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Quantitative two-photon imaging of fluorescent biosensors

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Fluorescent biosensors are now routinely imaged using two-photon microscopy in intact tissue, for instance, in brain slices and brains in living animals. But most studies measure temporal variation — for example, calcium transients in response to neuronal activity — rather than calibrated levels of biosensor occupancy (and thus levels of the sensed analyte). True quantitative measurements are challenging, since it is difficult or impossible to calibrate a sensor's dose–response *in situ*, and difficult to compare the optical signals from tissue to those during *in vitro* calibration. Ratiometric measurements (at two wavelengths) are complicated by variations in laser power and by wavelength-dependent attenuation in tissue. For some biosensors, fluorescence lifetime imaging microscopy (FLIM) provides a valuable alternative that gives well-calibrated measurements of analyte levels.

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Introduction

Genetically encoded optical tools are providing fantastic new methods for manipulation and measurement of brain cells (and many others) in real time and with cellular specificity. Optical measurement gives a dramatic report of episodic activities: bursts of stimulus-triggered action potentials are brilliantly apparent as flashes in the fluorescence of highly optimized calcium sensors [1]. But for many important biological signals, a temporal pattern is not enough: a more intricate quantitative assessment of an optical reporter's signal is needed. And such quantitative measurement can be especially challenging in the context of brain imaging, both because the imaging involves two-photon excitation, and because the usual methods of signal calibration by chemical manipulation are difficult or impossible. This review considers optimal approaches to quantitative biosensor imaging in this context, using either optical ratiometric or fluorescence lifetime imaging.

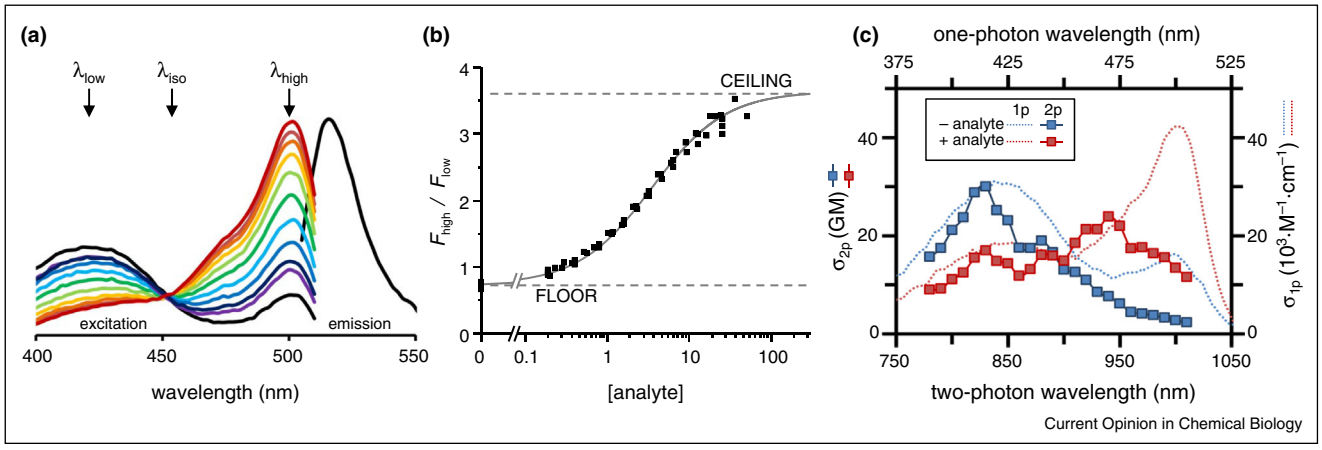
What is required for translating the fluorescent output of a biosensor into a quantitative measurement of the sensed level? A biosensor controlled by analyte binding[1] gives a fluorescent report that is proportional to its occupancy — the empty sensor usually has non-zero fluorescence, and the occupied sensor has a fluorescence that is greater or less than the empty value. But the intensity of any fluorescent signal will vary also with the concentration of the biosensor itself. To infer the occupancy of the sensor (and thus the concentration of analyte), the fluorescent signal must somehow be normalized to learn where it sits between the minimum and maximum values (Figure 1b). In some situations it is possible to measure these 'floor' and 'ceiling' values for each experiment — for instance, by permeabilizing the cells containing the biosensor and depleting or flooding the cell with the analyte. Ideally it is even possible to construct an *in situ* calibration curve for the sensor by observing the fluorescence response to known intermediate concentrations of the analyte.

