Avoiding bias in fluorescence sensor readout

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Fluorescent indicators can provide quantitative insights into the spatiotemporal dynamics of signalling molecules released by brain circuits. However, a mismatch between the experimental context and the experimental imaging settings often introduces unexpected errors and biases in such measurements. Appreciating this mismatch should help to arrive at unbiased estimates.

Genetically encoded fluorescent sensors have opened a new horizon in our understanding of the molecular machinery underpinning the activity of brain cells and circuits. The biochemical signals that such indicators encode possess certain spatiotemporal characteristics, which could vary widely among experimental paradigms. In a previous Comment article, I discussed the typical misinterpretations of fluorescence recordings, which arise from the spatiotemporal mismatch between the sensor and its target[1]. The need to find the imaging settings by which the unbiased measurements can be achieved is no less important in addressing this mismatch. In many cases, however, the settings of a fluorescence microscope are simply tuned to obtain the best possible image quality using industry-approved calibration standards, which are not necessarily related to the actual experimental paradigm. Likewise, standard measures of indicator's sensitivity in vitro do not necessarily provide an unbiased reference for the signalling scenarios in situ. In this Comment article, I consider several characteristic examples illustrating how the experimental conditions of fluorescence brain imaging could bias its quantitative outcome.

[H1] Ligand release transiency dictates sensor saturation

A standard dose-response test for newly developed fluorescent sensors involves measuring the sensor emission intensity at different concentrations of its ligand. The resulting S-shape curve thus represents steady-state or equilibrated conditions of the ligand-sensor reaction. For instance, with the sensor kinetics typical of fast fluorescent indicators for Ca^{2+} or glutamate ($k_{on} \approx 10^7$ $M^{-1}s^{-1}$, $k_{off} \approx 50$ s^{-1})[2,3] the ligand concentration 0.1 µM would correspond to a ~95% saturated fluorescent signal in such tests (Fig. 1a). However, during ion channel opening or synaptic vesicle fusion, a ligand concentration surge lasts for only low-millisecond or sub-millisecond periods. In such cases, a basic non-stationary ligand-sensor kinetic model predicts that brief 0.1 µM ligand transients will generate essentially sub-saturation fluorescence responses, while displaying strong sensitivity to the transient duration (Fig. 1a). Only when the concentration transient lasts for more than several milliseconds (this cut-off time depends on the sensor's kinetic), the fluorescent signal readout approaches the values reported by the steady-state dose-response curve; for instance, during relatively slow extracellular waves of GABA[4].

[H1] Ratiometric readout depends on the imaging depth

Brain tissue is a 'turbid medium' that exhibits, unlike translucent in vitro preparations, substantial light scattering. Thus, for any fluorescent indicator, significant signal attenuation occurs with greater depth in tissue. Generally, concomitant fluctuations in emission intensity could be minimised or cancelled out by using ratiometric indicators that respond to the reaction under study by changing the ratio between two chromatic bands in their emission spectrum [5], for instance, green and red. However, light scattering increases sharply with shorter wavelengths: in live brain tissue, the depth at which light intensity drops by e (~2.72) times is ~500 µm for green (500 nm) and ~750 µm for red (750 nm) light [6] (Fig. 1b). Thus, the green/red signal ratio will decrease as the imaging depth increases (Fig. 1b). Such changes have to be corrected for in ratiometric readout at different depths.

[H1] Laser scanning mode can miss short-lived fluorescent signals

Frame scan is among the most common regimes of a laser-scanning fluorescence microscope. As it normally takes the laser beam 0.5–2 ms to complete individual one-pixel-wide lines forming the frame, a high-resolution frame image usually takes 100–300 ms to generate (Fig. 1c). This implies that recorded frames could entirely miss brief

local fluorescent signals that occur intermittently in the frame area (Fig. 1c). The recoded frame sequence could thus report the fluorescence signalling dynamics that are reduced in frequency and intensity compared with real activity. Identifying loci of brief signals across the frame series should help to correct for missed activity. In some cases, a hardware solution could be provided by using a resonant scanner that may achieve a 1–2 kHz frame rate[7].

[H1] Recorded peak values depend on the pixel size

It is often important to compare the amplitudes of fluorescent indicator responses among different areas or specimens. In many instances, the fluorescent signal is generated by a sub-microscopic or nanoscopic source of the signalling molecule or ion. With the conventional optics operating at light diffraction-limited resolution, the peak signal will be spread over the sub-microscopic point-spread function of the microscope[8]. To retain its amplitude during image registration, pixel resolution of the system has to be similar to or higher than the point-spread function (or the diffraction limit). With larger pixels, the peak signal will be diluted over the larger pixel area, thus reducing its value compared with the 'true' amplitude (Fig. 1d). It is therefore important to make sure that for amplitude comparison purposes, the image pixel size should usually be below 0.2 x 0.2 μm^2.

[H1] Concluding remarks

Important information conveyed by fluorescent sensor measurements could be heavily biased when the experimental imaging settings do not fully match the purposes and the conditions of the experimental paradigm. Addressing such disparities should help to arrive at the correct quantitative estimates, as illustrated by the characteristic examples presented here.

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Competing interests

The author declares no competing interests.

