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”

Randall Lindquist and Raluca Niesner
Deutsches Rheuma-Forschungszentrum (DRFZ), Berlin, Germany

Understanding mechanisms of physiologic and pathophysiologic processes requires their investigation at the cellular and molecular level within the genuine environment—the living organism. In the past two decades, two-photon laser-scanning microscopy (TPLSM) has become, because of the increased tissue penetration and lesser scattering of the longer wavelengths used in two-photon excitation (3), one of the most versatile tools to study cellular behavior in vivo. Currently, TPLSM performed in various organs of living animals, especially in mice, gives information about typical cellular motility patterns and cell-cell interactions (9), but relatively little quantitative functional information.

This has been problematic, as information on cellular and tissue morphology and dynamics must be complemented by information on cellular function to draw meaningful conclusions. The most reliable way to quantify cellular function using fluorescence-based techniques has proved to be Förster resonant energy transfer (FRET) (2). FRET as a photo-physical phenomenon occurs by the immediate propagation of excessive energy from the previously excited “donor” molecule to the “acceptor” molecule, exciting it to a higher energy level (Fig. 1). The donor molecule relaxes to its ground state without emitting any radiation, while the acceptor molecule relaxes to its ground state by emitting its characteristic fluorescence. The FRET efficiency strongly depends on the distance between the donor and acceptor molecules (the FRET pair) and on the energetic states of both molecules, as illustrated schematically in Fig. 1. If the donor and acceptor fluorophore are in the same molecule, bridged by a protein region whose conformation is sensitive to a factor of interest, conformational changes that lead to changes in the relative distance or orientation of the donor and acceptor fluorophores will therefore lead to large changes in FRET efficiency.

In this issue of American Journal of Physiology-Cell Physiology, Richard Day and colleagues thoroughly discuss the challenges of performing FRET quantification in intravital TPLSM and offer an excellent practical road map for other researchers to calibrate their own FRET constructs for reliable intravital FRET experiments (7). The first generations of FRET probes used CFP and YFP as donor and acceptor fluorophores, respectively, which were well-suited for cell culture studies, in which tissue penetration and autofluorescence are relatively minor concerns. Further, as imaging of cultured cells is best performed with a confocal microscope, most publications to date focus on the photophysical characterization of these FRET-pairs after single-photon excitation, leaving the optical nonlinear effects typical for TPLSM mostly unstudied.

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 auto-fluorescence 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

The Deutsche Forschungsgemeinschaft (DFG) is acknowledged for supporting the present work under grant TRR130, central project C01 and under grant Ni1167/3-1, JIMI to R. Niesner.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.L. and R.N. prepared figure; R.L. and R.N. drafted manuscript; R.L. and R.N. edited and revised manuscript; R.L. and R.N. approved final version of manuscript.

REFERENCES