Fluorophore-drug conjugates to unravel the mechanisms of action of therapeutic assets

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Fluorescent probes have become pivotal tools in biochemical research, enabling longitudinal tracking of biomolecules and cells under physiological conditions. Fluorescence-based technologies have progressed to create exciting avenues in molecular imaging, where multiple biological events can be simultaneously visualized *in situ* in living organisms. Fluorophores can be broadly categorized into labeling or activatable probes, depending on their spectral properties. Initially, fluorophores were mainly employed as bright labels to monitor the localization of specific proteins or cells. More recently, fluorescent probe development has focused on the design of activatable fluorophores that 'turn-on' in well-defined environments, enhancing signal-to-noise ratios and boosting sensitivity.[1] In addition to being powerful analytical tools to detect metabolites and/or discriminate subpopulations of cells, activatable fluorophores have started to be employed in mechanistic studies for drug discovery. These range from assays at the molecular level to map drug-binding sites in target proteins[2] to profiling assays in therapeutically-relevant subsets of cells.[3]

Among many immune cells found in our body, macrophages display a broad range of functions and a remarkable capacity to adapt to their environment. Macrophages play critical roles in tissue repair and remodelling, including the tumour microenvironment and the orchestration of the host immune response to infection. The activity of macrophages is regarded as a biomarker for diagnostic and therapeutic exploitation in many pathologies, and several fluorescent probes have been designed as macrophage reporters.[4] Such probes, however, provide generic readouts of macrophage activity and cannot modulate the function of subpopulations of cells. The diversity of phenotypes of macrophages represents a major challenge for chemists since macrophage-targeting probes must discriminate between closely-related subpopulations that coexist in the same tissue. This becomes particularly important when subpopulations of macrophages have divergent *in vivo* functions (e.g., proinflammatory vs anti-inflammatory macrophages in tissue regeneration). Such studies had not been possible to date with non-encoded chemical probes because of the challenge of producing molecules that target only defined macrophage subsets *in vivo*.

The recent prodrug-fluorophore conjugates combining small molecule drugs and activatable fluorophores represent a remarkable addition to our chemical toolbox for studying the

mechanisms of action of drugs *in vivo*.[5] These prodrug-fluorophore conjugates exploit the hydrolytic activity in the phagosomes of macrophages as the bioresponsive trigger to distinguish between proinflammatory and anti-inflammatory macrophages. Macrophages enclose a relatively high number of intracellular phagosomes, with a broad range of pH values (i.e., from 6.5 to 4.5) depending on their maturation state and phagocytic activity. The combination of activatable profluorophores and prodrugs responding to the phagosomal pH rendered conjugates that released bright fluorophores and cytotoxic drugs only in proinflammatory M1 macrophages. This approach has advantages over conventional strategies: 1) from the therapeutic point of view, improved efficacy because prodrugs are only active in targeted subpopulations with fewer side effects; 2) for mechanistic studies, the simultaneous release of fluorophores and drugs enables to image the action of the drugs in real time.

The first example of these conjugates combined a pH-activatable BODIPY profluorophore and the drug doxorubicin (i.e., a DNA-intercalating cytotoxic agent) (Figure 1A). Their coupling through a pH-sensitive *N*-acylhydrazone group afforded poorly fluorescent and inactive conjugates. However, when these molecules localized in the acidic phagosomes of M1 macrophages, they released cytotoxic doxorubicin and a green fluorophore detectable by flow cytometry and confocal microscopy. The selectivity of the conjugates for M1 macrophages vs other macrophages was validated in cell mixtures. More importantly, the conjugates were used to image and modulate macrophage function *in vivo,* targeting proinflammatory macrophages in a zebrafish model of tissue repair, whereby the tail fin was amputated and its regeneration was monitored over time. The targeted release of the 'fluorophore + drug' cocktail highlighted the proregenerative role of specific macrophages *in vivo* (Figure 1B). Another remarkable feature of these molecules is their potential for translational studies in human cells. This is critical in macrophages since notable differences have been reported between murine and human species. *Ex vivo* assays in monocyte-derived macrophages from human peripheral blood confirmed the application of these conjugates for future translational studies.

