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The Potential of Bioorthogonal Chemistry for Correlative Light and Electron Microscopy^{*}

Introduction

Correlative light and electron microscopy (CLEM) is an imaging technique that combines the virtues of light microscopy (LM) with those of electron microscopy (EM). With this technique specific molecular and cellular structures in a cell can be identified with LM, after which ultrastructural information about their subcellular location and context can be obtained. CLEM studies that involve fluorescence microscopy may benefit from fluorescent markers that can be attached to molecules of interest to allow their identification and localisation. To date, this has most readily been done by fluorescent fusion proteins, by fluorescent antibody labelling or by the chemical modification of a protein with a fluorescent detection group.¹⁻³ As well as these fluorescent detection moieties, structures must be present in the CLEM sample that are both EM- and LM- detectable in order to correlate (overlay) the LM image with the EM image. Examples of such

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EM/LM detectable structures are fluorescently-labelled cellular structures that are suitable to be identified by EM through their distinct morphology (e.g. stained nuclei), or fluorescently-labelled electron-dense particles (e.g. fluorescent microspheres).¹⁻⁴

The above-mentioned labelling approaches have been very successfully applied to CLEM imaging of specific proteins in their cellular context. However, they carry some limitations. First, the use of fluorescent fusion proteins requires genetic manipulation of the cell, which can be difficult and can affect the function of the protein of interest.⁵ An alternative to genetic manipulation is antibody labelling. However, for specimens prepared for CLEM, antibody labelling is an elaborate procedure, of which the success rate is notoriously low due to lack of functional antibodies.⁶ Finally, all these labelling approaches do not readily allow imaging of non-templated biomolecules, such as glycans and lipids.

Bioorthogonal chemistry is a powerful new labelling tool that circumvents the disadvantages mentioned above and allows for the imaging of a wide range of biomolecules. Its mechanism⁷ relies on the introduction of a small abiotic chemical group (one that is non-reactive with other chemical functionalities found in the cell) into a biomolecule of interest which can be specifically reacted with a detection moiety using a so-called 'bioorthogonal' chemical reaction: a reaction of the tag with a detectable group that is essentially background free in biological systems (Fig. 1A).⁸ As this labelling strategy makes use of a small chemical group to tag a biomolecule of interest it minimally interferes with the structure of the labelled biomolecule and as such minimally affects cellular biochemistry.⁹ Since the initial development of the Keto-oxime and Staudinger-Bertozzi-ligations, bioorthogonal labelling chemistry has evolved rapidly. Currently, a wide-ranging chemical toolkit is available of both tags for incorporation into biomolecules and reactions for subsequent labelling of these tags.¹⁰ The choice of tag and modification chemistry can therefore be optimised and tailored for the specific biological hypothesis.⁷

The introduction of a bioorthogonal tag into a biomolecule of interest occurs most readily by the metabolic incorporation of a tagged biomolecule building block. One of the approaches that exemplifies the metabolic incorporation strategy was reported by Saxon et al.¹¹ They synthesised a cell-permeable azide-tagged N-

acetylmannosamine analogue (Ac₄ManNAz) which was administered to mammalian cells during cell culture.¹² Inside these cells the acetyl groups were removed from Ac₄ManNAz, after which it was passed on to the steps of the sialic acid biosynthetic pathway where it was converted to N-azidoacetyl sialic acid (SiaNAz). After conversion to the nucleotide sugar CMP-SiaNAz, SiaNAz is incorporated into various glycoconjugates by sialyltransferase enzymes. With this approach Saxon et al. produced cells containing azide-tagged sialoglycans and visualised these using the Staudinger-Bertozzi reaction (Fig. 1B).

