Quantifying colocalization: The case for discarding the Manders overlap coefficient

Jeremy Adler | Ingela Parmryd

Department of Medical Biochemistry and Cell Biology, University of Gothenburg, Gothenburg, Sweden

Correspondence
Ingela Parmryd, Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Box 440, 405 30 Göteborg, Sweden.
Email: ingela.parmryd@gu.se

Funding information
Vetenskapsrådet, Grant/Award Number: 2015-04764

Abstract
Colocalization measurements aim to characterize the relative distribution of two molecules within a biologically relevant area. It is efficient to measure two distinct features, co-occurrence, the extent to which the molecules appear together, and correlation, how well variations in concentration of the two molecules match. The Manders overlap coefficient (MOC) appears in most colocalization software but the literature contains three interpretations of its measurements: (a) co-occurrence, (b) correlation, or (c) a combination of both. This is surprising given the simplicity of the underlying equation. Testing shows that the MOC responds both to changes in co-occurrence and to changes in correlation. Further testing reveals that different distributions of intensity (Gaussian, gamma, uniform, exponential) dramatically alter the balance between the contribution from co-occurrence and correlation. It follows that the MOC’s ability to differentiate between different patterns of colocalization is very limited, since any value is compatible with widely differing combinations of co-occurrence, correlation, and intensity distribution. To characterize colocalization, we recommend reporting both co-occurrence and correlation, using coefficients specific for each attribute. Since the MOC has no clear role in the measurement of colocalization and causes considerable confusion, we conclude that it should be discarded.

Keywords
colocalization analysis, co-occurrence, correlation, fluorescence, MOC, quantification

1 | INTRODUCTION

Colocalization refers to the relative pattern of distribution of two molecules and is assessed when studying biological phenomena such as gene transcription, vesicular transport, cell signaling, and pharmacokinetics. However, precisely what is meant and, therefore, what should be measured, is hard to pin down. This lack of clarity is demonstrated by the large number of colocalization coefficients, with each encapsulating a different definition.

Over a decade ago, we recognized that the assorted definitions of colocalization form two clusters [1]. One around correlation, the relationship between the variation in concentration of the two molecules. The second cluster reports a conceptually simpler measure, co-occurrence, the extent to which the signal from both molecules appears in the same pixels. Consider two molecules within a biologically meaningful region of interest (ROI), their co-occurrence could range from nil, when the molecules occupy different subdomains, to complete, when they are never separated. Similarly, the correlation could range from zero (unrelated) to a perfect, positive or negative match, when the variations in concentration of one tightly tracks variations in the second. Co-occurrence and correlation are completely independent i.e. one does not predict the other. Therefore, both are valuable measurements.
Some coefficients fall outside our bipolar scheme and instead merge the two primary attributes into a single value. These we categorize as hybrids which we suggest includes the Manders Overlap Coefficient (MOC) [2, 3] and the \( H_{\text{Coeff}} \) [4, 5].

Our initial case that the MOC is a hybrid coefficient was based on examination of the equation and simulations. These simulations covered the complete range of correlation, the critical observations were that a maximal MOC required perfect positive correlation and complete co-occurrence, reductions in the correlation progressively reduced the MOC, but even a perfectly negative correlation had a substantial residual value—attributable to co-occurrence [3]. We concluded that at best the MOC presents “a highly (intensity) weighted measure of co-occurrence, also affected by correlation and sensitive to offset”. There are coefficients that exclusively measure co-occurrence, the M1 and M2 pair that report the fraction of the total intensity (above background) of one molecule where the other molecule also is present. Other coefficients exclusively measure correlation, the Pearson correlation coefficient (PCC), the Spearman rank correlation coefficient (SRCC) and Kendall’s Tau. We therefore recommended avoiding the MOC. Correlation measurements (PCC, SRCC, and Kendall’s Tau) predate their use in quantitative colocalization analysis [6–8]. By contrast, the MOC was created specifically to measure colocalization but has not been acknowledged as a measure correlation by other disciplines.

We anticipated that our previous study, the only substantive examination of the MOC’s performance, would establish its status as a hybrid (mixed) coefficient and discourage its use. However, two mutually incompatible alternative interpretations of the MOC subsequently appeared in review articles, (i) the MOC is a measure of co-occurrence [6] or (ii) the MOC is an alternative measure of correlation [9].

