
Article:
Cordelieres, F. and Peckham, M. orcid.org/0000-0002-3754-2028 (2025) Commentary on critique and rebuttal of 'On the pixel selection criterion for the calculation of the Pearson's correlation coefficient in fluorescence microscopy'. Journal of Microscopy, 299 (2). pp. 91-93. ISSN: 0022-2720

https://doi.org/10.1111/jmi.70006

Commentary on critique and rebuttal of “On the pixel selection criterion for the calculation of the Pearson's correlation coefficient in fluorescence microscopy”

Fabrice Cordelieres[1] and Michelle Peckham[2]

[1]Bordeaux Imaging Center, UAR 3420 CNRS - Université de Bordeaux - US4 INSERM, *Traitement et analyse d'images*, Centre Broca Nouvelle-Aquitaine, 146, Rue Léo-Saignat 33077 Bordeaux
fabrice.cordelieres@u-bordeaux.fr
ORCID: 0000-0002-5383-5816

[2] School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT
m.peckham@leeds.ac.uk
ORCID: 0000-0002-3754-2028

We recently published an article in the Journal of Microscopy entitled “On the pixel selection criterion for the calculation of the Pearson's correlation coefficient in fluorescence microscopy” by Sergio Lopez and co-authors (Lopez *et al.*, 2025). The article was published as part of a themed issue, and was reviewed by 3 independent reviewers, none of whom raised major criticisms.

We subsequently received a letter from Adler and Parmyrd “Quantifying colocalisation by correlation: use pixels with both fluorophores within biologically meaningful ROIs”, which was very critical of the original paper, and raised a number of key issues.

The journal is, of course, open to criticisms raised of papers we have published, so we have considered this letter seriously. However, we thus feel it is important to point out that while a reasonable discourse is important in science, it is important to raise potential issues in a respectful and scholarly tone. Scholarly dialogue should be constructive and not make any personal attacks. No research paper is perfect, but in specifically critiquing an article, it is important to remain respectful and acknowledge that mistakes can inadvertently be made.

The Journal of Microscopy is published by Wiley on behalf of the Royal Microscopical Society, and we have a code of conduct: https://www.rms.org.uk/code-of-conduct.html that expects professional conduct, that all members and non-members must treat everyone with dignity and respect, and should not bully or abuse others. Unfortunately, the tone and language used by Adler and Parmyrd in their original letter did not meet this code. We thus strongly encouraged Adler and Parmyrd to write their critique in a more respectful way.

The authors of the original article have provided a robust response. While acknowledging a flaw in their analysis (Figure 2), they point out that this error is not unique and also occurs in the ImageJ plugin JACoP (Bolte & Cordelieres, 2006).This is useful for everyone to know (and thus avoid). JACoP (http://rsb.info.nih.gov/ij/plugins/track/jacop.html) groups the most important intensity correlation coefficient-based (ICCB) analyses, together with an ImageJ (FIJI) based plugin for object-based colocalization analysis. Faulkner and colleagues have now provided an update and comment on this calculation in their response, thus further clarifying their approach to colocalization, as well as PCALL and its documentation. We believe this will be helpful for the community.

After several revisions of the original critique, together with responses from the original authors, we have now decided to publish the final versions of the critique and response, to raise awareness of the challenges in performing co-localisation analysis.

To help the non-specialist reader make more sense of the papers and the critique as well as the original paper, we make some comments below to help non-specialists to consider how they use this type of approach in estimating co-localisation:

This paper by Lopez *et al*. deals with the interpretation of the **Pearson's correlation coefficient (PCC)**, in the context of colocalization studies. The authors emphasize the existence of a single definition for the way the PCC is computed, but at least three ways to feed the formula with the image data. They differentiate $\mathbf{PCC_{ALL}}$, where all pixels are considered, from $\mathbf{PCC_{AND}}$ and $\mathbf{PCC_{OR}}$. The two latter PCCs are threshold based: only part of the intensities are used, lying above channel specific thresholds. In $\mathbf{PCC_{AND}}$, a pixel is used for calculation only if both its intensities are above its respective threshold. In $\mathbf{PCC_{OR}}$, a pixel is used for calculation if at least one of its intensities is above one of the channels' thresholds.

