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Bioorthogonal tools to study fatty acid uptake in immune cells

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General Introduction

Introduction

All living cells have an inherent need for exogenous metabolites to cover their energetic needs, and to provide building block for biosynthesis.¹ The same is true for immune cells. What has been largely underappreciated over the last decades, is how the availability of metabolites to immune cells can directly impact their immunological function, activation, and differentiation.¹ This growing field, called immunometabolism, has been gaining popularity in recent years.^{2,3}

This thesis focuses on the study of the metabolic requirements of T cells during their activation. T cell activation is part of the *adaptive immune response*, which is mounted by antigens presented on the surface of antigen-presenting cells (APCs) that in turn are recognised by specific T cell receptors (TCRs) on the surface of T cells. The binding of the TCR to the presented antigens, in combination with relevant costimulatory signals, forms the basis of the activation and differentiation of T cells, from quiescent cells to highly specialised effectors/orchestrators of the anti-pathogen immune response.⁴

In response to the TCR binding, a plethora of signalling cascades inside the T cells are stimulated to mount the appropriate immune response.^{5,6} This includes a substantial metabolic reprogramming to, amongst others, support their increased growth and proliferation during the clonal expansion phase, and their conversion from small featureless cells to large effector cells.^{7,8} Quiescent, naïve T cells maintain low biosynthetic levels and their cellular energy demands are mostly maintained by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS, Figure 1A).⁹ Conversely, effector T cells have a large biosynthetic burden where the synthesis of nucleotides, amino acids, and fatty acids for DNA, proteins, and lipids, respectively, are needed.¹⁰ Therefore, these T cells will upregulate their aerobic glycolysis, which will dwarf the contribution of OXPHOS to their metabolism (Figure 1B). Aerobic glycolysis generates energy faster, albeit less efficient, than OXPHOS, and is an important source for essential biosynthetic building blocks.¹

The metabolic reprogramming of T cells is further supported by an increased uptake of exogenous nutrients such as glucose, amino acids, and fatty acids (FAs, Figure 1B). The local concentration of these nutrients in the cell's direct microenvironment is therefore a key factor in the activation of T cells. Low or high availability of these nutrients can directly modulate T cell activation and thus the immune response.^{11–15} The cellular uptake of exogenous glucose and amino acids in T cells is defined by well-known, distinct membrane transporters, such as GLUT1 for glucose, and the LAT-family of solute carriers for the amino acids.^{16–18} The expression of these transporters is therefore closely linked to the uptake of the nutrients, and thereby also to T cell activation.^{13,19} It has been demonstrated that the knockout of these nutrient transporters impairs T cell survival and effector differentiation, highlighting their importance for proper T cell function.^{13,19} However, transporter levels are not the only determining factor in how much nutrient a cell takes up. The competing transporter activity of its surrounding cells and other factors affecting local nutrient concentrations also determine the uptake (and hence activation) of T cells.²⁰