The MOC was one of a number of new coefficients launched in a seminal article on the quantification of colocalization [2]. The MOC was presented as an alternative to the PCC, a plus being that its range (zero to one) “avoids the negative values of the correlation coefficient (PCC, range −1 to +1) that are difficult to interpret when the degree of overlap is the quantity to be measured”. Note this description predates our division of colocalization into co-occurrence and correlation, so the meaning is a little unclear. The MOC was further described as “the numerator is proportional to the number of colocalising objects” and “an overlap equal to 0.5 implies that 50% of both components of the image overlap with the other part of the image ... one condition: the number of objects in both components of an image has to be more or less equal”. Overall, this melange suggests the MOC might be a replacement for or a supplement to the PCC or a measure of overlap. However, the requirement for similar number of objects greatly restricts its utility as a measure of overlap.

If this was not sufficiently confusing, an extension to Manders et al.’s own interpretation was later presented “If the image has an overlap (MOC) of 0.5 it implies that 50% of its pixels overlap. A value of zero means there are no overlapping pixels.” [10]. The switch from “components” to “pixels” is an appreciable change, with the description of the MOC now resembling the area fraction (Ar%)—the co-occurrence by area [11]. The MOC was later described by the same authors as representing “the true degree of colocalization” with values “from 0.6 to 1.0—values indicating colocalization” [12]. Why the MOC reported the true colocalization and why values below 0.6 do not to show differing degrees of colocalization were not explained.

The correlation based interpretation of the MOC is “0 corresponds to negative correlations, 0.5 to no correlation and 1.0 to full correlation” [9] effectively compressing and offsetting the range of the PCC, while retaining its meaning.

That the MOC follows changes in co-occurrence was noted by us [3] and later Dunn et al. stated “it is primarily sensitive to co-occurrence, the fraction of pixels with positive values for both channels” but did not recommend its use, noting it “only indirectly and somewhat unpredictably measures co-occurrence” [13]. Nonetheless, the MOC was recently promoted by another set of authors as a measure of co-occurrence, “The MOC only expresses the degree to which two structures spatially overlap, in an intensity weighted manner” [6]. The review recapitulated many of the findings in our 2010 paper but ignored our major finding, that the MOC responds to both co-occurrence and to correlation [3].

To avoid confusion between the MOC with the M1 & M2 pair or the Ar%, all of which measure overlap, we have abided by common practice and have replaced the MOC’s original designation as the “overlap coefficient”, with the eponymous Manders Overlap Coefficient.

Since there are widely differing opinions about the MOC and because it appears even in new colocalization software [14, 15], we have extended our earlier examination with a greater emphasis on co-occurrence.

## 2 | METHODS

### 2.1 | Colocalization coefficients

\( R \) and \( G \) are the intensities after background subtraction of two different molecules in individual pixels within a ROI of two aligned images.

**Manders Overlap Coefficient**

\[
\text{MOC} = \frac{\sum (R \cdot G)}{\sqrt{\sum (R^2) \cdot \sum (G^2)}} \tag{1}
\]

All pixels within the ROI are conventionally included in calculating the MOC. We also examined a variant, \( \text{MOC}_{\text{cooc}} \), calculated using only pixels where both molecules are present.

**Pearson Correlation Coefficient** [16]

\[
\text{PCC} = \frac{\sum (R - R_{av}) \cdot (G - G_{av})}{\sqrt{\sum (R - R_{av})^2} \cdot \sum (G - G_{av})^2} \tag{2}
\]
$R_{av}$ and $G_{av}$ are the mean intensities of the two molecules. Only pixels in which both R and G exceed background are used.

**M1 & M2**

\[ M1 = \frac{\sum R_g}{\sum R} \]  \hspace{1cm} (3)

$R_g$ is the intensity above background of pixels where the G molecule is also present

\[ M2 = \frac{\sum G_r}{\sum G} \]  \hspace{1cm} (4)

$G_r$ is the intensity above background of pixels where the R molecule is also present.

M1 and M2 report the fraction of the total intensity of each molecule found in the presence of the other molecule.

**Area Fraction**

\[ Ar\% = 100 \times \frac{N_{rg}}{N} \]  \hspace{1cm} (5)

$N$ is the number of pixels in the ROI and $N_{rg}$ is the number where both molecules are above background. $Ar\%$ is the percentage by area of co-occurrence.

### 2.2 | Testing the MOC

We created an ImageJ macro (MOC-InteractiveScatterPlot.ijm) that calculates the MOC, its numerator and denominator, the PCC, M1 & M2 and the $Ar\%$ from data that can be interactively altered in a scatterplot (Supporting information). We also provide an Excel spreadsheet (MOC, PCC, M1 and M2) showing intermediate calculations (MOCColocSpreadsheet).

### 2.3 | Simulations

The generation and analysis of datasets used macros within the FIJI version of ImageJ [17]. Distributions, except the half Gaussian, were generated using a macro (MakeColocImagePair.ijm, Supporting information) calling the RandomJ plugin created by Erik Meijering. The half Gaussian distribution was originally a Gaussian distribution with a mean of zero, altered by using the absolute (sign-free) values, producing a skewed distribution.

