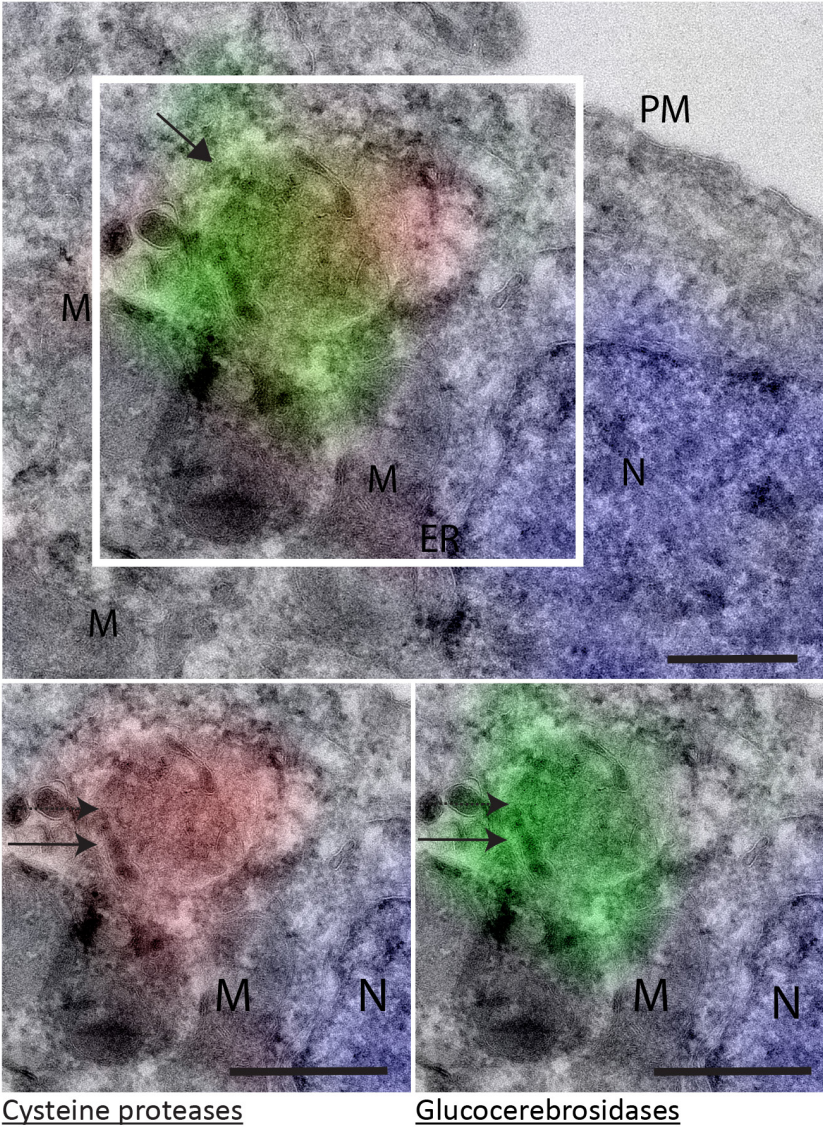


Figure 9: Whole-cell and on-section confocal microscopy of MelJuSo cells incubated for 2 h with 10 μ M E-64-BODIPY-TMR (red) and 100 nM INHIBODY-Cy5 (green). Cells were additionally stained with DAPI (blue). Scale bars, 25 μ m.

