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Introduction

The immune system is the defence mechanism that protects the human body against illness and infection. Through a series of steps called the immune response, it attacks pathogens and potentially harmful commensals. When rapid and effective, the infection will be eliminated quickly and disease will not occur. However, many bacterial pathogens have evolved strategies to subvert and exploit the immune response in order to enter and replicate in eukaryotic cells.

A prime example of such a bacterial pathogen is *Salmonella enterica* (*Salmonella*). *Salmonella* is a Gram-negative facultative intracellular pathogen that infects both humans and animals.\(^1\) More than 2500 *Salmonella* serotypes exist, which can be broadly grouped in non-typhoidal and typhoidal.\(^2\) Non-typhoidal *Salmonella* serotypes cause localised gastrointestinal infections in healthy human adults,

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whereas typhoidal serotypes have evolved to enter and exploit intestinal macrophages and cause life-threatening systemic infections.\textsuperscript{3, 4}

Typhoidal \textit{Salmonella} establishes a systemic infection upon the temporal and spatial injection of bacterial proteins into macrophage cells.\textsuperscript{5} This temporal and coordinated delivery of effector proteins modulates the functions of the macrophage cell organelles and promotes \textit{Salmonella} replication and its subsequent dissemination throughout the host.\textsuperscript{6} \textit{Salmonella} can enter macrophages by several endocytic routes, which are triggered by the secretion of several effector proteins via both pathogenicity island 1 (SPI1) and non-SPI1 type III secretion systems (T3SS) (Figure 1, (1)).\textsuperscript{7} Once intracellular, \textit{Salmonella} remains inside the spacious phagosome (SP) (Figure 1, (2)). This vacuolar compartment shrinks over minutes to hours to form an adherent membrane around one or more bacteria, which is then referred to as the \textit{Salmonella}-containing vacuole (SCV) (Figure 1, (3)). Upon the injection of effector proteins \textit{Salmonella} modulates the interaction of the SCV with the endocytic environment and the ER-Golgi network and as such ensures the maturation of the SCV and its own replication (Figure 1, (4)).\textsuperscript{3}

Understanding the mechanisms by which \textit{Salmonella} modulates and interacts with host cell organelles is thus of key importance for the understanding of \textit{Salmonella}-virulence mechanisms in the development of systemic \textit{Salmonella} infections.\textsuperscript{6} To unravel these mechanisms injected and secreted bacterial proteins need to be labelled and traced.

Proteome-wide labelling of injected and secreted bacterial proteins can be established using bioorthogonal non-canonical amino acid tagging (BONCAT). With BONCAT bioorthogonally functionalised amino acid analogs such as azidohomoalanine (Aha) and homopropargylglycine (Hpg) are incorporated in the proteomes of prokaryotic and eukaryotic cells (Figure 2A). These bioorthogonal amino acid analogs are incorporated by wild-type methionyl-tRNA synthetase (metRS) at the sites where naturally methionine amino acids are incorporated. After incorporation, bioorthogonally functionalised amino acids can be labelled with a detection group using chemical ligation (Figure 2C).\textsuperscript{8, 9}
Recently, Grammel et al. reported that the BONCAT labelling strategy can even be used to selectively label and study the proteome wide expression levels of bacterial pathogens inside mammalian immune cells. By mutating the methionyl-tRNA synthetase (NLL-MetRS) of bacteria, non-canonical amino acid such as azidonorleucine (ANL) and 2-aminoocytynoic acid (AOA) (Figure 2B) were specifically incorporated in Salmonella Typhimurium and Yersinia enterocolitica whilst present in the environment of a host cell. Recently, Mahdavi et al. reported that this bioorthogonal labelling approach even enabled the identification of distinct secretion profiles for intracellular and extracellular bacteria, and showed that it allows the analysis of the temporal order of bacterial protein expression during different stages of infection.