Datasets, 32-bit floating point numbers with $500 \times 500 \times 3$ pixels, were generated with different frequency distributions; Gaussian, half Gaussian, uniform, exponential or gamma. Each dataset contained three images with the same distribution of intensities, two generated independently and the third the inverse of the first. The first two images were inherently uncorrelated (PCCs in the range $-0.003$ to $+0.003$), the first when compared to itself was perfectly positively correlated. The first and third images were negatively correlated, illustrated in scatter plots.

Initially all values in the distributions exceeded zero, with zero used as the background. The initially complete co-occurrence was then progressively reduced by setting an increasing fraction of the pixels in one or both images to zero.

Symmetrical distributions (uniform and Gaussian) were inverted using the ImageJ operation “invert”. Asymmetric distributions (gamma, exponential, half Gaussian) required a more complex inversion method: swapping the values of the most intense pixels with the mean value of the same number of pixels at the opposite end of the intensity range (MakeInverseNonSym.ijm). The inverted image retains the original distribution and mean. See Supporting information, Generating negatively correlated image pairs, for an extended description.

Measurements used ColocMOCMeasure.ijm, see Supporting information.

### 2.4 | Statistics

To examine the variability inherent in the simulations, eight paired datasets ($2 \times 500 \times 500$ pixels) each with a Gaussian distribution were run with an area fraction ranging from $100\%$ to $10\%$, producing 110 MOC measurements from each pair. The largest standard deviation was 0.18% of the mean ($n = 8$), justifying running the simulations once (Supporting information, Statistics). Measurements of the PCC showed similarly small variation as the number of pixels used in the calculation was reduced (Supporting information, Statistics).

### 2.5 | Data and software availability

The four macros and the Excel spreadsheet will be made available on github upon acceptance of this manuscript.

### 3 | RESULTS

#### 3.1 | The equation

The MOC resembles the PCC, but uses the raw intensity above background, without subtracting the mean, in the numerator and the denominator. An operational difference is that the MOC uses every pixel in the ROI while the PCC should be calculated using only pixels where both molecules are present [18, 19]. These two alterations create a profound difference.

The MOC’s numerator is the sum of the pixel-by-pixel product of the intensity of the two molecules. Since intensities cannot be negative, the numerator has a minimum value of zero, when one or both of the values in each pixel is zero (0-molecule or 1-molecule pixels). This measures co-occurrence since only 2-molecule pixels contribute to the numerator. But the numerator also depends on which intensities
are paired and hence reflects correlation, since pairing similarly ranked intensities produces a larger numerator than pairing high with low ranking intensities.

The MOC’s denominator is the maximum possible sum of the product of the pairs of intensities, but unlike the numerator, it is a global calculation, from the sum of the squared values for each molecule, rather than pixel-by-pixel. Dividing the numerator by the denominator restricts the MOC’s range to a maximum of one.

For a given population of intensities, rearranging which intensities are paired alters the numerator but leaves the denominator unchanged.

3.2 | Small dataset simulations

Using scatterplots with a small number of datapoints, we set out to examine the MOC when the underlying data is clear and can be incrementally altered. The PCC, a measure of correlation, plus M1, M2, and Ar%, three measures of co-occurrence, were also calculated. The ImageJ macro, MOC-InteractiveScatterPlot, is described in the Supporting information.

First, we examined which combinations produced a MOC of zero. The simplest is the absence of both molecules, with one or more 0-molecule pixels (Figure 1(A)), which might be expected to produce a MOC of zero, instead NaN (not a number) is returned. NaN appears when the denominator is zero, since dividing by zero is problematic. The only informative measurement is the zero returned by the Ar% (no co-occurrence by area).

Adding a 1-molecule pixel to the two 0-molecule pixels (Figure 1(B)) still produces NaNs, with the denominators for the MOC, PCC, and M2 at zero. However, the M1 coefficient now becomes calculable, the total intensity (the denominator), is no longer zero, producing zero rather than NaN. Inserting an additional 1-molecule pixel makes no difference (Figure 1(C)).

When both molecules are present though not together, the MOC is finally calculable (Figure 1(D)), since the denominator now exceeds zero although the numerator is still zero. The zero now correctly shows the absence of co-occurrence. M2 also changes from NaN to zero. The Ar% still correctly shows the absence of co-occurrence.

Discarding the 0-molecule pixels (Figure 1(E) compared to Figure 1(C) and Figure 1(F) compared to Figure 1(D)) does not affect the MOC (still NaN), but is reported by the Ar%. In other words, the MOC is unaffected by 0-molecule pixels.