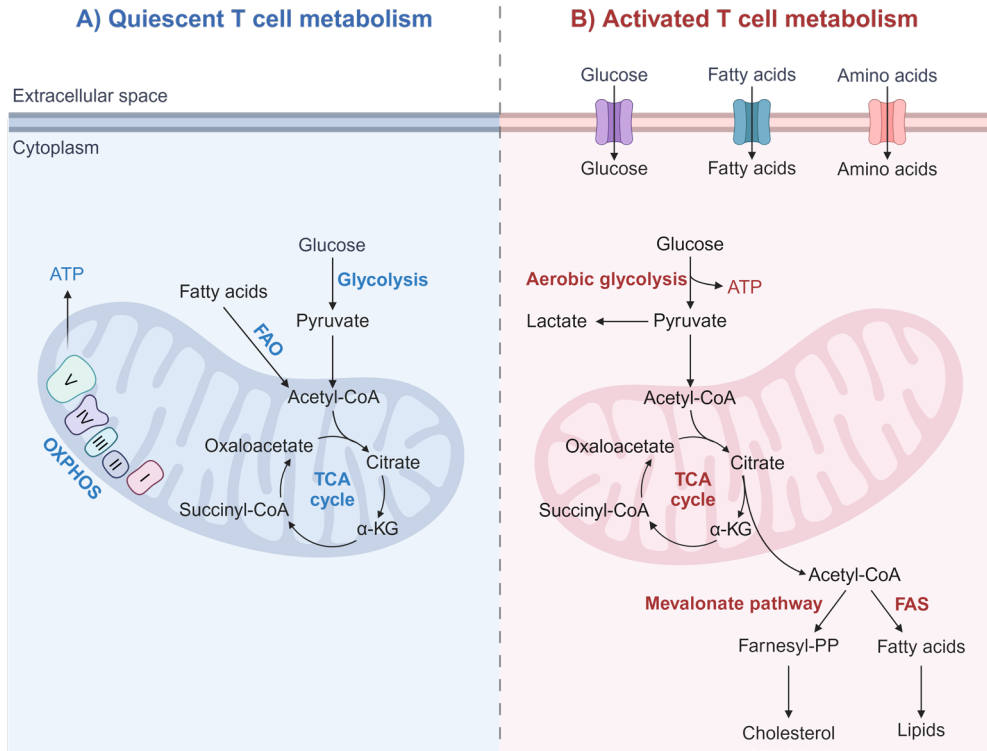


Figure 1: Metabolic pathways that support T cells. **A)** Quiescent T cells break down glucose, via glycolysis, or fatty acids, via fatty acid β -oxidation (FAO), to acetyl coenzyme A (CoA). Acetyl-CoA enters the tricarboxylic acid (TCA) cycle in the mitochondria, which generates reducing equivalents for oxidative phosphorylation (OXPHOS) through several enzymatic reactions. Complex V in the electron transport chain generates ATP, the molecular currency of energy in the cell, from OXPHOS. **B)** Activated T cells undergo metabolic reprogramming, where aerobic glycolysis becomes increasingly important for ATP production. The uptake of extracellular nutrients such as glucose, fatty acids, and amino acids are also increased. Additionally, products of the TCA cycle form important precursors for the synthesis of several complex biomolecules which are needed for activation and proliferation. For example, citrate is transported from the mitochondria to the cytoplasm where it is converted to acetyl-CoA. Acetyl-CoA is the basis of fatty acid synthesis (FAS), leading to the biosynthesis of cellular lipids. Simultaneously, acetyl-CoA supports the production of farnesyl pyrophosphate (farnesyl-PP) via the mevalonate pathway, a precursor for cholesterol biosynthesis. The figure is made with BioRender.

T cells also require FAs during their activation.¹⁵ However, the uptake, intracellular transport, and function of this class of nutrients is much less defined. While there have been indications that FAs can enter T cells via passive diffusion²¹, the contribution of transporters such as CD36, fatty-acid binding proteins (FABPs), or fatty acid transport proteins (FATPs) appears to be significant to control cellular FA uptake.²² In addition to FA uptake, T cells also cover their FA need by *de novo* fatty acid synthesis (FAS), complicating the matter further.²³

FAs are important for a number of physiological pathways in cells. They can be used as energy storage, as energy substrates that are degraded via lipolysis and fatty acid β -oxidation (FAO), or as structural membrane components in the form of phospholipids and cholesterol.²⁴ Additionally, they can play important roles in intracellular signal transduction²⁵, as messengers between cells²⁶, or as modulators of gene transcription.²⁷ However, changes in FA homeostasis and lipid storage can also lead to the accumulation of free FAs (FFAs) in cells, causing lipotoxicity.²⁸ The complexity and diversity of FA biology, as well as its importance for T cell function and activation, highlights the relevance of studying this class of biomolecules.

This Chapter will further focus on current knowledge of FA uptake and metabolism in different T cell subsets, emphasising the importance of the correct function and regulation of FA-related metabolic pathways for these essential immune cells. Extra attention will be given to the immunomodulatory FA oleic acid (OA), as this is one of the better-studied examples of a FA that is key during T cell activation. Lastly, the molecular tools to study exogenous FA uptake will be discussed.