Figure 1: Schematic overview of interactions between Salmonella and host macrophage cells. Salmonella gets internalised by endocytosis upon secretion of effector proteins trough the T3SS (2). Subsequently Salmonella is enclosed in a spacious phagosome (SP) that is formed by membrane ruffles (2). Later, the phagosome fuses with lysosomes, acidifies and shrinks to become adherent around the bacterium. This is called the Salmonella-containing vacuole (SCV) (3). Upon the injection of effector proteins through the T3SS Salmonella modulates the interaction of the SCV with the endocytic environment and the ER-Golgi network and as such ensures the maturation of the SCV and its own replication (4).
Figure 2: BONCAT for the identification of secreted bacterial effector proteins. (A) Structures of natural amino acid Met (1) and bioorthogonal amino acid analogs Aha (2) and Hpg (3) that can all be charged to tRNA_Met by the wild-type MetRS. (B) Structure of Anl, which can be utilised only by a mutant synthetase like MetRS_NLL and can therefore be incorporated cell-specifically in the bacterial proteome during infection. (C) Reaction scheme of Cu-catalyzed cycloaddition reaction between proteins that contain (azide- or alkyne-) modified amino acids with an azide- or alkyne-functionalised reporter.
Towards Ultrastructural Imaging of Salmonella-Host Interactions using Bioorthogonal Labelling and Super-Resolution CLEM

BONCAT is thus a very powerful tool to unravel the temporal expression of Salmonella effector proteins. However, to investigate the interaction of these effector proteins with the host-environment, tools are needed that allow for their detection within the context of the hostcell. Recently, an approach was reported that enables the visualisation of BONCAT-labelled bacteria in the context of a host cell with correlative light and electron microscopy (CLEM). This correlative transmission electron microscopy (TEM) technique allows for the wide field navigation to areas of interest with fluorescence microscopy and provides narrow field high-resolution information on the interior of the cell with TEM, and would thus allow for the visualisation of BONCAT-labelled Salmonella in the context of host cell organelles. However, the resolution of the fluorescence detection within this strategy, which is around 250 nm, is not sufficient to unravel the existence of secreted virulence factors, which can be around 2-20 nm in diameter.

Recent developments in the field of CLEM have shown that upon implementing super-resolution imaging in CLEM, the detection resolution of fluorescent probes can be improved 10-fold, resulting in a more accurate and sensitive ultrastructural localisation of fluorescent labels. Since its initial inception it was shown that, depending on the research question, super-resolution imaging strategies can be combined with TEM in various ways. It has been reported that upon lowering osmium tetroxide (OsO₄) concentrations and optimisation of embedding resin, fluorescence quenching could be prevented and fluorescence was preserved. This sample preparation technique was used with both PALM (photoactivated localisation microscopy) and STED (stimulated emission depletion) microscopy. Another example described the use of PALM, or STORM (stochastic optical reconstruction microscopy), after Tokuyasu sectioning and subsequent OsO₄ contrast enhancement prior to scanning electron microscopy measurements. Since this latter technique uses Tokuyasu sample preparation as also reported for the BONCAT-CLEM strategy, it could be implemented in the CLEM strategy for the visualisation of secreted virulence factors. The aim of this study is therefore to apply the BONCAT-CLEM strategy for the imaging of BONCAT-labelled Salmonella virulence factors upon implementation of STORM imaging in the CLEM imaging sequence.
Results

In order to monitor *Salmonella*-host interactions with bioorthogonal amino acid tagging and CLEM, it was first tested whether bioorthogonal amino acids could be incorporated into the *Salmonella* proteome without the need of tRNA/tRNA synthetase engineering. To this end, the incorporation of the bioorthogonal amino acid Hpg into the bacterial proteome was monitored, analogous to the method outlined for *E. coli* in Chapter 3.\(^{25}\)

It was decided to explore the incorporation of Hpg into the *Salmonella* model bacterium *S. Typhimurium*, as it represents a useful model to characterise the molecular mechanisms that underpin the interactions between typhoidal *Salmonella* species and phagocytic cells.\(^3\) Incorporation into the proteome was optimised with respect to cell viability and incorporation levels as assessed using SDS-PAGE analysis. In line with previous findings, extended incubation times resulted in reduced viability (Figure 3A), suggesting negative effects on protein expression upon prolonged exposure to bioorthogonal amino acids.\(^{10, 14}\) Surprisingly, lowering the concentration of the bioorthogonal amino acid Hpg resulted in a higher incorporation level (Figure 3B). Thus, optimal conditions in terms of viability and incorporation levels were 30 minutes incubation with 0.4 mM Hpg, and these conditions were used for all further imaging studies.