A single 2-molecule pixel produces a MOC (Figure 1(G)), 1.0, regardless of the intensities, since the denominator must equal the numerator, hence 1.0. Adding a second 2-molecule pixel reduced the MOC (Figure 1(H)), despite the PCC becoming both measurable and maximal.

The PCC is similar to the MOC in the first five distributions (Figure 1(A)–(E)) in that neither is calculable, because their denominators are zero. The PCC remains incalculable (Figure 1(F) and (G)) failing in Figure 1(F), because its calculation should employ only 2-molecule pixels [18, 19], since 0-molecule, 1-molecule and 2-molecule pixels do not form part of the same distribution [5]. However, in Figure 1(G) the single pixel shows co-occurrence, but the PCC is still returns NaN, the mathematical explanation being that subtracting the mean from a population of one produces a denominator of zero. Hence, the minimum requirement for the PCC is a pair of 2-molecule pixels, which produces the highest possible correlation, one, (Figure 1(H)), or minus...
one (not shown). NaN is still possible, in the unlikely event that the intensity of one molecule in every pixel is identical since, as each equals the mean, the denominator would become zero.

3.3 | Positive correlations

As noted above, a single 2-molecule pixel produces a maximal MOC. Adding further 2-molecule pixels that are aligned and form a line passing through the origin maintains the maximal MOC and the PCC reports a perfect correlation (Figure 2(A)–(C)). Note, adding low intensity 2-molecule pixels has little effect on either the numerator or the denominator (Figure 2(B)), while adding high intensity 2-molecule pixels substantially increase both (Figure 2(C)). The addition of 0-molecule pixels has no effect on the MOC, leaving the denominator and numerator unchanged (Figure 2(D)).

Adding four 1-molecule pixels reduces the MOC by increasing the denominator but leaves the numerator unaltered (Figure 2(E)). Changing a 2-molecule pixel into two 1-molecule pixels that preserve the denominator, has little effect for a low intensity 2-molecule pixel (Figure 2(F)). By contrast altering a high intensity 2-molecule pixel markedly reduces the MOC, since the numerator falls while the denominator is unchanged (Figure 2(G)), an intensity weighted measure. Reducing the correlation reduces the MOC (Figure 2(H)).

In summary, the upper limit of the MOC requires complete co-occurrence and a perfectly positive correlation. However, a perfect positive correlation, measured by the PCC, is not always sufficient for a maximal value of the MOC (Figure 2G), which also requires that the datapoints are aligned with the origin.

3.4 | Negative correlations

It has been suggested that a perfect negative correlation should produce a MOC of zero [9]. Testing found that for one perfect negative correlation the MOC is just above its midpoint (0.54) (Figure 3(A)). Decreasing the co-occurrence by changing the two extreme 2-molecule pixels, while preserving the denominator, produces only a small reduction in the MOC (Figure 3(B)), whereas changing two midrange 2-molecule pixels reduces the MOC appreciably (Figure 3(C)). Reducing the co-occurrence by adding two 1-molecule pixels with a high value for one molecule reduces the MOC by increasing the denominator (Figure 3(D)). A similar addition of two midrange 1-molecule pixels (Figure 3(E)) produces a smaller increase in the denominator and no change in the numerator, hence a smaller decrease in the MOC than (Figure 3(D)).

Adding pixels that alter the correlation changes the MOC. A single 2-molecule pixel with high values for both molecules increases the MOC while making the PCC less negative (Figure 3(F)). A similar increase in the MOC follows the addition of four midrange 2-molecule pixels, but this addition only has a minor effect on the PCC, as the values are clustered around the mean (Figure 3(G)). The addition of one low value 2-molecule pixel increases the PCC, but has only a marginal effect on the MOC, since it does not appreciably alter either the denominator or the numerator of the MOC (Figure 3(H)). Analogously to the situation with a positive correlation, adding a pair of medium-intensity 1-molecule pixels to (D) produces a small increase in the denominator and no change in the numerator, hence a smaller decrease in the MOC than (Figure 3(D)).

![Figure 2](image-url) The MOC and positive correlations. A series of scatterplots show the effect of adding or moving a small number of pixels on the MOC, its numerator (num) and denominator (dnm), the PCC, M1 & M2 and the Ar%. The axes show zero, values below are treated as zero. (A) A perfect positive correlation going through the origin. Panels B-G also have a perfect positive correlation going through the origin. (B) Adding a pair of low-intensity 2-molecule pixels. (C) Adding a pair of high-intensity 2-molecule pixels to B. (D) Adding two 0-molecule pixels to C. (E) Adding four medium-intensity 1-molecule pixels to D. (F) Splitting a low-intensity 2-molecule pixel from C into two 1-molecule pixels. (G) Splitting a high-intensity 2-molecule pixel from C into two 1-molecule pixels. (H) Moving a low-intensity 2-molecule pixel and a medium-intensity 2-molecule pixel from C, lowers the correlation.
correlation, (Figure 1(D)), adding 0-molecule pixels to a negative correlation does not alter the MOC (not shown).