Fatty acid and lipid metabolism in different T cell subsets

During metabolic reprogramming upon activation, effector T cells upregulate their FA-related anabolic pathways, including *de novo* FAS, accompanied by the aforementioned switch to aerobic glycolysis (Figure 1B). The increase in FAS supports the production of cellular lipids, such as ceramides and phospholipids, to sustain increased proliferation and growth.²⁹ The biosynthesis of other cellular lipids such as isoprenoids, cholesterol, and cholesterol derivatives is also upregulated in effector T cells.²⁹ The biosynthetic precursors of these lipids are products of the mevalonate pathway. It is therefore unsurprising that the knockout of the key enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) from this pathway ablates proper effector T cell functions.³⁰ At the same time, T cells take up exogenous lipids and cholesterol, either as FFAs¹⁵, or from low-density lipoprotein (LDL) particles via the LDL receptor (LDLR), which is upregulated in early activating T cells.³¹ The expression of LDLR is essential for the early activation of effector T cells, highlighting that the endogenous lipid and cholesterol biosynthesis is insufficient to cover the cell's metabolic needs during differentiation.³¹

During the early activation of CD4⁺ effector T cells, FA metabolism is governed by two distinct signalling pathways¹⁵ resulting from the activation of mammalian target of rapamycin complex 1 (mTORC1). This leads to the activation of the transcription factors peroxisome proliferator-activated receptor gamma (PPAR γ) and that of sterol regulatory element-binding protein 1 (SREBP1), which upregulate the expression of genes related to FA uptake and *de novo* FAS, respectively.¹⁵ If either of these two signalling pathways are inhibited, the T cells suffer from reduced activation and proliferation, indicating that both FA uptake and FAS are essential for proper activation of effector T cells.¹⁵ SREBPs have also been implicated as essential for the metabolic reprogramming and activation of CD8⁺ T cells, by regulating the lipid biosynthesis program, further supporting the essential role of these metabolic pathways for effector T cell differentiation.³²

The picture is further complicated by the differential reliance on FA uptake and FAS

by the different T cell subsets. After activation, CD4⁺ effector T cells can turn into different sub-types, each with their own function in the immune system.³³ To support their immunological functions, T helper 1 (Th1), Th2, and Th17 cells show unique changes in FA metabolism. Th17 cells are heavily dependent on *de novo* FAS, and inhibition of the rate-limiting enzyme, acetyl-coenzyme A carboxylase 1 (ACC1) leads to a decline in the differentiation of this T cell subset.^{34–36} *De novo* FAS is also important for Th1 and Th2 differentiation, but to a lesser extent than for Th17 cells.^{35,37} Interestingly, the expression of PPAR γ has been shown to be important for the function and proliferation of Th2 cells.^{37,38} This could indicate that the uptake of exogenous FAs plays a more important role for Th2 cells, contrary to Th17 cells where *de novo* FAS plays a bigger role. However, the precise roles of FA uptake and FAS to the biology of T cell subsets remains to be fully elucidated. Regulatory T cells (Tregs), which can suppress immune responses in an antigen-specific manner³⁹, show a particularly intriguing lipid uptake and FAS phenotype.²⁹ Tregs show low levels of glycolysis.^{40,41} Yet, they are not metabolically quiescent.^{8,9} They show an increased FA uptake, and use FAO to support their immunosuppressive functions (Figure 1), distinguishing them from other T cell subsets.^{8,9,42} The role of this difference compared to effector T cells is, as of yet, unknown, nor is it known whether this could be exploited therapeutically, for example in treatments aimed to reactivate the immune system to fight cancer.^{43,44}

After the end of the effector phase, T cells can form long-lasting memory cells that can reactivate quickly in response to encounters with the same antigen.⁴⁵ These memory T cells (both central and peripheral) are different again in their metabolic behaviour. They are metabolically quiescent, but instead of breaking down glucose to fuel OXPHOS, memory T cells rely heavily on catabolic FAO (Figure 1A).^{1,7,9,46} This utilisation of FAO is believed to support memory T cell persistence and longevity by providing a stable ATP source⁹, and increasing mitochondrial spare respiratory capacity.⁴⁷ Additionally, it is believed that FAO yields less oxidative damage to the cells, minimising the damage the cells accumulate over their (long) lifetimes.⁴⁸

