Avoiding interpretational pitfalls in fluorescence imaging of the brain

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Fluorescent sensors of molecular activity have revolutionised our knowledge of the brain. However, their signals report a reaction between the target and the sensor molecules rather than the activity of interest per se. Thus, understanding location, sensitivity and imaging environment of a sensor should help avoid misinterpretation of its readout.

Molecular optical sensors have become a tool of choice in our quest to understand how the brain works. Delivered to the cells or cellular compartments of interest, through genetically encoded expression or other means, they are capable of unveiling intricate behaviours of their otherwise invisible molecular targets. However, the interpretation of fluorescent signals emitted by sensor molecules is far from trivial.

Fluorescent signals from optical sensors report an interaction between the sensor and the target molecule rather than what the latter molecule is doing on its own. The dynamic profile of recorded fluorescence also depends on the location and mobility of the sensor molecules themselves. In addition, the fluorescence readout can vary with the optical conditions of the tissue and the imaging settings involved. As a result, the spatiotemporal characteristics of the signal reported by an optical sensor can deviate significantly from the spatiotemporal features of the molecular activity under study. Here, I discuss and illustrate several basic principles that might help avoid bias or misinterpretation of optical data generated by fluorescent sensors.

Sensor location

Gene-targeting techniques are rapidly emerging that enable the expression of optical sensors for neurotransmitters and neuromodulators at highly specific subcellular loci, such as synaptic connections or axonal compartments[1]. Thus, the recorded fluorescence signal could be highly localised, and this could be interpreted as the highly localised release of the target molecule. In fact, the molecule of interest could be released elsewhere and over a large tissue area[2], before reaching the sensor's location (Fig. 1a). Specific control experiments should help clarify this uncertainty. For instance, analysing the signal gradient, or restricting extracellular diffusion of the studied molecule (e.g., by applying its molecular scavenger / buffer) may shed light on its spatial origin.

Binding kinetics of the sensor

Fluorescent sensors normally change their emission properties — and thus generate a useful signal— upon binding their target molecule. Most sensors have very fast binding kinetics (in the sub-millisecond range) whereas unbinding can last from several to hundreds of milliseconds[3], during which time the fluorescent signal will persist. Similar logic applies to sensors that trigger an emission-generating molecular reaction, such as G-protein-coupled receptor-based sensors[4]. Thus, a very brief action of a target molecule could generate a response that is orders of magnitude longer (Fig. 1b). Understanding the relationship between the two, the effect of noise and diffusion escape, often requires modelling, similar to what has been developed for Ca^{2+} imaging[5] or fast-scan cyclic voltammetry[6].

Sensor expression

In baseline conditions, the emission intensity (brightness) of a sensor scales with its local expression level, which may vary many-fold across the tissue. Such heterogeneity might prompt an investigator to consider the darkest sensor-labelled areas as less informative. However, the molecular target's response amplitude is conveyed by the relative, rather than the absolute, change in the sensor's fluorescence[7], and the largest relative change may occur where the sensor expression is relatively low (Fig. 1c). To avoid overlooking the key areas of molecular response it would help, firstly, to ensure a full dynamic range of imaging and, secondly, to explore the areas of low sensor expression with increased laser power or pixel dwell-time.

False-positives in Ca^{2+} imaging

Ca^{2+} imaging using wide-field illumination (one-photon excitation) is becoming a universal tool in monitoring neuronal network spiking activity in freely moving animals[8]. However, light absorption and scattering vary across brain tissue regions, affecting fluorescence readouts. In particular, nerve cell bodies are much more translucent than the surrounding neuropil and therefore are likely to relay, at least partly, fluorescent signals of the neighbouring cells without carrying out any activity on their own. This may result in false-positive detection of neuronal activity (Fig. 1d), especially when the emission occurs across the tissue volume, as in most mini-scopes. Revealing the exact synchrony of fluorescent signals among neighbouring cells might help detect such false-positives.

Surface-to-volume ratios

In neuronal Ca^{2+} imaging, fluorescent transients are often considered to reflect Ca^{2+} channel activity in the excitable cell membrane. In fact, such transients report a change in the intracellular Ca^{2+} concentration. This implies that, under the same Ca^{2+} channel activity in the membrane, the fluorescent Ca^{2+} signal will depend directly on the local surface-to-volume ratio [9]. As a result, cell compartments with a low surface-to-volume ratio may appear inactive, which would be a false-negative reading (Fig. 1e). The high-affinity membrane-tethered, as opposed to cytosolic, Ca^{2+} indicators, and a full understanding of the cell morphology should help avoid such a misinterpretation.

Concluding remarks

Experimental recordings generated by fluorescent sensors rarely mirror exactly the molecular processes they are designed to report. Reliable, unbiased interpretation of such recordings involves a good understanding of the basic principles of fluorescent imaging, as illustrated by the characteristic examples presented here.

