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

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Summary and Future Prospects

In this thesis the combinatorial use of bioorthogonal labelling and Electron Microscopy (EM)-based imaging techniques is explored to enable observations of specific molecular targets in their ultrastructural context within the cell. In **chapter 1** the principles of EM imaging for biological research are discussed, including two different types of electron microscopes and various techniques to prepare specimens for transmission electron microscopy (TEM). **Chapter 1** additionally describes several labelling strategies that can be employed to identify biomolecules within EM-revealed structures. One of these approaches; correlative light and electron microscopy (CLEM) imaging, is explained by means of two generally used CLEM strategies; live-cell CLEM and on-section CLEM.

In **chapter 2** the importance and potential of bioorthogonal chemistry for CLEM imaging is emphasised. In this review an overview is given of frequently used bioorthogonal ligation strategies for imaging, including the copper-catalysed Huisgen cycloaddition (ccHc), the strain-promoted cycloaddition, the inverse

electron-demand Diels-Alder cycloaddition, and the photoclick reaction. **Chapter 2** further highlights the advances that have been made towards CLEM imaging of bioorthogonal functionality, including azide-modified gold particles, bioorthogonally-functionalised selenide/zinc sulphide core-shell quantum dots and bioorthogonally-functionalised fluorophores that are capable of photooxidising diaminobenzidine (DAB).

Chapter 3 describes the development of two methods for EM detection of bioorthogonal labels. The first method is a gold labelling strategy that allows for the direct detection of bioorthogonal tags in the EM. The second method is a CLEM-based imaging method in which bioorthogonal tags are detected with fluorescence microscopy (FM), after which EM imaging is performed and images of both FM and EM are correlated. Both methods show that bioorthogonal labelling can be used to selectively label and localise the presence of bioorthogonal tags with EM in a non-homogenous sample of tagged and untagged *E. coli* bacteria.

The methods described in **chapter 3** are based on the ccHc-reaction. This chemical ligation strategy is well-known for its high reaction rate and selectivity and makes use of a very small bioorthogonal ligation handle, which minimally perturbs the structural integrity of the tagged biomolecule. Nevertheless, as stated in **chapter 2**, a variety of alternative labelling strategies exist, which might also be amenable to the bioorthogonal labelling of ultrathin cryosections. Investigation into these alternatives is desirable, since application of different bioorthogonal labelling strategies within one single specimen allows for the simultaneous bioorthogonal labelling of multiple different biomolecules.[1] One of the most promising alternative bioorthogonal labelling strategies that can serve this purpose is the reaction between a tetrazine and a strained alkene. In this type of reaction an electron-deficient diene, a tetrazine, is reacted with a strained alkene such as norbornene. This reaction does not interfere with thiol groups and has evolved in both selectivity and fast kinetics. Moreover, the tetrazine reaction has been well exploited and optimised for several *in situ* and *in vivo* applications.[2, 3]

In **chapter 4** an in-depth analysis of gold and fluorescence labelling for EM-imaging is presented. Upon comparison of both labelling strategies it is shown that, if epitopes of interest are found in various structures within the same

specimen, there exist inherent discrepancies between the fluorescence signals and the distribution of gold particles. However, since gold labelling has great advantages regarding its punctate readout, it is desirable to look into alternative methods that can reach the punctuality benefits of the gold particles. One of these alternative strategies; the implementation of super-resolution imaging in a CLEM imaging sequence[4], has been demonstrated in **chapter 6**, where it is shown that stochastic optical reconstruction microscopy (STORM) imaging can be succesfully perfomed on ultrathin cryosections without affecting EM-detectable cellular ultrastructures. In addition to this STORM-CLEM strategy, labels capable of polymerising DAB could also serve as a suitable alternative for gold particle labelling. Recently Ngo et al. have reported the use of a DAB polymerisation strategy for the EM detection of bioorthogonally-labelled biomolecules.[5, 6] Ngo et al. labelled bioorthogonal groups with fluorophores capable of photoconverting triplet oxygen to singlet oxygen. These singlet-oxygen species can precipitate DAB which results in the formation of insoluble DAB precipitates within four nanometers of the original fluorophore. Due to the close proximity at which DAB precipitation occurs, biomolecules -tagged with bioorthogonal functionality- are labelled with high resolution, making this strategy a suitable alternative to gold labelling (Figure 1).[6] However, since Ngo et al. use resin polymerisation for their study, this strategy is not compatible with on-section CLEM, precluding any type of additional on-section (immuno)-labelling and the ability to retrieve the exact same region of interest with both fluorescent and EM detection.