An example – the myriad of roles of oleic acid in T cell function

Oleic acid (OA) is a monounsaturated non-essential FA (C18:1, ω -9, Figure 2).⁴⁹ It comprises 13% of the circulating FFAs in the blood stream⁵⁰, with its serum concentration being between 30 μ M and 3.2 mM in healthy adults.⁵¹ Its main dietary source is olive oil (of which OA constitutes ~70%)⁵², and like most other dietary FAs, OA can be stored in lipid droplets, metabolised by FAO, or used in the biosynthesis of other lipids.⁵³

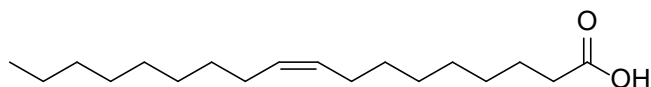


Figure 2: Structure of the monounsaturated fatty acid oleic acid (C18:1, ω -9).

In addition to the purely metabolic properties, OA has been described to have direct immunomodulatory properties, and it is often described as anti-inflammatory.^{54–56} OA treatment has been shown to influence immunological functions in diseases like

asthma⁵⁷, sepsis⁵⁸, and cancer.^{59–64}

In an ovalbumin-induced BALB/c asthma mouse model, for example, daily oral administration of 250 mg/kg OA has been shown to reduce symptoms *in vivo* by reducing cytokine secretion, reducing the amount of inflammatory cells present in the lung, and alleviating the overall pathological changes in the lung (such as epithelial cell proliferation, inflammatory cell infiltration, and mucus hypersecretion).⁵⁷

In an experimental sepsis mouse model, the daily oral administration of 0.28 mg OA was also shown to mitigate inflammation by increasing production of the anti-inflammatory cytokine IL-10, as well as decreasing production of the pro-inflammatory cytokines IL-1 β and TNF α .⁵⁸ OA also decreased systemic corticosterone levels, as well as decreasing neutrophil migration and accumulation at the site of infection. These factors were likely contributing to the increased bacterial clearance observed upon treatment with OA, and could indicate a beneficial effect of OA on sepsis outcome.⁵⁸

Rather surprisingly, OA has also been implicated in a beneficial reduction in cancer progression and growth in cancer types such as pancreatic cancer⁵⁹, esophageal cancer⁶⁰, lung carcinomas^{61,62}, tongue squamous cell carcinomas⁶³, and human hepatocellular carcinoma cells⁶⁴. The molecular mechanism is not known, but changes in autophagy and apoptosis have been suggested as possible mechanisms for the anti-tumour effect of OA.^{62–64}

Since OA appears to impact the immune system in a wide variety of ways, and shows beneficial effects in its combat of multiple diseases, a more in-depth understanding of the cellular and molecular basis of these immunomodulatory effects are needed. Since T cells are important contributors of the immune system, and represent cells of the adaptive immune system, the uptake and use of OA by T cells is described below.

Oleic acid affects T cell proliferation, metabolism, and differentiation

OA has been shown to increase the rate of T cell proliferation both *in vitro* and *in vivo*.^{65–69} However, at high concentrations, OA decreased both T cell proliferation and viability via apoptosis^{65,70}, indicating a dose-dependent effect of the FA. A proposed mechanism for the increased proliferation of OA-treated T cells is the incorporation of OA into membrane lipids, such as phosphatidylcholine, which appears to facilitate an increased calcium flux through the membrane.⁶⁷ There were no indications of exogenous OA altering metabolic pathways such as glycolysis and OXPHOS, suggesting OA is not used as a catabolic energy source. No changes in T cell receptor (TCR) signalling were detected either.⁶⁷ However, the exposure of T cells to OA did significantly increase the expression of several genes related to FAO, as well as genes related to cholesterol biosynthesis and *de novo* FAS.⁷¹ This could indicate that OA serves as a substrate to generate acetyl-CoA, which can in turn be fed into biosynthetic pathways generating cholesterol, and long-chain FAs.⁷¹

