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Context-dependent intravital imaging of therapeutic response using intramolecular FRET biosensors

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ABSTRACT

Intravital microscopy represents a more physiologically relevant method for assessing therapeutic response. However, the movement into an *in vivo* setting brings with it several additional considerations, the primary being the context in which drug activity is assessed. Microenvironmental factors, such as hypoxia, pH, fibrosis, immune infiltration and stromal interactions have all been shown to have pronounced effects on drug activity in a more complex setting, which is often lost in simpler two- or three-dimensional assays. Here we present a practical guide for the application of intravital microscopy, looking at the available fluorescent reporters and their respective expression systems and analysis considerations. Moving *in vivo*, we also discuss the microscopy set up and methods available for overlaying microenvironmental context to the experimental readouts. This enables a smooth transition into applying higher fidelity intravital imaging to improve the drug discovery process.

Contents

Abbreviations: GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; IVM, intravital microscopy; 2D, two-dimensional; 3D, three-dimensional; FP, fluorescent protein; ECM, extracellular matrix; SHG, second harmonic generation; THG, third harmonic generation; PDAC, pancreatic ductal adenocarcinoma; FRET, Förster resonance energy transfer; FLIM, fluorescence lifetime imaging microscopy; IHC, immunohistochemistry; FRAP, fluorescence recovery after photobleaching; AAV, adeno-associated virus; AF, autofluorescence; BiFC, bimolecular fluorescence complementation; cps, counts per second; IRF, instrument response function; RMCE, recombinase-mediated cassette exchange; PLIM, phosphorescence lifetime imaging microscopy; CARS, coherent anti-stokes Raman scattering; OPO, optical parametric oscillator; Ti:Sapphire, titanium sapphire; MP, multiphoton; GLCM, gray level co-occurrence matrix; TAM, tumor-associated macrophage; LUT, look-up table; TVB, time-varying background.

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1. Introduction

The ability to monitor cellular processes in real time has proved revolutionary. Since the discovery of green fluorescent protein (GFP), innumerable variants have been applied to track macroscopic growth, subcellular organelles and even single-molecules. The advent of intravital microscopy (IVM) increases the biological fidelity of such imaging, without the need to infer from a two- or three-dimensional (2D/3D) scenario. Here we will discuss the various considerations required to move research into this more physiologically relevant setting.

One of the most common uses of fluorescent proteins (FPs) is for tagging a protein of interest, where spatial information is then informed by fluorescence microscopy. However, this spatial information alone is often insufficient for drug validation studies, where details about protein-protein interactions and activity are necessary to demonstrate drug response [1,2]. An increasingly common technique for high-throughput protein-protein interaction studies is bimolecular fluorescence complementation (BiFC), whereby two non-fluorescent halves of a FP re-associate upon interaction, forming a complete fluorescent molecule [3–5]. However, BiFC is less common *in vivo* [6], with more studies favoring dynamic fluorescence techniques, such as Förster resonance energy transfer (FRET), where a transient interaction between two FPs is measured, or fluorescence recovery after photobleaching (FRAP), where a powerful laser pulse is applied and recovery of fluorescence into the bleached region is monitored.

Live monitoring by these techniques using IVM can allow a more faithful readout of drug response, compared to standard 2D or 3D techniques. However, this increase in authenticity also requires some additional considerations. There is a well-established link, for instance, with tumor hypoxia and increased chemoresistance, radioresistance and metastasis [7–9]. Similarly, the burden of a dense, fibrotic extracellular matrix (ECM) is known to hamper response to therapeutics [10–13], while stromal and immune interactions with the tumor can similarly favor growth [14–19]. This microenvironmental context for drug validation studies is a necessary addition, as it is a lack of such context that is contributing to the increasing attrition rates of lead compounds in the pharmaceutical industry [20–23]. Here we will demonstrate IVM techniques that facilitate both live monitoring of therapeutic response and provide microenvironmental context for this data, ultimately improving the likelihood of success in later clinical trials.

2. Using fluorescent reporters to monitor therapeutic response

The first consideration for assessing therapeutic response by IVM is the live readout. Many fluorescent reporters exist for this purpose.

2.1. Fluorescent and bioluminescent reporters

Fluorescent and bioluminescent measurements of tumor volume have become standard in the field, with bioluminescent proteins, such as luciferase, providing high sensitivity for even small cell populations [24]. These proteins have tremendous versatility for creating reporters of complex cellular processes, such as measuring kinase activity through intracellular shuttling [25], microRNA activity through degradation of a luciferase construct [26] or ER stress by changes in protein folding [27]. When sufficient cellular or subcellular resolution is obtained, these reporters are readily translated to an intravital setting [28]. For example, utilizing the inherent photoconversion properties of specific FPs, real time tracking of cell populations by IVM is possible [29,30]. This population tracking has extended to lineage tracing [31–33] and cell cycle dynamics [34,35], where we can begin to tease out the role of single cells in the formation of resistant populations [36,37]. Similarly, we recently demonstrated the power of such imaging by tracking the onset of metastasis with a GFP-tagged E-cadherin reporter using FRAP by IVM [38,39]. By observing the increased rate of recovery of the fluorescent E-cadherin, we could demonstrate that the onset of metastasis involves 'unzipping' of the more rigid cell-cell junctions, exemplified by a lower E-cadherin mobile fraction in these stable junctions. This is only a small taste of the readouts achievable through the application of IVM, where more extensive reviews are available [2,40,41].

2.2. Intramolecular FRET biosensors

When it comes to assessing drug response, many fluorescent reporters provide accurate spatial information, but fail to inform on protein activity; essential for live monitoring of small molecule