To address whether the alkyne-*S. Typhimurium* bacteria could be imaged within a host cell using bioorthogonal labelling and confocal microscopy analysis, mouse bone marrow-derived dendritic cells (BM-DCs) were incubated with alkyne-modified *S. Typhimurium* expressing the fluorescent protein DsRed.\(^{26}\) Subsequently, cells were washed, fixed and labelled with AlexaFluor-488 azide using the optimised copper-catalysed Huisgen cycloaddition (ccHc) reactions described in Chapter 3 of this thesis.\(^{14}\) Confocal analysis revealed colocalisation of the DsRed-expressing *S. Typhimurium* and the bioorthogonal AlexaFluor-488 label (Figure 4). However, the signal of the AlexaFluor-488 was faint, which is most probably a result of the presence of endogenous Met that competes with Hpg for its incorporation. Despite this competition, these results show that the alkyne-*S. Typhimurium* bacteria can be labelled detectably with AlexaFluor-488-azide in the environment of a host cell using a ccHc reaction.
Figure 3: Hpg incorporation in S. Typhimurium A) S. Typhimurium cells were grown to an OD$_{600}$ of 0.3-0.5. Cultures were then incubated with the indicated Hpg or Met concentrations. OD$_{600}$ were measured at indicated time points and relative generations were calculated (first generation was set at 1 at timepoint 0). B) Top: Fluorescence gel of AlexaFluor-647 alkyne-labelled S. Typhimurium cells grown in the presence of the indicated concentrations of Hpg and Met. Bottom: Coomassie-staining loading control shows relative amounts of total protein per sample.
For analysis of alkyne-\textit{S. Typhimurium} bacteria on ultrastructures, bioorthogonally tagged \textit{S. Typhimurium} has to be specifically labelled after Tokuyasu sample preparation and cryosectioning. To test the feasibility of this approach BM-DCs were again incubated with alkyne-\textit{S. Typhimurium} and subjected to Tokuyasu sample preparation: after cryosectioning, the samples were transferred to an EM grid and labelled with AlexaFluor-488-azide using a ccHc-reaction. These sections were then imaged in the confocal microscope. This revealed a very low signal-to-noise ratio of the AlexaFluor-488-azide (Figure 5A), in line with the whole cell confocal microscopy findings.

In an attempt to increase the intensity of fluorescence of the bioorthogonal handles, an additional labelling step was included to enhance the signal. First the sections were incubated with anti-AlexaFluor-488 IgG, which was then visualised using the IgG-binding protein A conjugated with AlexaFluor-647. This approach resulted in an improvement of the signal-to-noise ratio and provided sufficient fluorescence to perform CLEM analysis (Figure 5B). After this successful confocal imaging, the sections were embedded in methylcellulose with uranylacetate and subjected to EM imaging. Correlation of the confocal and EM images was
performed using nuclear DAPI stain (Figure 6). Images were successfully correlated and morphological information obtained from the EM images showed that the alkyne-positive foci located on intact *S. Typhimurium*, as well as smaller non double-membrane containing structures (Figure 6D/E, yellow arrows). These results show that, upon use of a combination of bioorthogonal labelling and CLEM, ultrastructural information of *S. Typhimurium* and of the host phagocyte could successfully be obtained.