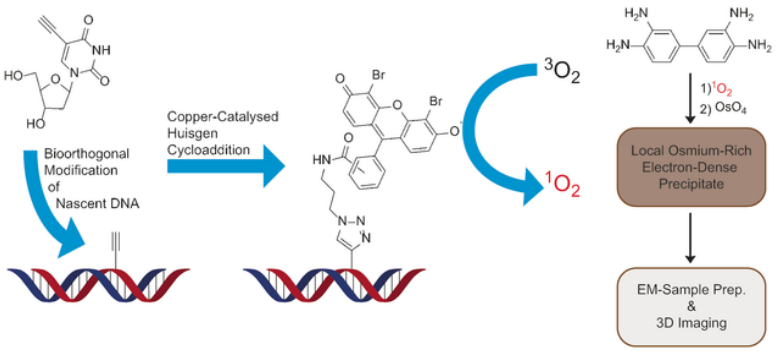

Figure 1: Detection of bioorthogonal groups in resin embedded EM samples: A) First, a bioorthogonal uridine analogue is metabolicially incorporated in nascent DNA. This handle was modified at the end of the experiment with dibromofluorescein, which can efficiently convert triplet oxygen into singlet oxygen. This singlet oxygen can then convert a 3,3'-diaminbenzidine (DAB) substrate into an insoluble polymer with high affinity for osmium. This osmium is electron dense and can thus be used to visualise the original bioorthogonal group.

In **chapter 5** the CLEM detection of bioorthogonal labels described in **chapter 3**, is applied to the imaging of bacterial degradation in the phagolysosomal system of phagocytic cells. The in situ study of bacteria in the phagocytic pathway is very difficult, as genetic modification is complicated for certain pathogens and -if successful- only allows tracking of pathogen phagocytosis until the point where the genetically-introduced reporter proteins are degraded by the proteolysis that is the hallmark of normal phagosomal maturation. In **chapter 5** it is shown that detection of bioorthogonal groups by CLEM allows one to obtain high resolution information on the subcellular location of the degrading bacteria, even after the signal of the genetically encoded protein reporters has gone.

In **chapter 6** the CLEM strategy reported in **chapter 5** is applied to the imaging of bioorthogonally-labelled *Salmonella Typhimurium* upon implementation of STORM super-resolution imaging. This strategy has great potential to elucidate the temporal and spatial injection of *Salmonella* virulence factors and their interactions with host cells organelles. To further characterise the effects of single virulence factors on host-organelle organisation and morphology it is desirable to additionally apply this strategy on host cells infected with *Salmonella* mutant strains, such as Δssej and ΔsopB that are known for their attenuated virulence.[7, 8] Of additional importance would be to unravel how *Salmonella* affects the architecture of host-cell organelles. Although the in **chapter 6** reported CLEM strategy can provide valuable information on the ultrastructural 2D architecture of the host-cell organelles, it does not provide comprehensive information on relative organelle organisation and their alterations upon *Salmonella* infection. This information can only be obtained upon reconstructing a volumetric 3D image of the obtained cellular ultrastructures. The classic approach for doing this, is by the reassembly and 3D reconstruction of TEM images from sequential sections.[9] This classic 3D reconstruction strategy is compatible with the here presented CLEM imaging of bioorthogonal labels; however, with this method only a limited number of sections can be reconstructed into a 3D volume. Other approaches, such as serial block-face (SBF) and focused-ion beam (FIB) milling and scanning electron microscopy (SEM) might therefore serve as good alternatives. With these approaches faster reconstruction can be performed of large volumes with a resolution that is close to that obtained with TEM.[9] However, the main disadvantage of these approaches is that they are poorly compatible with fluorescence labelling and imaging due to chemical fixation, resin embedding and block staining.[9] In case fluorescence imaging is desirable, it must therefore be

performed prior to SBF- and FIB-SEM imaging.[10] Staining upon DAB precipitation, as presented by Ngo et al., therefore serves as a good staining alternative for SBF- and FIB-SEM imaging, albeit that with this type of labelling only one single biomolecule (class) of interest can be labelled per specimen.[5, 6]

In **chapter 7**, CLEM imaging of active enzyme populations is explored. CLEM imaging of active cysteine protease populations are demonstrated using both two-step and direct activity based probe (ABP)-labelling approaches. It is shown that with both strategies active populations of cysteine proteases can be labelled in a similar fashion with high selectivity and efficiency. In addition it is shown that with direct ABPs multiple enzyme populations can be simultaneously CLEM imaged. Further improvement of the ultrastructural detection of active enzyme populations could be achieved upon use of ABPs that become fluorescent upon binding of the enzymatic product. These quenched ABPs (qABPs) are intrinsically quenched, but fluorescently label the target enzyme upon binding by a mechanism-based nucleophilic displacement of the quencher group. In this manner background levels of unbound ABP are lowered, resulting in an improved signal to noise ration. The development of such probes has been initially described by Blum et al., who developed qABPs to dynamically image cysteine protease activity in real time with decreased background levels.[11, 12] Since the development of these probes a variety of qABPs have been generated, including 2,3,5,6-tetrafluoro phenoxymethyl ketone (PMK)-modified probes, with greatly improved in vivo stability. An example of such a PMK probe is the fluorescently quenched pan-cathepsin probe BMV109, reported by Verdoes et al.[13] Verdoes and co-workers additionally showed that upon modification of the peptide scaffold of BMV109, non-peptidic analogs could be developed with a high selectivity for individual cathepsins, such as cathepsin S-specific probes.[14, 15]

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