Since this first inception, the applications and classes of biomolecules that can be labelled with this approach have expanded rapidly. For instance, Salic & Mitchinson nicely demonstrated that bioorthogonal tags can be incorporated into newly synthesised DNA of both cultured cells and mouse tissues after metabolic incorporation of the tagged nucleic acid 5-ethynyl-2'-deoxyuridine.¹³ An additional example is the metabolic incorporation of bioorthogonal tags into lipids in order to study protein lipidation and lipid trafficking (thoroughly reviewed in ¹⁴). Likewise, metabolic incorporation of bioorthogonal chemical tags has been reported for proteins.¹⁵ Kiick et al. showed the incorporation of bioorthogonal tags in the proteome of *Escherichia coli* (*E. coli*) cells upon addition of the tagged amino acid azidohomoalanine. They showed that azidohomoalanine could be incorporated at sites where the amino acid methionine naturally resides. Hatzenpichler et al. showed that tagging of proteins using this abiotic amino acid is a successful approach to study newly synthesised proteins in individual microorganisms within environmental samples.⁹ In addition to the proteome-wide metabolic incorporation of bioorthogonal tags, single proteins can also be modified by using amber codon suppression (reviewed in ¹⁶). Although genetic modification is needed for this approach –with the same downsides as other genetic techniques– it is a great addition to the bioorthogonal toolkit. Attachment of abiotic tags to covalent enzyme inhibitors even allows to selective visualise active populations of enzymes in a complex mixture.¹⁷⁻¹⁹ Moreover, it can be applied to the tagging of biomolecules in living multicellular organisms, such as *C. elegans*²⁰, zebrafish (*Danio rerio*)^{21, 22} and mice (*Mus musculus*)²³.

There are now also numerous bioorthogonal reactions available for labelling these tags (thoroughly reviewed in ²⁴ and ¹⁰). Examples of the most often used labelling strategies are illustrated in Fig. 1C-F. The copper-catalysed Huisgen cycloaddition

(ccHc) is well-known for its high reaction rate and selectivity and is often used on fixed sample material, as copper is toxic to cells (Fig. 1C).²⁵ The strain-promoted cycloaddition reaction is a faster alternative of the Staudinger ligation and allows in vivo labelling as there is no need of copper catalysis during this reaction (Fig. 1D).²⁶ The inverse electron-demand Diels-Alder cycloaddition is a fast bioorthogonal reaction that does not require catalysis. An example of such a reaction is the cycloaddition of s-tetrazine and trans-cyclooctene derivatives (Fig. 1E).²⁷ It is also possible to use photo-activatable chemical groups for so-called 'photoclick'-reactions. Nitrile imine mediated [1,3]-dipolar cycloaddition reaction and has been employed to selectively functionalise an alkene genetically encoded in a protein inside *E. coli* cells.²⁸ The reaction procedure was reported to be simple, straightforward, and nontoxic to *E. coli* cells (Fig. 1F). This variety in labelling strategies and chemical reactions highlights the versatility of the approach as it can be altered upon experimental settings. It is even possible to 'multiplex' different bioorthogonal reactions;²⁹ i.e. to first label one class of biomolecules (in this case a proteasome subunit) using one bioorthogonal reaction, then perform an additional bioorthogonal reaction on a second class and a third reaction on a third class of bioorthogonal groups. This approach shows that the reactions are not just bioorthogonal, but also mutually orthogonal to one another.

Bioorthogonal chemistry is anticipated to become a powerful and useful addition to the CLEM labelling toolkit. It would allow the imaging of non-protein biomolecules and it precludes the need for genetic tagging and antibody labelling. Furthermore, the fate of biomolecules labelled by these approaches can even be monitored during the degradation process. For example, a protein labelled with bioorthogonal amino acids can be imaged, even when it is proteolytically degraded, as - unlike reporter proteins - the tags survive this catabolic pathway.³⁰ At the start of this project, bioorthogonal reactions had not been combined with CLEM-imaging. This is surprising, since fluorescent imaging of bioorthogonal tags has become commonplace over the last decade and a half.⁷ In this review, some of the inroads that have been made towards the CLEM-imaging of bioorthogonal reactions will be highlighted.