A MeJuSo



B MeJuSo

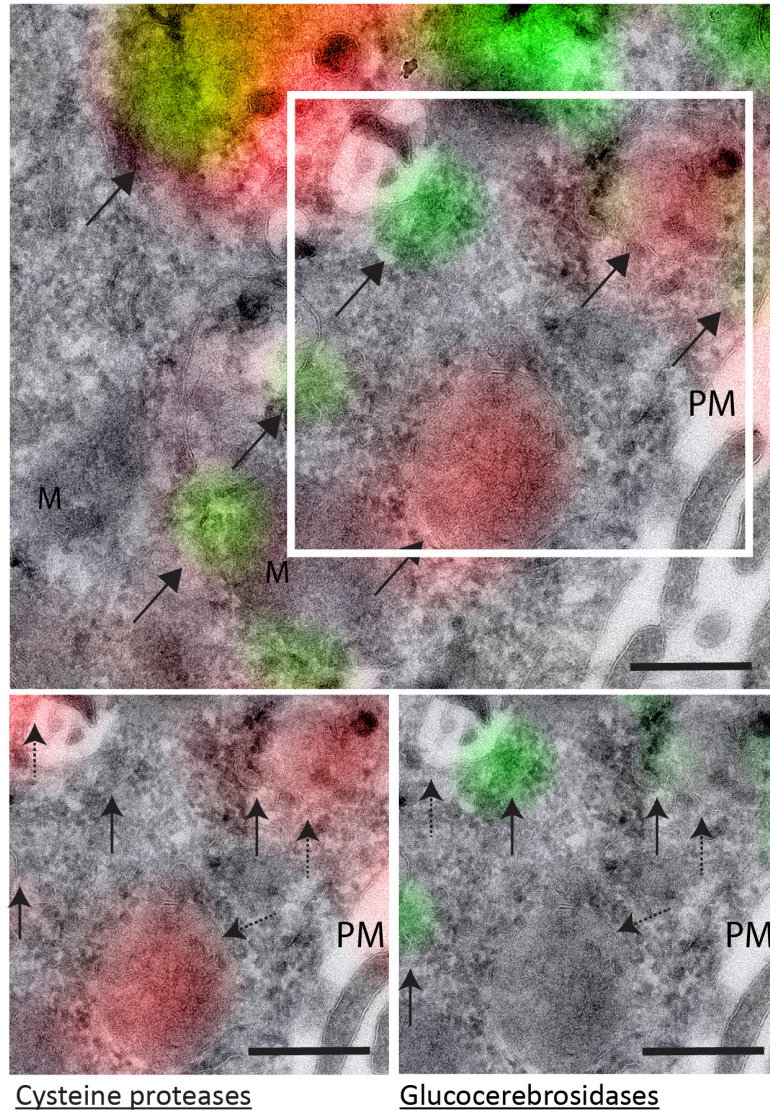


Figure 10 A/B: CLEM imaging of cysteine proteases and glucocerebrosidases (GBAs) in MeJuSo cells using direct ABP labelling. MeJuSo cells were incubated for 2 h with 10 μ M E-64-BODIPY-TMR (red) and 100 nM INHIBODY-Cy5 (green). Cells were fixed in 2% PFA, subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were additionally stained with DAPI (blue). Upper; CLEM image of cysteine proteases (red) and GBA (green) in HeLa cells; arrows indicate locations of cysteine proteases and GBA labelling. Scale bar 500 nm. (Lower left) High magnification detail of upper image showing ultrastructural context of E-64-BODIPY-TMR labelling of cysteine proteases (red, arrows). M, mitochondria; N, nucleus, scale bar, 200 nm. (Lower right) High magnification detail of upper image showing GBA (green, arrows). M, mitochondria; N, nucleus; PM, plasma membrane. Scale bar 200 nm.

Conclusion

In this chapter the first examples of ABP-CLEM are described. Firstly, populations of active cysteine proteases were imaged within the ultrastructural cellular context of dendritic cells using both a two-step and a direct ABP approach. It was shown that cysteine proteases reside inside membrane-limited lysosome-like structures of around 50-250 nm in diameter. It was additionally demonstrated that with both direct and two-step labelling enzyme activity can be labelled with high selectivity and efficiency, indicating that bioorthogonal labelling does not suffer from section penetration limits that have been reported for on-section antibody labelling strategies.¹⁹

CLEM imaging of multiple enzyme classes shows that HeLa cells have a complete overlap in ultrastructural cellular location of cysteine proteases and GBA, whereas in MeJuSo cells a partial colocalisation could be observed. This partial colocalisation of GBA with E-64-BODIPY-TMR reactive cathepsins is not surprising as it is well established that there is a fundamental difference in the transport of newly synthesised cathepsins and of GBA to lysosomes. Cathepsins acquire mannose-6-phosphate (M6P) moieties in their N-linked glycans in the Golgi apparatus, which ensures the mannose-6-phosphate receptor (M6PR)-mediated transport to late endosomes/lysosomes.²⁰ In sharp contrast, GBA enzymes depend on binding to the membrane protein LIMP-2 in the endoplasmic reticulum for their transport to lysosomes.^{21, 22} Upon LIMP-2 binding, newly formed GBA is transported to acidic late endosomes/lysosomes where low pH dependent dissociation of the complex occurs. It is therefore that distinct vesicular structures are reported to be involved in transport of cathepsins/M6PR and GBA/LIMP-2.²⁰ ABP-CLEM may provide new insights into the routing biology of these enzymes, and this information is crucial for research on enzyme activity in the lysosomal storage disorder Gaucher disease. It is foreseen that ABP-CLEM can be applied to monitor various Gaucher disease hallmarks and can be used for both diagnostic and therapeutic applications whereby information of GBA activity relative to the activity of other enzyme populations is of interest.

