Intravital FRET: comprehending life at single-molecule level. Focus on “A practical method for monitoring FRET-based biosensors in living animals using two-photon microscopy”

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In contrast, the publication of Day and colleagues places particular emphasis on the photophysical characterization of the most promising cyan/yellow fluorescent proteins as FRET-pairs in intravital (two-photon excitation) experiments. Special attention is dedicated to the largely unknown two-photon excitation spectra of these, enabling to determine mCerulean3 or mTurquoise and mVenus, respectively, based on their brightness, photostability, and minimal overlap in their two-photon excitation spectra to be most suitable for intravital FRET quantification. Additionally, a thorough set of calibration experiments is performed, including the linkage of the donor molecule of choice (mTurquoise) with a nonfluorescent acceptor (mAmber) as well as linking the FRET pair mTurquoise-mVenus by linkers of different length, to fully characterize the FRET efficiency behavior. The meticulous calibration is an excellent and generally valid tool for biomedical researchers intending to perform FRET quantification in vivo.

In intravital imaging, the depth of penetration of the excitation light is the major factor limiting the imaging volume. Increasing the brightness of the fluorophores, by using more recently developed FPs like mCerulean3 instead of ECFP, is one way to improve this; the other way is to use longer-wavelength light, which is scattered less in tissue. This has already led to the development of fluorescent proteins emitting further in the red part of the spectrum as also demonstrated by Day and colleagues elsewhere (5). The development of new excitation sources for TPLSM—such as optical parametric oscillators—which moved the excitation to the infrared (above 1,100 nm) (4) has accompanied this trend in fluorescent protein design. Additionally, these proteins show negligible background from tissue autofluorescence as compared to their cyan/yellow counterparts. This trend towards red and infrared fluorescent proteins is expected to extend to the design of FRET constructs in the next years.

Next to the choice of adequate fluorescent proteins pairs for FRET, the development of appropriate linkers, or more general, appropriate constructs is of central relevance. A large variety of highly elaborate FRET constructs have been developed in the past decades (1, 8, 10). The key properties necessary for effective FRET quantification in vivo are high FRET efficiency to yield a large dynamic range, specificity, low cross talk with other cellular processes and fast response time, since intravital experiments aim at monitoring the highly dynamic motion of cells in living organisms. Day and colleagues demonstrate in vitro and, even more striking, in virally transduced hepatocytes in vivo that their A-kinase activity reporter AKAR4.1 rapidly and highly reliably responds to glucagon signaling.

Among the large variety of methods to quantify FRET, only a few are truly suitable for intravital TPLSM imaging. The ratiometric FRET technique presented here based on single-wavelength excitation is definitely the fastest and currently
most practical, although it may still suffer from several limitations regarding the mostly uncontrollable signal-to-noise ratios of the donor and acceptor, respectively, in tissue as well as their different photobleaching behavior. Fluorescence lifetime imaging of the donor counteracts the limitations of the ratiometric methods, since it acquires only the fluorescence of the donor; however, it remains much slower than the ratiometric method owing to its need for higher photon counts (2, 7). In this regard, existing efforts (2, 6) aiming at the development of faster, more sensitive FLIM systems should be strengthened.

As the microscopy community moves towards fluorescence imaging experiments at redder wavelengths as well as towards functional imaging measurements, we would do well to follow the meticulous optimization and calibration of FRET reporter constructs demonstrated by Day and colleagues, especially for investigations performed in living animals.

GRANTS

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

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