Overall negative correlations result in lower values for the MOC than positive correlations but the values are appreciably above zero.

### 3.5 Sensitivity to gain

Colocalization measurements would be incoherent if the relative gain or exposure time of each image had to be factored in. A reported virtue of the MOC is its insensitivity to changes in gain or to photobleaching [2], a much repeated claim. We have previously reported that both the MOC and the PCC are insensitive to changes in gain [3]. Now we demonstrate that also the M1, M2 and Ar% coefficients are insensitive to gain (Figure S1).

### 3.6 The interplay between correlation and co-occurrence

We next examined large populations with well-defined distributions. In images of biological specimens, the possible distribution of intensities covers a very broad range, ultimately depending on how, within a ROI, the molecules interact with different components that may be distributed inhomogenously or homogenously. Accordingly, we used a wide range of distributions, three degrees of correlation covering the widest possible range and progressively altered the co-occurrence.

Initially we chose a distribution (gamma order 5) with a midrange peak and skewed distribution favoring high intensities (Figure 4(A)). Scatterplots for three different correlations covering a wide range are shown (Figure 4(B)), as are images representative of the three different correlations (Figure 4(C)). The scatterplot for the negatively correlated pair is curved, and therefore does not have a PCC of \(-1\), because the gamma 5 distribution is asymmetric. However the correlation is perfectly negative when measured with the SRCC, a ranked version of the PCC [7, 18].

The MOC follows changes in both correlation and co-occurrence with a maximal value when co-occurrence is complete and accompanied by a perfect positive correlation going through the origin (Figure 4(D)). Switching to a negative correlation reduces the MOC by 30%. The changes in the numerator and denominator (Figure 4(E)) show the numerators falling linearly as the co-occurrence is progressively reduced, while the denominator, which is the same for all the correlations, falls nonlinearly, explaining the curvatures seen in Figure 4(D). Since the MOC is affected by both co-occurrence and correlation it is unable to differentiate between widely differing patterns of colocalization; for instance a midrange MOC, 0.5, is compatible with combinations from a correlation of 1.0 with a co-occurrence of 0.25 to correlation of \(-1.0\) with a co-occurrence of 0.58, making it impossible to differentiate between extremely different patterns of colocalization.

The changes in co-occurrence in Figure 4(D) and (E) were produced by altering only one of the two images (Red), the second (Green) occupying the whole image. To examine a greater variety of co-occurrence, the fraction of the area was changed in both images for positive and negative correlations (Figure 4(F) and (G)). They follow the pattern seen...
in Figure 4(D), but greatly increase the variety of co-occurrence and correlation combinations compatible with a MOC of 0.5.

**3.7 Different distributions**

Since intensity distributions vary widely in images of cells we examined additional distributions using the same distributions for each image (Figure 5). Overall, the six distributions show a similar pattern, the MOC depends on both co-occurrence and correlation, but the balance between the two varies dramatically. With a gamma order 1 distribution (Figure 5(A)) or an exponential distribution (Figure 5(B)) the MOC is reduced by 80% as the correlation switches from positive to negative and is much less influenced by co-occurrence, while with a Gaussian distribution (Figure 5(F)) the MOC drops by only 20% as the correlation is altered. When the different distributions are considered a MOC of 0.5 now covers an even wider range of combinations of co-occurrence and correlation.

The frequency distribution histograms explain the varied effects of correlation changes. In the negatively correlated Gamma order 1 distribution high values from one image are paired with low values from the second, producing a small numerator, but when the correlation becomes positive high–high and low–low pixels result, and the
numerator dramatically increases. At the other extreme, a distribution clustered around a tight peak like the Gaussian, the effect is smaller. Even though low–high pixels still become either high–high or low–low pixels, the change in the product of the intensities for the majority of the pixels is smaller than for a distribution with a wider spread like the Gamma order 1 population.

3.8 | Offset

We have previously demonstrated that the MOC is sensitive to offset for Gaussian distributions [3]. An extension of this investigation supported the initial conclusion (Figure S2). We additionally examined the effect of offset for gamma and uniform distributions. The effect was mixed; the MOC for positive correlations fell while for negative correlations even small offsets increased the MOC (Figure 6(A) and (B)). Overall, the MOC from datasets with different correlations converge with increasing offset, since the numerator increases linearly for the three correlations while the denominator increases nonlinearly (Figure 6(C) and (D)). Note after offsetting, a perfectly positive correlation no longer produces a maximal MOC.

