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LETTER TO THE EDITOR

Re-analysis on geometric energy

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Davies et al. raise cautions regarding the broad application of our methods (Makino et al. 2016), and propose alternative approaches to measure the spatial distribution of neurons. We appreciate those more generally valuable measurements, but their comments might not fully apprehend some of the core messages in our paper.

Dichotomization

In many experimental systems, signals, for example the immunohistochemical fluorescent intensities measured in our study, often take continuous values and thus cannot be dichotomized without an artificial threshold. Although dichotomization may benefit some analyses, we would note that dichotomizing methods also neglect signal intensities. In particular, if there are a lot of cells near the threshold value, any subtle change in the threshold employed may cause an unexpected iceberg effect. Thus, in our recent paper (Makino et al. 2016), we devised two new parameters to tame this statistical variation in continuous variables: the geometric energy, E_g, and the geometric entropy, H_g. In this context, the question posed by Davies et al. about whether we would "have reached similar conclusions if neuron intensities were dichotomized into low and high expression values" seems to be out of focus; note that, even if the conclusions are inconsistent, this fact might merely indicate that previous methods using arbitral thresholds led to erroneous conclusions. In the case of our paper, however, the conclusions are consistent both with and without dichotomization (Fig. 1a). Specifically, we separated all neurons into immunochemically positive and negative cells at three arbitrary thresholds of mean, mean + 2 × SD, and mean + 5 × SD of their fluorescence intensities, and labelled them '1' and '0', respectively. Irrespective of the thresholds used, the E_g values of the real data were consistently higher than those of the corresponding surrogate data, providing robust evidence of spatial clustering in the artificially dichotomized data. Compared to the original comparison (Figure 3C in Makino et al. 2016), the difference is small. This is due simply to the use of '1' for positive cells. If the positive cells are assigned a larger number, such as 10 or 100, the difference becomes larger.

Outliers

Davies et al. claim that "the resulting value of this statistic is likely to be highly sensitive to outliers which have a real potential to adversely dominate results". There seems to be another misunderstanding of our message. Our parameter E_g is indeed designed to be sensitive to outliers. The immunochemical signal is usually distributed with a long tail, and the signal intensities of so-called "positive" cells are often tens of times stronger than those of "negative" cells (see Figure 2A in Makino et al. 2016). Thus, a good measurement has to sensitively incorporate those outliers into its value. On the other hand, it is intriguing that our parameter E_g can evaluate the spatial clustering even without such outliers; when the fluorescence intensities

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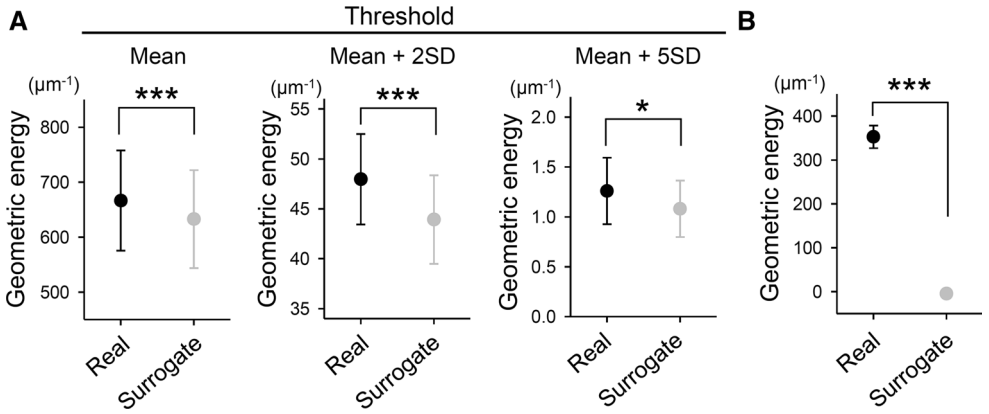

Fig. 1 Geometric energy analyses. **a** For the same dataset as that used in Figure 3C of Makino et al. (2016), geometric energy (E_g) values were computed after dichotomization at three different thresholds: mean, mean + 2 × SD, and mean + 5 × SD, indicating that, in all cases, the positive cells were significantly clustered. *Error bars* SEM of nine mice. Mean $^{***}P = 2.0 \times 10^{-4}$, $t_8 = 6.44$; Mean + 2 × SD $^{***}P = 2.1 \times 10^{-5}$, $t_8 = 8.87$; Mean + 5 × SD $^{*}P = 1.1 \times 10^{-2}$, $t_8 = 3.28$; paired *t* test versus surrogate. **b** E_g was computed using logarithmically scaled fluorescence intensities. Even under conditions where outliers were reduced, E_g detected significant clustering of c-Fos positivity. *Error bars* SEM of nine mice. $^{***}P = 6.8 \times 10^{-7}$, $t_8 = 13.9$, paired *t* test versus surrogate

were expressed in the logarithmic scale to decrease outliers, the E_g values still led to the same conclusion (Fig. 1b).

Cluster sizes

We agree with Davies et al. that a shortfall of our E_g analysis is that it cannot identify either the number, position, or shape of the clusters. We introduced the post hoc H_g analysis and estimated 'mean' cluster size. As Davies et al. suggest, the H_g analysis may suffer from edge effects and boundary bias. To address this point, instead of tiled grids, the use of randomly placed circles with different diameters may reduce the edge-effects of the aligned grids, which we will report in another methodological paper. Finally, Davies et al. elegantly demonstrate that BMRF, GMRF, and KDE overcome these problems. The methods follow traditional routes and are not too novel, steadily allowing the estimation and visualization of the clusters under study. We encourage future research on how to choose the most appropriate analyses depending on data type.

Compliance with ethical standards

Conflict of interest We declare no conflict of interest.

Reference

Makino K, Funayama K, Ikegaya Y (2016) Spatial clusters of constitutively active neurons in mouse visual cortex. Anat Sci Int 91:188–195