**Figure 5**: Protein A-AlexaFluor-647 enhancement of bioorthogonal labelling. BM-DCs were incubated with alkyne-*S. Typhimurium* and washed with PBS to remove unbound/non-internalised *S. Typhimurium*. Cells were fixed, subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. A.i) Sections were reacted with AlexaFluor-488 azide using ccHc-conditions (green), and stained with DAPI (blue). B.i) To enhance the fluorescence signal of the bioorthogonal label sections were additionally labelled with Protein A-AlexaFluor-647 (green) using a rabbit anti AlexaFluor-488 antibody. (ii) Details of i. Scale bar: 7 µm.
Figure 6: CLEM imaging of phagocytosed alkyne-S. Typhimurium. BM-DCs were incubated with alkyne-S. Typhimurium and washed with PBS to remove unbound/non-internalised S. Typhimurium. Cells were fixed and subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were reacted with AlexaFluor-488 azide using ccHc-conditions (green) and stained with DAPI (blue). The fluorescence signal of the AlexaFluor-488 was enhanced with protein A AlexaFluor-647 using a rabbit anti-AlexaFluor-488 antibody. DAPI staining was used for correlation purposes. A) low magnification confocal image, B) high magnification confocal image, C) CLEM image of B correlated with EM image, D) detail of C, E) detail of D. Yellow arrows indicate label that derived from alkyne-S. Typhimurium. Scale bar 500 nm.

The resolution of the fluorescence signal that was used for the correlation of the bioorthogonal signal results in accuracies around 250 nm, due to the van Abbe diffraction limit of visible light. Since the main envisaged goal of the method development was to reveal the location of secreted virulence factors that are around 2-20 nm in size\textsuperscript{16}, the detection level of the fluorescence signal had to be improved. It was therefore explored whether the photoactivated localisation microscopy (PALM)-CLEM imaging of fluorescent proteins initially reported by Betzig and co-workers\textsuperscript{17}, could be modified to the use of detecting the
bioorthogonal labels. For this the related Nikon N-STORM was explored on the Tokuyasu-sections that were previously imaged by confocal microscopy (Figure 6). Sections were now labelled with AlexaFluor-647 and embedded between a glass slide and coverslip with a glucose oxidase (GLOX) buffer. The use of STORM imaging on the sample sections resulted in a drastic improvement of both the accuracy and detection levels of the fluorescently labelled alkyne-<i>S. Typhimurium</i> (Figure 7). Additionally, distinctions in label intensities could be observed within the individual bacteria. Since alkyne handles were incorporated prior to infection, non-labelled regions within the bacteria might represent regions where newly synthesised, untagged, proteins are formed.<sup>27</sup>

![Figure 7: Super-resolution N-STORM image of 75 nm cryosection of BMDCs incubated with alkyne-<i>S. Typhimurium</i>. BM-DCs were incubated with alkyne-<i>S. Typhimurium</i> and washed with PBS to remove unbound/non-internalised <i>S. Typhimurium</i>. Cells were fixed and subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were reacted with AlexaFluor-647 azide using ccHc-conditions (red). A.i) Wide field low resolution image of alkyne-<i>S. Typhimurium</i> A.ii) Super resolution N-STORM image of alkyne-<i>S. Typhimurium</i>. Scale bar: 500 nm.

Sample sections were subsequently treated as for the previously reported CLEM strategy and EM structure were analysed (Figure 8). It seemed that STORM imaging did not affect the appearance of the ultrastructures. Membranes were intact and no structural alterations could be observed between STORM-imaged regions compared to non-imaged ones. Moreover, host cell organelles were still recognisable by their distinct morphological appearances. Upon correlation of the STORM images, signals were primarily present on the <i>S. Typhimurium</i> bacteria.
Moreover, small labels surrounding the bacteria were present on small structures with a diameter of around 10-20 nm, which were both present on membranes of vesicular host structures and were seemingly unattached within the spacious phagosome.