3.9 | MOC_{cooc}

Conventionally the MOC is calculated from every pixel in the ROI including both background and foreground pixels, although some software (Zen from Zeiss and colocalization finder (imagejdocu.tudor.lu/plugin/analysis/colocalizationfinde)) allows the exclusion of low intensities. We examined whether calculating the MOC on the same basis as the PCC, that is, only using 2-molecule pixels, would produce a better measure of correlation, since excluding the 0-molecule and 1-molecule pixels eliminates the influence of co-occurrence. The MOC_{cooc} measurements are the right hand set of datapoints, those with complete co-occurrence, in Figures 4(C) and 5, and they remain unchanged in our simulations as the area of co-occurrence is reduced and the MOC_{cooc} is based on a progressively smaller subset of the two images. All measurements for the highest negative correlations

FIGURE 5 The MOC and the interplay between correlation and co-occurrence for different distributions. The MOC and six different distributions; (A) Gamma order 1, (B) Exponential, (C) Half Gaussian, (D) Gamma order 3, (E) Uniform and (F) Gaussian. The histogram at the top left of each panel shows the distributions. The co-occurrence was incrementally altered for three different correlations: (i) perfectly positive (Pos), (ii) uncorrelated (Un) and (iii) strongly negative (Neg), by setting a differing fraction of the pixels of one image to zero, while in the second image all pixels are above zero and unchanged. In this instance, the numerical value of the co-occurrence is the same as the nonzero area of the red image, the Ar% and M2.
are appreciably above zero, with three of the six distributions shown in Figure 5 exceeding the MOC’s midpoint. A MOC of zero thus cannot be equated with a perfectly negative correlation.

4 | DISCUSSION

Despite being created over 27 years ago, a consensus has not emerged about the interpretation of the MOC, instead there is increasing disagreement. In seeking to unequivocally establish the MOC’s role, we examined each proposal by testing the MOC’s response to controlled changes with well-defined datasets.

There are currently three incompatible interpretations (i) the MOC is an alternative to the PCC as a measure of correlation [9], (ii) the MOC is a valuable measure of co-occurrence [6, 20] or (iii) the MOC merges co-occurrence and correlation into a confusing single measure, our position [3, 21]. Depending on which interpretation you accept, a change in the MOC has substantially different biological meanings.

The backdrop is our proposal that patterns of colocalization are efficiently described by measuring both the co-occurrence and the correlation, the two being intrinsically unrelated, with each reporting a distinct and informative feature of colocalization. Taken together co-occurrence and correlation locate a particular pattern of colocalization within the wide range of possible patterns.

Testing by making incremental changes in correlation and co-occurrence demonstrates that both affect the MOC. The degree of co-occurrence is always influential while the effect of changes in correlation is strongly influenced by the intensity distributions. Changes in correlation within distributions with a broad spread of intensities have a major impact on the MOC, while for tight distributions with a small spread and a large offset, more biologically unlikely, the effect is reduced.

The correlation interpretation of the MOC is clear, essentially the PCC but with a compressed and offset numerical range without negative values [9]. However, its proponents did express some legitimate skepticism about the utility of the MOC’s exclusively positive numerical range for reporting correlation when, with their interpretation, values below 0.5 flag negative correlations. Remarkably, these authors had previously presented the MOC almost as a measure of co-occurrence “the former (zero) corresponding to nonoverlapping images”, quite correct, while, “the later (one) reflecting 100% colocalization between both images” [22]. Our current simulations, previous work and the equation itself shows that a zero value only occurs when the two molecules never co-occur. This is quite different from a perfect negative correlation where high values for one would be associated with low values for the second and vice versa, together with an intermediate population with more similar values [5]. We have previously demonstrated that populations with perfect negative correlations can have values at the high end of the MOC’s range [3, 13] and here demonstrate that even a perfect positive correlation plus complete co-occurrence does not always produce a maximal MOC.

The case for the MOC as an effective measure of correlation is weak; the proponents do not include or cite any supporting material, leaving an argument from authority [9]. Our simulations demonstrate

![FIGURE 6 The MOC and offset. (A) A gamma order 1 distribution and (B) a uniform distribution, with the change in the MOC shown for three different correlations; perfectly positive (Pos), uncorrelated (Un) and strongly negative (Neg) and complete co-occurrence. One image of the pair was progressively offset while the other was unchanged. Histograms showing each distribution and the maximal offset are inserted. (C and D) Changes in the numerators and denominators corresponding to (A) and (B). Denominators for the three correlations are almost identical (Table S1) and the y-axis shows the values of the numerator and denominator on a linear scale.](image-url)
that while the MOC is affected by the degree of correlation, the changes in correlation account for a highly variable fraction of the measurement. In addition, the MOC’s numerical range from a perfect positive to the largest negative correlation varies widely for different distributions. In an attempt to improve the MOC we restricted the calculation to pixels in which both molecules are present, a necessity for the PCC since 0-molecule, 1-molecule and 2-molecule pixels are not part of the same distribution [5, 23]. The MOC is a better measure of correlation than the MOC, but negatively correlated datasets still have high and therefore quite misleading values and never approach zero. The PCC and our preference, the SRCC, should be used for correlation [18, 24].