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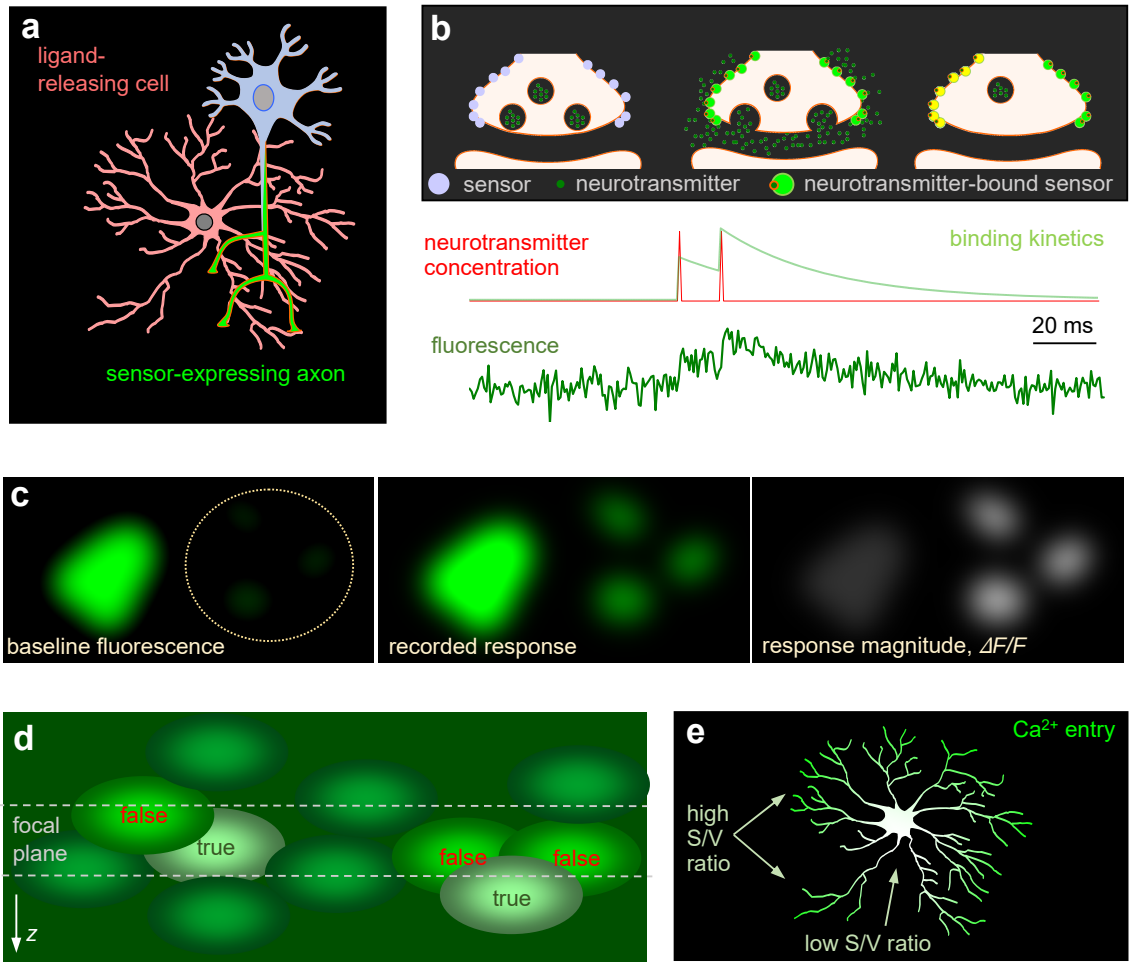
a
ligand-releasing cell
sensor-expressing axon
b
sensor
neurotransmitter
neurotransmitter-bound sensor
neurotransmitter concentration
binding kinetics
20 ms
fluorescence
c
baseline fluorescence
recorded response
response magnitude, ΔF/F
d
focal plane
false
true
false
false
true
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e
Ca2+ entry
high S/V ratio
low S/V ratio

Figure 1 **| Characteristic examples of fluorescence imaging settings prone to misinterpretation. a |** A signal molecule released from one cell could be reported by its fluorescent sensor expressed locally in the neighbouring cell. **b |** Neurotransmitter sensor fluoresces upon binding the released neurotransmitter, and remains fluorescent long after the latter has diffused away (top). The schematic traces show the typical time course of local neurotransmitter concentration (red), neurotransmitter-bound sensor level (light green) and the corresponding noisy fluorescence recording (dark green). **c |** Areas of low sensor expression (left, dotted oval) responding to the target stimulus (middle) may reveal a much greater action than the areas of high expression (right). **d |** Relatively translucent cell bodies (blurred ovals) could relay fluorescence of adjacent active responders (true), thus generating a false-positive readout (false). **e |** A high surface-to-volume ratio boosts Ca^{2+} signals in local cell compartments even when Ca^{2+} channel activity in cell membranes is similar throughout the cell.