REPLY TO PISUPATI ET AL.:

Evaluating single subunit counting data to find the correct stoichiometry

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single subunit counting | single molecule fluorescence | voltage-gated K channels

In their Letter (1), Pisupati et al. comment that they found a different Kv2.1:Kv6.4 stoichiometry (2) than we report in Möller et al. (3). Our manuscript was still under review when their article was published. Realizing that the results were inconsistent, we added an objective evaluation algorithm calculating relative probabilities for the different stoichiometry models (3).

The authors of the Letter (1) speculate that our lower probability of fluorescence (p_f), an intrinsic property of the green fluorescent protein (GFP), is due to prebleaching or too fast bleaching rate. We can exclude both possibilities. We handle our preparations exclusively under low light with a wavelength of >600 nm. After focusing with lower light intensities, we record from an adjacent region not exposed to light previously. Similarly, the probability of two-step bleaching during one image is negligible (0.0025%), and our automated step-detection algorithm (4) detects double-bleaching events.

But even with a different p_f, the data are intrinsically consistent, as our objective algorithm uses the probability obtained from our controls to evaluate that a 2:2 stoichiometry is more likely than a 3:1 stoichiometry ($P > 99.99\%$, $N \approx 9{,}000$) (3).

The question remains, what reasons could have led to the diverging results of the two studies?

1) Model evaluation is imperative. It is important to evaluate not only the chosen but also alternative models. Contrary to Pisupati et al., we evaluated alternative models for both single-subunit-counting and electrophysiological measurements.
2) Expression level has to be adjusted appropriately. When comparing one-step versus two-step stoichiometries, the distribution only contains two single data points, while being fit with two free parameters. Such a distribution is extremely susceptible to contaminations, and two different mixtures with altered p_f would result in identical distributions. Approaching it differently than Pisupati et al., we allowed colocalization. This not only led to six to eight data points per distribution, but also removed the ambiguity among different mixtures. The probability of fluorescence is obtained as an intrinsic control.
3) Quality control has to be applied. Recordings with too many of the following ambiguities should be discarded, since inclusion of artifacts and exclusion of a large fraction of visible spots question the meaningfulness of the remaining ones: (*i*) Coverslips have to be thoroughly cleaned to prevent contaminations, typically displaying one-step bleaching; (*ii*) analysis of all visible spots is imperative, as user selection leads to (subconscious) bias, distorting the results; (*iii*) rapidly moving spots are indistinguishable from one-step bleaching events; and (*iv*) almost all spots should be fully bleached. The automated detection algorithm used in our study analyzes all spots and automatically rejects movies with too many exclusions (4). In the example shown by Pisupati et al. (2), only 14 of ~75 spots were included in the analysis.
4) Experimental parameters have to be chosen carefully. Bleaching rate and exposure time must match such that almost all spots are fully bleached. A high ratio of bleaching time constant to total exposure time leads to preferential exclusion of spots with more bleaching steps, distorting the distribution and biasing it toward one-step bleaching events. The more bleaching steps, the longer it takes to bleach. Pisupati et al. used a ~7-times-longer bleaching time constant, and 33% of the selected spots were excluded from analysis because bleaching was not complete.

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The authors declare no competing interest.

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