Bioorthogonal labelling for CLEM imaging

To allow EM imaging of bioorthogonal tags an electron-dense group is required that can be introduced using a bioorthogonal reaction. The most commonly used EM-detectable groups are gold nano-particles (GNPs).³¹ However, GNPs in

combination with bioorthogonal labelling have not been explored for this purpose. This is remarkable, since the inverse use of bioorthogonal chemistry – to synthesise protein/DNA-modified gold particles - has been reported.^{32, 33} In these studies, no change in GNP size was observed and hydrophobic, organometallic and hydrophilic moieties could be introduced onto the particles. Brennan et al.³⁴ used a similar biochemical approach to produce biomolecule-modified gold particles. They first produced azide-modified gold particles and 4-pentynoic acid modified lipase and reacted the two using a copper (II) catalyst with ascorbate reducing agent to generate the active Cu(I) species in situ. Under reducing conditions the thiol-gold linkage appeared stable and gold particles modified with lipase were observed. These examples indicate that GNPs have the potential to be used in combination with bioorthogonal chemistry as a labelling strategy for EM samples.

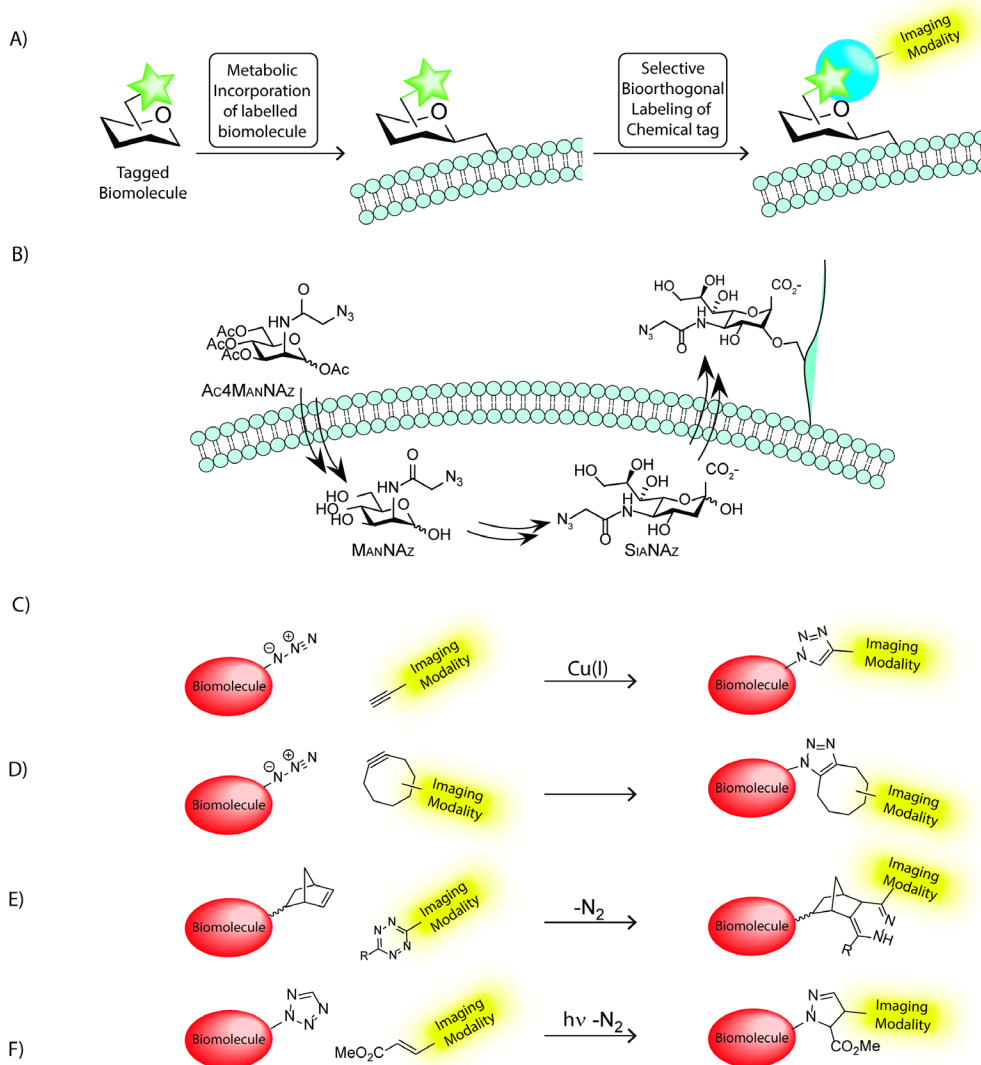


Figure 1: Bioorthogonal chemistry for imaging. A) General approach: a biologically inert group is incorporated into a biomolecule class in a living cell and selectively visualised using chemistry specific for this bioorthogonal group; B) An azide-labelled N-acetylmannosamine analogue is converted to CMP-sialic acid in vivo. Azido-sialic is then incorporated into the nascent glycoproteins. C-F) Recently applied bioorthogonal reactions for imaging; C) the copper-catalysed Huisgen cycloaddition²⁵, D) the strain-promoted cycloaddition²⁶, E) the inverse electron-demand Diels-Alder cycloaddition²⁷, F) or the photoclick reaction²⁸.

Instead of electron-dense EM-detectable particles, bioorthogonal fluorophore introduction could also be used for EM-imaging; namely in combination with

CLEM, especially considering that fluorescent bioorthogonal labelling strategies are very well established in the field. Additionally, to perform CLEM with this particular strategy, detectable moieties are required that are visible in both imaging modalities (e.g. fluorescent electron-dense nano- or micro-particles).

One approach to circumvent this would be to directly react the bioorthogonal tags with such electron-dense fluorophores. Quantum dots (Qdots) are suitable candidates for this purpose.³⁵ As with GNP modification, significant development has gone into the modification of Qdots with biomolecules using bioorthogonal strategies. Again, they have not yet been used for introducing these fluorophores for CLEM. In the case of CLEM labelling, one difficulty with the modification of Qdots using 'classic' copper-catalysed Huisgen reaction (Fig. 1C) is fluorescence quenching.