The case for the MOC responding to changes in co-occurrence is stronger. In 2010, we described the MOC as a highly (intensity) weighted measure of co-occurrence with the caveat that it is also affected by correlation and therefore unsuitable for measurements of colocalization either by correlation or co-occurrence [3]. Dunn et al. later noted that the MOC only indirectly and somewhat unpredictably measures co-occurrence [13]. Despite these major reservations, it was asserted in a recent review on colocalization that the MOC is nonetheless a valuable measure of co-occurrence [6]. The authors devoted considerable attention to the MOC, largely reproducing our findings, but without commenting on our very different conclusion, despite familiarity with our 2010-study [3]. Their review featured two cells with very different scatterplots, widely differing co-occurrence, shown by the M1 and M2 pair, and widely differing correlation. However, the MOC reported that the colocalization in the two cells was almost identical. The authors noticed this difficulty, but instead of concluding that the big difference in correlation balanced the big difference in co-occurrence to create almost identical MOC-values, they argued that the MOC’s failure to correctly report the widely differing co-occurrence could be remedied if the M1 and M2 coefficients, accepted measures of co-occurrence were also considered. So the MOC, as a measure of co-occurrence, additionally requires that two other measures of co-occurrence are made and somehow factored in. Reporting co-occurrence with the M1 and M2 pair plus the Ar% has the virtues of simplicity and clarity.

We responded by presenting a dataset demonstrating that the MOC is affected by incremental changes in both correlation and co-occurrence, directly challenging the MOC’s utility as a measure co-occurrence [21]. Aaron et al. simply ignored this data and instead decided that we disagreed over an uncontroversial assertion, that “there is no one superior colocalization coefficient” [20]. In support, they offered two demonstrations showing that the MOC responds to changes in co-occurrence while the PCC does not, concluding “we cannot accept the claim of Adler and Parmryd that PCC is simply superior under all conditions”—rebuking a nonexistent claim. Interestingly, when the images used in their second demonstration were first published, the original authors did not use the MOC to measure co-occurrence [25]. Aaron et al’s observation, that PCC is unaffected by changes in co-occurrence while the MOC responds, is not surprising, since the PCC only measures correlation, which in both their demonstrations was constant.

Meanwhile, the actual disagreement with Aaron et al. centers on the MOC’s, response to changes in correlation. If the MOC is appreciably affected by changes in correlation it cannot reliably and consistently measure co-occurrence. Aaron et al., despite conducting an otherwise plausible range of tests on the MOC, omitted this most relevant test and, when presented with our results, ignored them [20, 21]. The MOC is unarguably affected by changes in correlation and the effect is variable but never minor.

The problem with the MOC is interpretation—what in terms of colocalization do the numbers mean. There is no straightforward interpretation of the MOC since, as we demonstrate, a given value can arise from widely differing combinations of co-occurrence, correlation and distribution. Declaring that the colocalization for any MOC-value is biologically equivalent is therefore misleading.

Once it acknowledged that the MOC combines co-occurrence and correlation, a potentially new role suggests itself, as a summary measure of colocalization, like body mass index (BMI) [20], BMI is functionally a ratiometric combination of mass and height. It works because human weight and height are strongly correlated and therefore outliers flag departures from the normal range. However, co-occurrence and correlation have no inherent relationship and the MOC does not combine them in an intelligible way.

To describe colocalization, we have consistently recommended reporting both co-occurrence and correlation. Each is a clearly defined and understandable measure of the relative distribution of a pair of molecules. Together the two complimentary measures place each pattern of distribution within the huge range of possible distributions. Clearly two measures do not provide a complete description and, depending on the biological question, further measurements may be relevant [26], for instance how the areas of co-occurrence are distributed and do their shape and size vary.

Overall, the MOC is demonstrably a hybrid coefficient and the two alternative interpretations of the MOC are untenable. Considering the evidence presented in this study and after examining the evidence behind alternative interpretations, the only change to our original assessment that the MOC is not suitable for making measurements of colocalization either by correlation or co-occurrence is to add: or as a summary measurement. The MOC lacks a clearcut role in quantifying colocalization and has caused considerable confusion.

ACKNOWLEDGMENT
Funding was provided by The Swedish Research Council (2015-04764).