Experimental

Cell culture

Mouse bone marrow derived dendritic cells (BM-DCs) were generated from B57BL/6 mouse bone marrow essentially as described²³ 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 (FCS, Greiner), 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. BM-DCs were incubated for 2 h with E-64-azide²⁴ (final concentration of 10 μ M) or E-64-TMR-azide after which the cells were washed with PBS and kept for 2 h in fresh medium.

HeLa cells were cultured in DMEM (Gibco) supplemented with 7.5% FCS (Greiner). MelJuSo (human melanoma cell line) cells were cultured in IMDM (Sigma Aldrich) supplemented with 7.5% FCS (Greiner). HeLa and MelJuSo cells were incubated for 2 h with 100 nM INHIBODY-Cy5 and 10 μ M E-64-BODIPY-TMR from stock solutions in DMSO. After incubation cells were washed and subjected to further analysis.

Whole cell confocal microscopy

Cells were seeded (7×10^4) on a 12-well removable chamber slide (Ibidi) and left to grow O/N. The following day activity based probes (ABPs) were added at the indicated time and concentration. Cells were fixed in 4% PFA for 15 minutes and kept in PBS at 4 °C until further analysis. In case of click-labelling with AlexaFluor-488, 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 CuSO_4 , 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM amino-guanidine, 5 μ M AlexaFluor-488 alkyne (Invitrogen)), and DAPI (1 μ g/ml). In case of one-step ABP labelling cells were only labelled with DAPI. 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.^{11, 25} Briefly, cells 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 0.1 M 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 of 75 nm were obtained essentially as described elsewhere.¹¹ Briefly, frozen samples were mounted in a cryo-ultramicrotome (Leica). Samples were 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 75 nm thick sections was produced that was retrieved from the cryo-chamber with the lift-up hinge method.²⁶ A droplet of 1.15 M sucrose was used for section retrieval.

Obtained sections were transferred to a specimen grid previously coated with formvar and carbon. Grids were additionally coated with 100 nm carboxylate-modified FluoroSpheres (350/440) (Life Technologies).

Sections that were click-labelled with AlexaFluor-488 were labelled as follows: thawed cryosections on an EM grid were left on the surface of 2% gelatin in phosphate buffer at 37°C for 30 minutes. 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 were then labelled with DAPI (5 minutes) (2 µg/ml) and additionally washed with PBS and aquadest. In case of one-step ABP labelling cells were only labelled with DAPI.

Microscopy and correlation

The Correlative Light and Electron Microscopy (CLEM) approach used has been described elsewhere.¹¹ Briefly, 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). After confocal microscopy the electron microscopy (EM) grid with the sections was removed from the glass slide, rinsed in distilled water and incubated for 5 min on droplets of an aqueous solution containing 2% methylcellulose and 6% uranyl acetate. Excess of methylcellulose/uranylacetate solution was blotted away and grids were air-dried. EM imaging was performed with a Tecnai 20 transmission electron microscope (FEI) operated at 120 kV acceleration voltage.

Correlation of confocal and EM images was performed in Adobe Photoshop CS6. In Adobe Photoshop, the fluorescence microscopy image was copied into the EM image as a layer and made 50% transparent. Transformation of the FM image was necessary to match it to the larger scale of the EM image. This was performed via isotropic scaling and rotation using 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 fluorescently labelled cells. At high magnification alignment was performed using fiducial beads.²⁷

Colocalisation analysis

The Pearson coefficient was determined on magnified confocal images (obtained in Adobe Photoshop CS6) using the Coloc2 function in ImageJ after background correction.

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