Figure 1 | **Schematic illustrations of fluorescence indicator readouts prone to errors**. a | A fluorescent sensor that is ~95% saturated by the 0.1 µM ligand concentration during steady-state dose-response testing (left) will still display high sensitivity to rapid ligand transients at this concentration (right; durations of a 0.1 µM ligand transient are shown and colour-coded; see text for the kinetic parameters used). b | Green light is scattered more intensely than red in brain tissue (left), which will reduce the (ratiometric) green/red ratio with greater imaging depths in tissue (right). c | Laser-scanning microscopy operating in conventional frame-scanning mode (left) will miss local fluorescence signals that are much shorter than the frame duration (right; *F* ordinate, fluorescent signal intensity). d | Increasing the image pixel size will reduce the detected signal peak amplitude of the fluorescent responses originating from sub-microscopic sources.
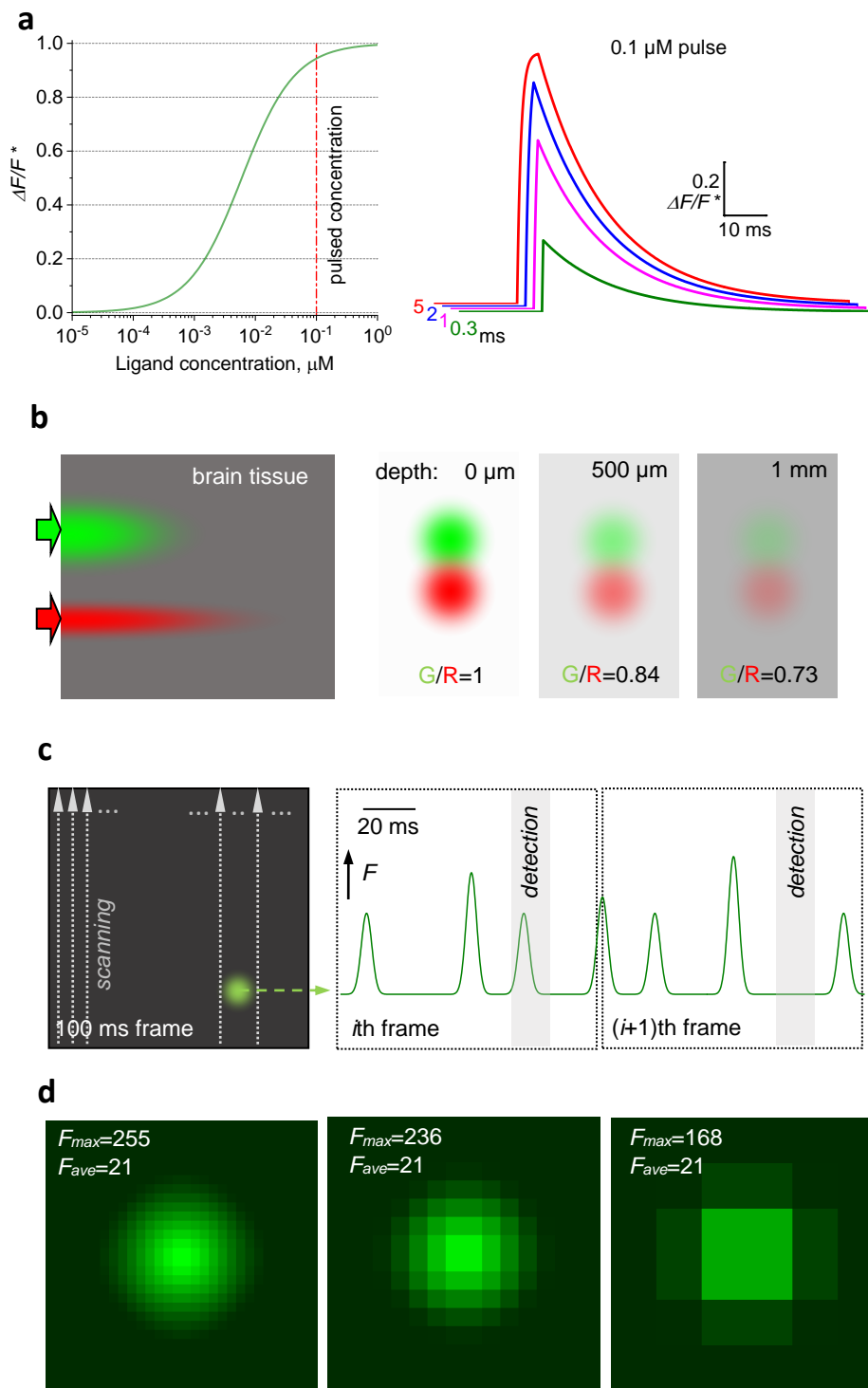
Figure 1 | **Characteristic examples of fluorescence indicator readouts prone to errors.**
a | A fluorescent sensor that is ~95% saturated by the 0.1 μM ligand concentration during steady-state dose-response testing (left) will still display high sensitivity to rapid ligand transients at this concentration (right; durations of a 0.1 μM ligand transient are shown and colour-coded). **b |** Green light is scattered more intensely than red in brain tissue (left), which will reduce the (ratiometric) green/red ratio with greater imaging depths in tissue (right). **c |** Laser-scanning microscopy operating in conventional frame-scanning mode (left) will miss local fluorescence signals that are much shorter than the frame duration (right; F ordinate, fluorescent signal intensity). **d |** Increasing the image pixel size will reduce the detected

signal peak amplitude of the fluorescent responses originating from sub-microscopic sources.