³⁶ Fluorescence quenching was circumvented by using either the strain-promoted [3+2]-cycloaddition reaction (Fig. 1D)³⁶, or the tetrazine-norbornene inverse electron demand Diels-Alder reaction (Fig. 1E).³⁷ In the former reaction, cyclooctyne-modified cadmium selenide/zinc sulphide (CdSe/ZnS) core-shell Qdots were modified with cyclooctyne groups and as such used to image the presence of azide-containing sugars on the surface of cultured CHO cells, analogous to the work performed by the Bertozzi group. However, in these experiments no CLEM was performed. Zhang et al.³⁸ recently used a similar approach to image the intracellular presence of viruses. CdSe/ZnS-Qdots were modified with an azide-containing outer coating. These particles were then reacted with dibenzocyclooctyne-modified viruses that had been used to infect GFP-expressing A549 cells. This approach allowed the imaging of viral infection in these cells with good selectivity. Recently, the same group published the *in vivo* imaging of virus infection using a near-infrared Qdot variant³⁹, highlighting the power of this approach.

A second alternative approach by which the subcellular location of fluorophores can be made EM-visible is by photoconversion of diaminobenzidine (DAB).⁴⁰ This approach uses fluorophores to photooxidise DAB, which results in precipitates after reaction with osmium. These precipitates are electron-dense and therefore EM-detectable. Such fluorophores are readily available as detecting agents for bioorthogonal reactions and numerous examples exist of the use of these to label biomolecules. However, again no examples have been reported of the approach

where first the fluorophore is used to image a bioorthogonal label followed by photoconversion of DAB to allow for EM-imaging.

A final example of an approach that has great potential for CLEM imaging of enzyme activities is the use of an aggregating probe. Ye et al.⁴¹ reported the use of a probe that upon cleavage by the apoptosis-related caspases 3 or 7 cyclises and precipitates to form insoluble fluorescent nano-aggregates. Rather than imaging these aggregates by CLEM, the authors imaged them by super-resolution microscopy and conventional confocal microscopy. They also showed that these probes could be applied to the *in vivo* imaging of tumour apoptosis after treatment with doxorubicin.

Conclusion

The examples and strategies discussed here highlight the power of bioorthogonal chemistry for the labelling of biomolecules in a cellular context. Bioorthogonal chemistry has not been explored for CLEM imaging, although many inroads have been made. It is anticipated that bioorthogonal chemistry will enable CLEM imaging of molecules for which the current toolkit is not amenable, such as non-genetically templated biomolecules, temporal subpopulations of proteins (those expressed in a given time window), or the imaging of enzymatically active subpopulation of a protein class.

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