Unfortunately, such *in situ* calibration is impossible when imaging the brain *in vivo*, and quite difficult even when imaging brain slices *in vitro*. Limited diffusional access combined with the cells' tendency to regulate the levels of all analytes of biological interest makes it impossible to control analyte concentration accurately.

The only alternative to *in situ* calibration is to use a calibrated optical measurement that can then be referred back to an *in vitro* calibration of optical response versus analyte concentration. The *in vitro* calibration would ideally be performed using protein samples or permeabilized cells viewed with the same microscope used for tissue imaging. Two imaging modalities can be used for this calibrated optical measurement: ratiometric imaging, and fluorescence lifetime imaging.

[1] For simplicity, we focus our discussion on biosensors that bind a particular analyte and report its concentration; other types of sensors exist (for instance those that report on their own phosphorylation state and thus indirectly on protein kinase and protein phosphatase activity) that present additional problems in quantitation. We have also focused on genetically encoded, fluorescent protein based sensors, though the same measurement principles apply to small molecule sensors.

Figure 1



Fluorescence behavior of a ratiometric biosensor. The examples here are for an ATP sensor, PercevalHR (adapted from [6]). **(a)** An excitation ratiometric sensor changes its excitation spectrum as analyte concentration is increased (from black to red). Relative fluorescence increases at some wavelengths (λ_{high}), decreases at others (λ_{low}), and often exhibits an isosbestic wavelength where there is no change (λ_{iso}). **(b)** The ratio varies predictably as a function of analyte concentration. **(c)** Comparison of the excitation spectra for one-photon and two-photon excitation, with and without analyte. The energy of a single photon of wavelength 425 nm is equivalent to the energy of two photons at 850 nm, but the two-photon excitation spectrum is not quite predictable from the single-photon excitation spectrum. Nevertheless, it is possible for this excitation-ratiometric sensor to be ratio-imaged using two-photon excitation (e.g. at 950 and 830 nm; see also [35]).

Ratiometric two-photon imaging

The principle of ratiometric imaging is simple: fluorescence is measured at two different wavelengths. Analyte binding somehow changes the relative fluorescence at the two wavelengths, so that the ratio can be used to infer the level of analyte. The level of the biosensor itself will scale the two fluorescence values equally, so that there is no change in ratio.

Excitation-ratiometric biosensors

For fluorescent protein (FP) based sensors, one common type of ratiometric sensor is excitation ratiometric. The original green fluorescent protein (GFP) from jellyfish always emits green (~500–550 nm) light, but it has two excitation bands around 405 nm (A band) and 495 nm (B band) [2]. The 'enhanced' GFP (EGFP) was cured of this problem — it has only the 495 nm excitation peak — but many GFP-based sensors exploit the two original GFP bands for ratiometric sensing using a single circularly permuted FP [3–5]. Binding of analyte shifts the resting state of the sensor between the two absorption bands, so that the relative response to the two excitation wavelengths is altered (Figure 1a,b). The switch between absorption bands can often be accomplished not only by analyte binding but also by environmental changes, particularly changes in pH. This is an important concern for the use of FP-based biosensors, requiring the simultaneous use of pH sensors for accurate calibration [6].

Excitation ratio measurements have long been used by biologists using standard one-photon excitation in the UV–visible range, particularly for the calcium-sensitive dyes such as the fura-2 dye introduced by Roger Tsien and colleagues in the 1980s [7]. As they described, the apparent affinity of the sensor varies systematically with the wavelengths chosen for the ratio measurement, and this important principle applies to all excitation-ratiometric measurements.

Each ratiometric image requires two separate exposures, using the two excitation wavelengths. For one-photon excitation, rapid wavelength switching can be accomplished using filter wheels, galvanometer-driven monochromators, or rapid switching of LED-based or laser light sources [8]. In the scanning two-photon microscope used for tissue and *in vivo* imaging, slow switching can be accomplished by tuning the pulsed excitation laser (typically a tunable Ti-sapphire laser) between two different excitation wavelengths. Even with modern integrated mode-locked lasers, tuning requires several seconds or more. Fast switching (on the millisecond time scale) requires a second (expensive) pulsed laser: each laser is tuned to a different excitation wavelength, and electronic shuttering (using electro-optical modulators) is used to allow sequential acquisition of signals evoked by the two lasers, often alternating by scan line [9].

An additional challenge for excitation ratio imaging with two-photon excitation is that two-photon excitation spectra often look very different from the one-photon spectra, because of the different rules for electronic transitions in response to one-photon versus two-photon excitation [10]. Nevertheless, selective two-photon excitation of the A-band and B-band of GFP, and thus excitation-ratiometric