There is further evidence that the metabolic reprogramming of OA-treated T cells appear to be the driving force of the differentiation into specific T cell subsets after exposure to OA.⁷¹ This is supported by the upregulated production of several key

cytokines upon treatment of cells with OA. IL-9, the signature cytokine of a Th9 subset, was upregulated in T cells upon treatment with 30 µg/mL of OA *in vitro*, as was IL-17A, the cytokine produced by Th17 cells. Treatment of cells *in vitro* with exogenous OA also increased production of IL-5, IL-13, and the transcription factor GATA3, which are all associated with the Th2 subset.⁷¹ OA-treated T cells were also shown to significantly increase their production of IL-2, IFN-γ, and TNF-α, as well as IL-4 and IL-10.^{66,67} This could indicate an enrichment of the Th1 and Th2 subsets.⁷² These results highlight a connection between OA uptake, cellular metabolism, and the differentiation of T cells into pro-inflammatory subsets. However, the broad subset of Th-types observed to be upregulated upon OA treatment suggest that it is a general factor in differentiation.

Regarding the immunosuppressive Treg subset, there is conflicting evidence for the role of OA in their activation. Both the decrease in Treg population upon OA-treatment^{66,71}, and an increase in Treg differentiation⁷³, have been reported. It was also shown that OA-treatment resulted in the enhanced suppressive function of Tregs.⁷⁴ Since Tregs generally rely on FAO-driven OXPHOS to generate energy for their suppressive functions^{8,40,75}, it could be the case that OA uptake amplified these metabolic pathways, which in turn increased FOXP3 expression and phosphorylation of STAT5.⁷⁴ This in turn enhanced the suppressive function of the OA-treated Tregs.

Although different papers describe different specific outcomes of the differentiation of T cells exposed to OA, it is clear that this FA has the potential of modulating T cell differentiation in either a pro- or anti-inflammatory manner. However, determining the exact outcome is influenced by the experimental setup, and still requires further research.

Molecular tools to study the uptake of exogenous fatty acids

Although there are substantial indications that OA, and other FAs, have strong immunomodulatory effects, it is still difficult to study the effect of FA uptake on cellular phenotype at a single-cell level.

To measure FA uptake, a FA molecule needs to be modified in a way that allows its detection and quantification. The most traditional way to achieve this would be to use ¹⁴C or ³H radioactively labelled FAs (Figure 3).^{76–79} The major advantage of this method is that the chemical modification of the FA is negligible, as the atomic composition of the isotope-labelled lipid is identical to that of the unlabelled lipid.⁷⁹ However, this method not only requires specialised laboratory setups, personnel training, and protective equipment, there are also no methods by which the uptake data from these experiments can be correlated to the biology of a single cell.⁷⁹

In order to obtain single cell data on FA uptake, several fluorescently labelled FAs were developed, where the FAs were covalently modified with a fluorophore such as BODIPY prior to FA uptake (Figure 3).^{80–85} This allowed for the major breakthrough of single cell FA studies, as it allowed the study of FA uptake by fluorescent microscopy^{80–84} and flow cytometry.^{81,82,84} Unfortunately, the modifications used in these experiments significantly alter FA structure (Figure 3), which in turn impacts their biochemical properties, uptake, and cellular distribution patterns.⁸⁶ There have

e.g. been observations of fluorescently labelled universal FA probes accumulating in specific organelles or cellular compartments.^{87,88}

Several other methods have been developed for measuring the uptake of FAs *in vivo* and *in vitro*. A bioluminescent FA probe was developed, where a long-chain FA was bound to luciferin via a cleavable linker.⁸⁹ Upon cellular uptake, the cleavable linker could be hydrolysed by intracellular glutathione which in turn released luciferin. By addition of the enzyme luciferase, luciferin emitted detectable light that was proportional to the FA uptake.⁸⁹ Another FA probe has been developed where FA uptake in T cells can be quantified using a FA-quantum dot conjugate (FA-Qdot).⁹⁰ Although both these FA probes have been demonstrated to be applicable *in vivo*, their large sizes could still lead to mismatched uptake and distribution patterns compared to the native FA molecules.⁸⁶

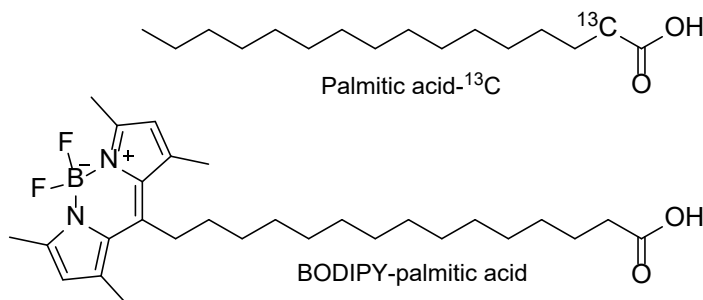


Figure 3: Structures of radioactively (palmitic acid-¹³C) and fluorescently (BODIPY-palmitic acid) labelled fatty acid analogues, using palmitic acid (C16:0) as an example.