This new chemical strategy opens multiple opportunities for biochemical studies in cells that can differentiate into various lineages or display variable phenotypes depending on their microenvironment (e.g., pluripotent cells). The expansion of this platform to other drugs and fluorophores will help to design targeted chemical immunomodulatory agents for specific subpopulations of cells, providing new insights on the mechanisms behind cell reprogramming and generating better therapies for immune-related disorders.

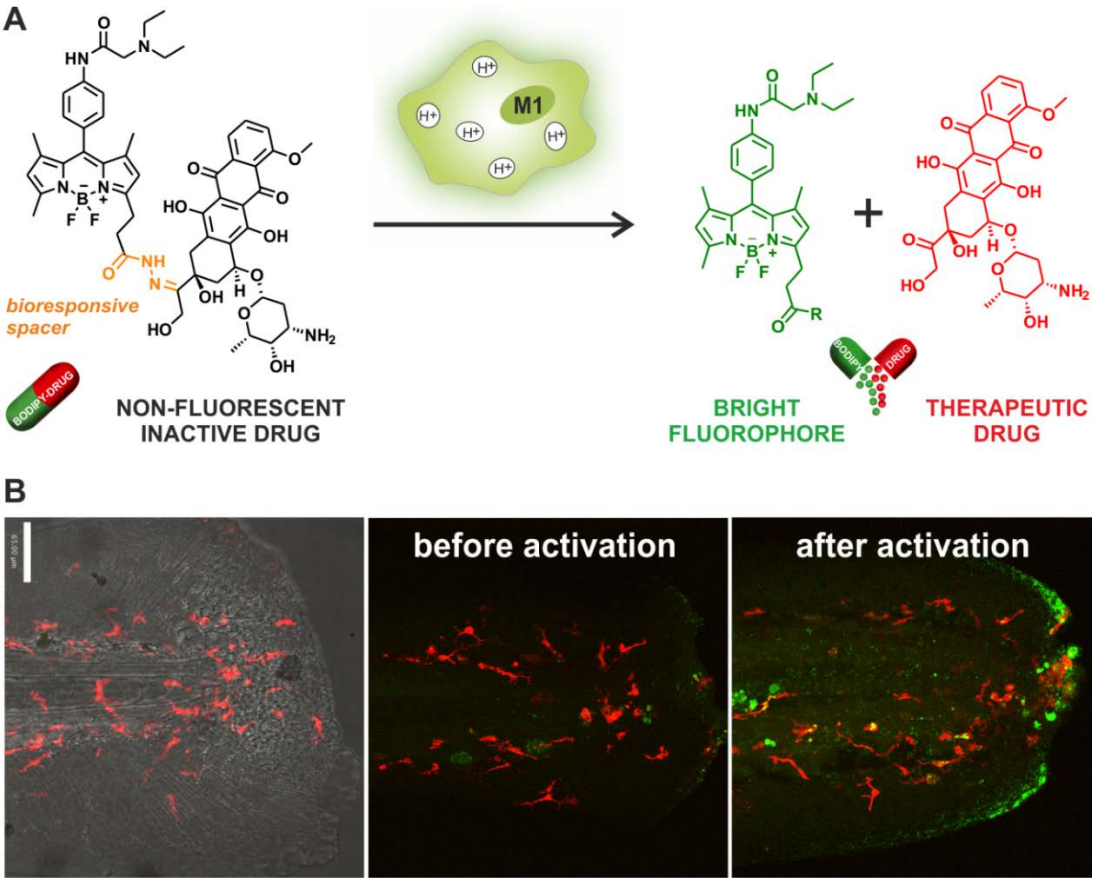

Figure 1. A) Chemical structures of prodrug-fluorophore conjugates and the outcome of their activation in M1 macrophages. B) Bright field (*left*) and fluorescence (*middle and right*) confocal microscope images of live transgenic zebrafish. All macrophages displayed red fluorescence (mCherry label), however green fluorescence was only detected in regenerating tissues where macrophages had been activated with lipopolysaccharide to induce the release of doxorubicin and the BODIPY fluorophore (*right*). Scale bar: 65 μm.

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