Figure 8: Super-resolution N-STORM-CLEM image of 75 nm cryosection of BMDCs incubated with alkyne-S. *Typhimurium*. BM-DCs were incubated with alkyne-S. *Typhimurium* and washed with PBS to remove unbound/non-internalised *S. Typhimurium*. Cells were fixed and subjected to Tokuyasu sample preparation and cryosectioned into 75 nm sections. Sections were reacted with AlexaFluor-647 azide using ccHc-conditions (red). A.i) Example 1 of super-resolution N-STORM image of alkyne-S. *Typhimurium*. A.ii) super-resolution CLEM image of A.i. B.i) Example 2 of super-resolution N-STORM image of alkyne-S. *Typhimurium*. B.ii) super-resolution CLEM image of B.i. Arrows indicate fragments of alkyne-S. *Typhimurium* that have been internalised by the host-cell. Scale bar: 250 nm.
Conclusion

The results presented show that upon implementation of STORM imaging in a CLEM setting, BONCAT-labelled *S. Typhimurium* can be imaged within the context of the host cell with high sensitivity and accuracy. This strategy has great potential to further elucidate the temporal and spatial injection of *Salmonella* virulence factors and their interactions with host organelles. In the presented experimental setup the *S. Typhimurium* bacteria were BONCAT labelled prior to their encounter with phagocytic host cells. This setup primarily allows for the monitoring of effector protein secretion during early stages of infection. However, since strategies have been reported to specifically BONCAT label a bacterium whilst interacting with a host cell, this imaging approach would also allow monitoring of effector protein secretion during later stages of infection. Moreover, due to the broad applicability of BONCAT labelling, this strategy can also be combined with several mutant strains that have been shown to have attenuated virulence. In this fashion information can be obtained on the host-interaction and secretion patterns of these attenuated strains. In addition, the site-specific incorporation of bioorthogonal handles in virulence factors of interest would even allow for a more targeted CLEM-imaging approach between virulence factors and host cell organelles. For example, a single virulence factor of interest can be specifically BONCAT-labelled and using the STORM-CLEM approach quantitative information can be extracted on their expression levels in the context of the infected host cell.
Salmonella Typhimurium culturing conditions

Salmonella Typhimurium expressing DsRed\textsuperscript{26, 31, 32} were grown overnight at 37°C in Lysogeny Broth (LB) medium. The following day cultures were diluted 1:33 in LB medium and grown at 37°C till an $\text{OD}_{600}$ between 0.3-0.5. Subsequently cells were collected and resuspended in Selenomet medium (Molecular Dimensions) and supplemented with either 0.04, 0.4 or 4 mM Homopropargylglycine (Hpg) (Chiralix) or 4 mM Methionine (Met) (Sigma-Aldrich). After 30, 60 and 120 minutes $\text{OD}_{600}$ were measured and cells were collected by centrifugation for gel analysis and bone marrow dendritic cell (BM-DC) infection experiments. Throughout culturing, cultures were supplemented with 100 µg/ml Ampicillin.

Mammalian cell culture conditions

Mouse BM-DCs were generated from B57BL/6 mice bone marrow essentially as described\textsuperscript{33} 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 µM 2-mercaptoethanol (Life Technologies), penicillin 100 l.U./mL and streptomycin 50 µg/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.

S. Typhimurium expressing DsRed and cultured in the presence of 0.4 mM Hpg for 30 minutes were added to the BM-DCs as suspensions in PBS at a multiplicity of infection (MOI) of 50. After 45 minutes of incubation unbound/non-internalised S. Typhimurium cells were washed away (2x PBS) and medium was replaced. Subsequently cells were subjected to confocal microscopy or Tokuyasu sample preparation.

SDS-PAGE analysis

At the indicated time points S. Typhimurium were collected and cells were lysed with lysis buffer (50 mM HEPES pH 7.3, 150 mM NaCl and 1% NP-40) and incubated on ice for 1 h. Subsequently protein concentrations were determined with a Quibit 2.0 fluorimeter (Life Technologies), after which 20 µg of the protein...
was incubated for 1 h with copper catalysed Huisgen cycloaddition (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-647 azide (Invitrogen)). Samples were then resuspended in 4x SDS Sample buffer (250 mM TrisHCl pH 6.8, 8% w/v SDS, 40% glycerol, 0.04% w/v bromophenolblue, 5% 2-mercaptoethanol) and incubated at 100°C for 5 minutes. After the samples were run through a Hamilton syringe multiple times to shear genomic DNA, samples were subjected to SDS-PAGE. Gels were then directly imaged with a Biorad Universal Hood III for in-gel visualisation of the fluorescent labelling. As a loading control gels were stained with Coomassie Brilliant Blue. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used as a protein standard.