Applying bioorthogonal (click) chemistry to explore fatty acid uptake

The aim of this thesis is to combine the favourable properties of radiolabelling (small labels) with the single-cell compatibility of fluorophore-labelled FAs using bioorthogonal chemistry. The term bioorthogonal chemistry, sometimes also referred to as click chemistry, was initially coined for the work started by Bertozzi in 2000 for labelling of cell surface glycans.⁹¹ The approach consists of the introduction of a small, biologically inert group into a biomolecule of interest. Then, after any biological phenomenon under investigation is complete, this group is reacted using chemistry that is selective for the introduced group and unreactive with all other chemistry found in the biological system.^{92–94} The biomolecule of interest can thereby be covalently labelled with a reporter molecule (e.g. a fluorophore, Figure 4). This approach has been highly successful for studying a wide variety of biomolecules (such as proteins, DNA, RNA, carbohydrates, and lipids).⁹³ Many excellent review articles have been published describing different bioorthogonal click reactions and their biological applications.^{92–98} The approach has also been extensively extended to the study of FA biology.^{86,87,99–101} In the remainder of this Chapter, the bioorthogonal investigations of FA biology, which are relevant for this thesis, will be discussed.



Figure 4: The principles of bioorthogonal (click) chemistry. A small chemical modification is introduced into the biomolecule of interest (diamond shape), allowing for a selective click reaction with a reporter molecule (e.g. a fluorophore, F). The figure is made with BioRender.

Bioorthogonal FA probes can be fitted with a bioorthogonal group, either through labelling of the fatty acid head group, or of the acyl tail. However, the former approach¹⁰², will remove the key acid moiety that lends the molecules their name. Here the focus will therefore lie on those approaches that label the FA acyl tail. Two bioorthogonal groups have been mainly used for tail-labelling: azides^{103–108}, and alkynes^{99,101,109–111} at the ω -position (Figure 5A). Since this renders the carboxylic acid moiety in its native state, these bioorthogonal FAs can be incorporated into endogenous biological processes such as phospholipid biosynthesis and protein lipidation, and the click reaction can be used to detect or visualise them.^{86,100,112}

The azide and alkyne modifications allow for detection via the copper(I)-catalysed alkyne-azide cycloaddition (CuAAC), where the reporter molecule contains the opposite modification, e.g. FA alkyne modification with an azide reporter molecule, or vice versa (Figure 5B).¹⁰⁰ CuAAC is widely used due to its relatively fast reaction kinetics, low unspecific reactivity, and the synthetic availability of alkyne and azide modifications to be incorporated into biomolecules.^{93–95} However, the major disadvantage of this reaction is the employment of cytotoxic copper(I) ions, which means CuAAC is not live cell nor *in vivo* compatible.^{93–95} Of the two groups, the alkyne is the better bioorthogonal label in FAs, as its lipophilicity is similar to that of the native, terminal ethyl group. The azide is more polar, thereby reducing the amphiphilic nature of the bioorthogonal FA analogue compared to the parent molecule. Interestingly, the alkyne/azide FAs have not yet been used to study FA uptake by activating immune cells.

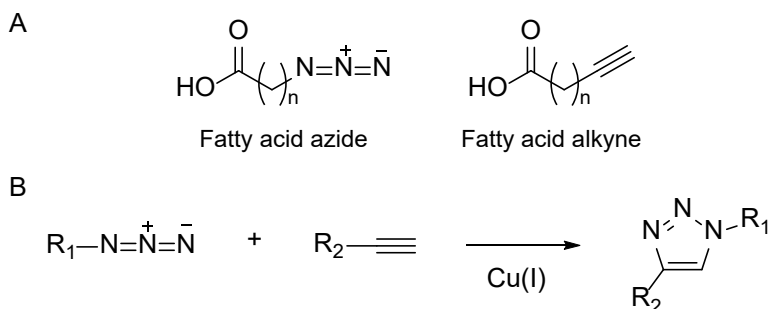


Figure 5: Structures of **A)** general bioorthogonal fatty acid analogues with terminal azide or alkyne modifications, and **B)** the copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) reaction.