AUTHOR CONTRIBUTIONS
Jeremy Adler: Conceptualization; formal analysis; methodology; project administration; writing-original draft; writing-review & editing. Ingela Parmryd: Conceptualization; funding acquisition; methodology; project administration; writing-review & editing.

CONFLICT OF INTEREST
The authors declare no conflict of interest.
REFERENCES

1. Adler J, Parmryd I. Recent review on colocalization seem to misunderstand the Pearson correlation coefficient. J Microsc. 2007;227:83 author reply 84-5.
2. Manders E, Verbeeck FJ, Aten JA. Measurement of co-localisation of objects in dual-colour confocal images. J Microsc. 1993;169:375–82.
3. Adler J, Parmryd I. Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Manders overlap coefficient. Cytometry A. 2010;77:733–42.
4. Herce HD, Casas-Delucchi CS, Cardoso MC. New image colocalization coefficient for fluorescence microscopy to quantify (bio-)molecular interactions. J Microsc. 2013;249:184–94.
5. Adler J, Parmryd I. Quantifying colocalization: thresholding, void voxels and the H(coef). PLoS One. 2014;9:e111983.
6. Aaron JS, Taylor AB, Chew TL. Image co-localization—co-occurrence versus correlation. J Cell Sci. 2018;131:jcs211847.
7. Spearman C. The proof and measurement between the association of two things. Am J Psychol. 1904;15:72–101.
8. Kendall M. A new measure of rank correlation. Biometrika. 1938;30:81–9.
9. Cordelieres FP, Bolte S. Experimenters’ guide to colocalization studies: finding a way through indicators and quantifiers, in practice. Methods Cell Biol. 2014;123:395–408.
10. Zinchuk V, Zinchuk O. Quantitative colocalization analysis of confocal fluorescence microscopy images. Curr Protoc Cell Biol. 2008;Chapter 4:Unit 4:19.
11. Adler J, Parmryd I. Colocalization analysis in fluorescence microscopy. Methods Mol Biol. 2013;931:97–109.
12. Zinchuk V, Grossenbacher-Zinchuk O. Recent advances in quantitative colocalization analysis: focus on neuroscience. Prog Histochem Cytobiol. 2009;44:125–72.
13. Dunn KW, Kamocka MM, McDonald JH. A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol. 2011;300:C723–42.
14. Ahmed M, Lai TH, Kim DR. colocr: an R package for conducting colocalization analysis on fluorescence microscopy images. PeerJ. 2019;7:e7255.
15. Sauvat A, Leduc M, Muller K, Kepp O, Kroemer G. ColocalizR: an open-source application for cell-based high-throughput colocalization analysis. Comput Biol Med. 2019;107:227–34.
16. Pearson K. Notes on regression and inheritance in the case of two parents. Proc R Soc London. 1895;58:240–2.
17. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9:676–82.
18. Adler J, Pagakis SN, Parmryd I. Replicate-based noise corrected correlation for accurate measurements of colocalization. J Microsc. 2008;230:121–33.
19. Barlow AL, Macleod A, Noppen S, Sanderson J, Guerin CJ. Colocalization analysis in fluorescence micrographs: verification of a more accurate calculation of pearson’s correlation coefficient. Microsc Microanal. 2010;16:710–24.
20. Aaron JS, Taylor AB, Chew TL. The Pearson’s correlation coefficient is not a universally superior colocalization metric. Response to ‘quantifying colocalization: the MOC is a hybrid coefficient—an uninformative mix of co-occurrence and correlation. J Cell Sci. 2019;132:jcs227074.
21. Adler J, Parmryd I. Quantifying colocalization: the MOC is a hybrid coefficient—an uninformative mix of co-occurrence and correlation. J Cell Sci. 2019;132:jcs222455.
22. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc. 2006;224:213–32.
23. A primer on TCR signaling. Nat Immunol. 2014;15:789.
24. French AP, Mills S, Swarup R, Bennett MJ, Pridmore TP. Colocalization of fluorescent markers in confocal microscope images of plant cells. Nat Protoc. 2008;3:619–28.
25. McArthur K, Whitehead LW, Heddleston JM, Li L, Padman BS, Oorschot V, et al. BAK/BAX macropores facilitate mitochondrial herniation and mtDNA efflux during apoptosis. Science. 2018;359:eaao6047.
26. Penarrubia PG, Ruiz FX, Galvez J. Quantitative analysis of the factors that affect the determination of colocalization coefficients in dual-color confocal images. IEEE Trans Image Process. 2005;14:1151–8.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Adler J, Parmryd I. Quantifying colocalization: The case for discarding the Manders overlap coefficient. Cytometry. 2021;1–11. https://doi.org/10.1002/cyto.a.24336