**Whole-cell confocal microscopy**

BM-DCs were seeded (7 x 10⁴) on a 12 well removable chamber slide (Ibidi) and left to grow O/N. The following day *S. Typhimurium* expressing DsRed and cultured in the presence of 0.4 mM Hpg for 30 minutes were added to the BM-DCs as suspensions in PBS at an MOI of 50. After 45 minutes of incubation unbound/non-internalised *S. Typhimurium* were washed away (2x PBS) and cells were fixed in 4% PFA for 15 minutes. Until further analysis cells were kept in PBS at 4°C. Fixed cells were incubated for 30 minutes with blocking buffer (1% BSA, 1% gelatin cold water fish skin, 0.3% Triton X-100), 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 µM AlexaFluor-647 Azide (Invitrogen)) and DAPI (1 µg/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. Briefly, BM-DCs infected with *S. Typhimurium* 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. Frozen samples were stored under liquid nitrogen.

Ultrathin cell sections were obtained as described elsewhere.\textsuperscript{34} 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 carboxylate-modified fluorescent fiducial beads (350/440)(Life Technologies).

Sections were labelled as follows: thawed cryosections on an electron microscopy (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 CuSO\textsubscript{4}, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM amino-guanidine, 5 μM AlexaFluor-488 Azide or AlexaFluor-647 Azide (Invitrogen) for 1 h and washed 6 times with PBS. In preparation for confocal microscopy the grids were blocked again with PBS/glycine containing 1% BSA after which the grids were incubated for 1 h with PBS/glycine 1% BSA supplemented with an AlexaFluor-488 antibody (Invitrogen). After washing with PBS/Glycine and blocking with PBS/glycine 0.1 % BSA, grids were incubated for 20 minutes on PBS/Glycine 1% BSA supplemented with AlexaFluor-647 Protein A (Invitrogen) to enhance the fluorescence signal. Sections were then labelled with DAPI (2 μg/ml), and additionally washed with PBS and aquadest.

**Microscopy and correlation**

The correlative light and electron microscopy (CLEM) approach used was adapted from Vicidomini et al.\textsuperscript{35} 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

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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.

In case of STORM imaging grids containing the sample sections were washed with glucoseoxidase (GLOX) buffer (100 µl PBS, 20 µl 50% glucose, 20 µl 1M MEA (monoethanolamine) and 2µl GLOX (0.7 mg/ml GLOX, 5 mg/ml catalase in PBS) supplemented with 30% glycerol (60 µL) and placed on glass slides. Grids were then covered with a small drop of GLOX buffer, after which a coverslip was mounted over the grid. STORM images were acquired using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. Excitation inclination was tuned to adjust focus and to maximise the signal-to-noise ratio. AlexaFluor-647 was excited illuminating the sample with a 647 nm (∼160 mW) laser line built into the microscope. Fluorescence was collected by means of a Nikon 100x, 1.4 NA oil immersion objective and passed through a quad-band-pass dichroic filter (97335 Nikon). 20,000 frames were acquired for the 647 channel. Images were recorded onto a 256 × 256 pixel region (pixel size 160 nm) of a CMOS camera. STORM images were analysed with the STORM module of the NIS element Nikon software.³⁰

After fluorescence or STORM microscopy the EM grid with the sections was removed from the glass slide, rinsed in distilled water and incubated for 5 minutes on droplets of uranylacetate/methylcellulose. Excess of uranylacetate/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.³⁶

Correlation of confocal and EM images was performed in Adobe Photoshop CS6. In Adobe Photoshop, the light microscopy (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.³⁷
Chapter 6

References

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