One downside of the CuAAC is that the copper catalyst used is toxic to cells, requiring the fixation of the cells prior to the click reaction.¹¹³ The copper catalyst is also incompatible with many fluorophores used in routine flow cytometry panels.¹¹⁴ These problems were negated by the development of the inverse electron-demand Diels-Alder (IEDDA) reaction – the reaction between an electron-poor diene, such as a tetrazine, and a strained or electron-rich dienophile – as a novel click reaction (Figure 6).^{115,116} This reaction has opened up a world of live-cell compatible chemistry with exceptionally fast reaction kinetics.⁹⁶ Since tetrazines absorb light at 500-530 nm, they have an added benefit of quenching fluorophores with absorbance wavelengths in a similar range.¹¹⁷ This presents an advantage for live-cell applications, where the resulting tetrazine-conjugated fluorophores only become fluorescent upon successful IEDDA ligation (Figure 6). This turn-on effect reduces background fluorescence and protects live cells from being destroyed by several washing steps.⁹⁶

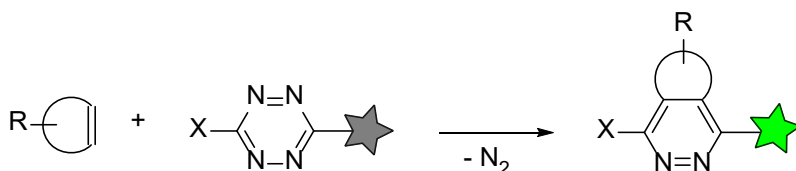


Figure 6: The inverse electron-demand Diels-Alder (IEDDA) reaction between an electron-rich dienophile and an electron-poor diene (here represented by a tetrazine-conjugated fluorophore).

While the IEDDA reaction has been used to study lipid biology before^{118,119}, its application to FA uptake has not been previously explored. This thesis describes the development of sterculic acid (StA) (Figure 7A) as a bioorthogonal FA analogue of OA. Contrary to the already established bioorthogonal OA analogues oleic acid alkyne (OAalk, Figure 7B) and oleic acid azide (OAaz, Figure 7C), StA was shown to react with tetrazines via an IEDDA reaction, therefore highlighting it as a live-cell compatible alternative to the azide- and alkyne-modified OA analogues (Chapter 2).

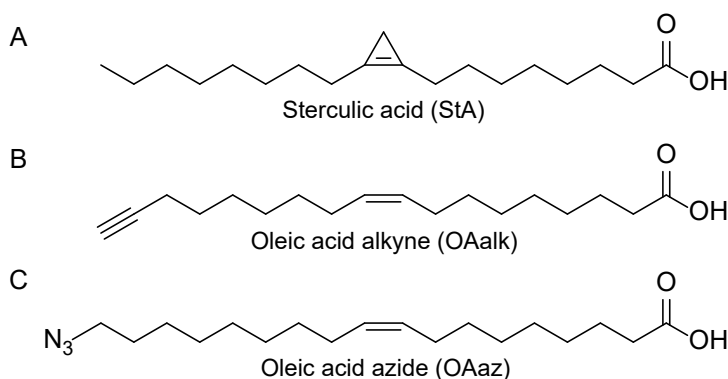


Figure 7: Structures of bioorthogonal oleic acid analogues **A)** sterculic acid (StA), **B)** oleic acid alkyne (OAalk), and **C)** oleic acid azide (OAaz).

Furthermore, StA was demonstrated as a versatile bioorthogonal OA analogue which could be incorporated into a plethora of methods to detect *in vitro* uptake and metabolism of OA in dendritic and T cells. This includes methods such as live-cell confocal imaging (Chapter 2), identification of post-translationally oleoylated proteins by mass spectrometry (Chapter 3), as well as the connection between phenotypic, transcriptomic, and proteomic differences, and a differential OA uptake at a single-cell level (Chapter 4).

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