The authors therefore revisit the PCC in three ways:
- Taking into account **all pixels ($\mathbf{PCC_{ALL}}$)**;
- Taking into account all possible **"object pixels"** (i.e., the ones that are above the threshold, $\mathbf{PCC_{OR}}$);
- Taking into account only **overlapping "object pixels"** (i.e., above the threshold in both channels, $\mathbf{PCC_{AND}}$).

1. Several terms are used that we redefine here:
   Colocalization is termed both as *"co-occurrence"* and *"correlation"*.
   Co-occurrence means "two proteins at the same place,"
   Correlation means "a certain idea of stoichiometry between molecular actors".
   PCC does not evaluate co-occurrence. To get a high PCC, both proteins should be found at the same location (**co-occurrence**) **AND** there should be a relationship between their intensities (correlation). Thus, it makes sense to expect that by restraining the areas in which PCC is computed for **co-occurrence spots**, it will be more likely to obtain a high value for PCC. Restraining the PCC computation to part of the image has been done before by others ((Costes *et al.*, 2004) (ref 38 of (Lopez et al., 2025)). This earlier work proposed to find thresholds, based on minimization of the PCC below the progressively decreasing thresholds.

2. **Colocalisation diagnostics should be presented relative to the actual resolution**. Care should be taken in drawing conclusions from sets of images that are not at all comparable (e.g. confocal, 10X/0.4 objectives) and super-resolved images (STED, SIM etc)). Implementation of the analysis can be done with widefield or confocal as well. However, at higher resolutions, the chances are that no colocalization occurs due to better separation of the proteins of interest. Thus, it is important to consider the resolution of the images in drawing any conclusions from data.

3. **Control localisation data:** In Figure 4A (Lopez et al., 2025), two antibodies to the same protein (Tom40/Tom40) are used to generate a control image that shows the highest possible colocalization level. However, it is worth remembering that two antibodies targeted at the same protein might compete if they are raised to the same epitope. This would result in an exclusion, but the resolution used might not show it. Readers should be aware about this potential limitation, when choosing control images.

4. **Thresholding:** Analysis of micrographs (and simulated data) in (Lopez et al., 2025) is based on a partitioning of the intensities relying on **Otsu thresholding**. This method aims at separating the intensities into two subsets, placing the threshold where the two subsets have a minimized variability. As a prerequisite, the histogram

should therefore display two populations of intensities. **We would advise readers to validate this approach and/or a display of the channels' histograms for this thresholding and to justify why a particular threshold method was chosen in their work.**

5. **For any normalisation of images**, it is helpful to report on how the intensities are normalised (e.g. Figure S1 (Lopez et al., 2025)), if it was done prior to or after thresholding. It is useful to explain any **need for normalization, and how the normalization method used was chosen**.

6. **Generation of a control dataset from original datasets**. This is commonly done by rotation of the original image and is efficient for sparsely distributed objects. In a more crowded environment, it is worth noting that this is less likely to work well (Costes et al., 2004).

7. **Simulation of microscopy data**. Again, this is a commonly used approach, to simulate what actually occurs within the microscope. However, it can be useful to include 1) blurring with different Gaussian figures to recapitulate the effect of different PSFs for the two channels; 2) adding noise to the image. The latter point is important as PCC is highly sensitive to noise.

8. **Z-stacks.** These are often used in co-localisation analysis and need the assumption that the shape/extent of the object is not modified from one experimental condition to the other.

9. **Thresholding or segmentation:** Faulkner and colleagues (Lopez et al., 2025) show that one can focus more on potential co-occurrence sites in order to get more significance out of the PCC_{xxx}. This approach could also be considered as segmentation. A drawback of thresholding is that it excludes intensities below thresholds. When computing a coefficient that is intensity-based, this might have a large influence on its value. Now that machine learning allows better segmentation, it could provide the region of interest where to look for colocalization, in a more intensity-independent fashion than threshold used to do.

Bolte, S. & Cordelieres, F. P. (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc,* **224,** 213-232.

Costes, S. V., Daelemans, D., Cho, E. H., Dobbin, Z., Pavlakis, G. & Lockett, S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J,* **86,** 3993-4003.

Lopez, S. G., Samwald, S., Jones, S. & Faulkner, C. (2025) On the pixel selection criterion for the calculation of the Pearson's correlation coefficient in fluorescence microscopy. *J Microsc,* **297,** 304-315.