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Perceptually Accurate Display of Two Grayscale Images as a Single Color Image

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Abstract

Life scientists often desire to display the signal from two different molecular probes as a single color image, so as to convey information about the probes relative concentrations as well as their spatial co-relationship. Traditionally, such color images are created through a merge display, where each grayscale signal is assigned to different channels of an RGB color image. However, human perception of color and grayscale intensity is not equivalent. Thus, a merged image display conveys to the typical viewer only a subset of the absolute and relative intensity information present in and between two grayscale images. The Commission Internationale de l'Eclairage L*a*b* color space (CIELAB) has been designed to specify colors according to the perceptually defined quantities of hue (perceived color) and luminosity (perceived brightness). Here, we use the CIELAB color space to encode two dimensions of information about two grayscale images within these two perceptual dimensions of a single color image. We term our method a Perceptually Uniform Projection (PUP) display and show using biological image examples how these displays convey more information about two grayscale signals than comparable RGB color space based techniques.

Lay Abstract—It is often useful to display the fluorescent signal from two different molecular probes as a single color image so that information about the probes' relative concentrations as well as their spatial co-relationship can be visualized. A common way to accomplish this effect is to assign each grayscale signal to a different channel of an RGB color image. However, this method fails to convey much of the information about the two grayscale images, because humans' perception of color and monochrome values is not equivalent. We have used the Commission Internationale de l'Eclairage L*a*b* color space (CIELAB) to display two grayscale images as a single color image. Our method encodes two dimensions information about the grayscale images as two, perceptually independent and uniform quantities - hue and luminosity - in the color image. Our technique, which we termed a perceptually uniform projection (PUP) display, conveys more information about two grayscale signals than comparable RGB color space based techniques. We

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Contributorship

A.T. conceived the PUP displays and wrote the paper. M.I., T.W., and K.H. prepared samples and provided images. T.C. provided discussion and suggested revisions and improvements. All authors have approved the final version.

apply this method to display several types of information commonly encountered in cell biology images.

Keywords

Merge display; color space; ratiometric display; fluorescence micrographs

Introduction

In fluorescence microscopy and other imaging modalities, it is often desirable to display as much information as possible about the signal from two different molecular probes within a single color image, so as to convey information about their relative concentrations and spatial co-relationship. A 'merge' image display is often used to visualize these features. In a conventional merge image display, the intensity values in each grayscale image are assigned to one (or two) of the three independent components ('channels') of the RGB (red, green, blue) color space, resulting in a single, color image. Using this method, the signals maintain a pure color at locations where they are present alone, while at locations where the signals overlap, their independent colors mix, producing a broad range of other colors. Such a merge image display is mathematically accurate, because at each pixel location, a pseudo-color value represents the pair of grayscale intensities present in the input signals (e.g. $R = \text{img1.int}$, $G = \text{img2.int}$, $B = 0$). However, color image displays are ultimately intended for human visual perception, and herein lies the problem: numerically equivalent pseudo-color and grayscale values are not perceptually equivalent.

As displayed on a standard computer monitor (using the sRGB color space), the most intense green (0,255,0) is perceived to be about 2x as bright as the most intense red (255,0,0), and 3x as bright as the most intense blue (0,0,255) (ITU-R, 2015). Thus, grayscale signals assigned to each of these color channels are not equally perceived in the color display. Mixed colors present similar issues: For example, a 2:1 red-to-green intensity ratio ('reddish orange') is perceived as less bright than a 2:1 green-to-red intensity ratio ('greenish yellow'), even though numerically the ratio is the same.

These perceptual consequences of RGB color space merge image displays may be either an advantage or a disadvantage depending on the application. When it is desirable for the viewer to mainly perceive only one of the two signals, a merge image can directly accomplish this effect by assigning the 'signal of interest' to the high brightness, green channel, and the 'less important signal' to the low brightness red (or blue) channel (for example, a protein of interest against a counter-stain that merely provides context). In other cases, it is desirable for the signal from both probes to be equally perceptible and to convey as much information as possible about how the intensities within and between the grayscale images are related. In these situations, a merge image display is lacking at the perceptual level, since the information within (or between) each grayscale image is not equally perceptible. One way to mitigate this loss at the perceptual level is to display each grayscale image separately, alongside the color merge display (North, 2006). However, this approach is unfortunately not ideal: The spatial co-relationships between the signals are not apparent

in the separate, grayscale images, while the relative intensities of the signals are not apparent in the merged image display.

In order to better convey in a single color image two dimensions of information about two grayscale images, it is necessary to understand, and account for, a typical human's visual perception. Visible light can be measured using two parallel systems: Photometry measures light in terms of its perceptual impact, while radiometry measures light in terms of its physical energy (Stimson, 1974). An RGB-color-space-based merge image display is radiometrically accurate but not photometrically accurate. Alternatively, the Commission Internationale de l'Eclairage $L^*a^*b^*$ (CIELAB) color space has been designed based on psychophysical experiments to quantify colors and brightness in a way that is photometrically accurate. The CIE system uses a quantity termed hue to represent perceived color and a quantity termed luminosity to represent perceived brightness. (Hue is symbolized by a pair of coordinates A,B, while luminosity is symbolized by a coordinate L). Both hue and luminosity are perceptually uniform, meaning that each level differs from its neighbors by an equivalent perceptual degree, and also perceptually independent, meaning that changing one quantity has no influence on the other (Schanda & János, 2007).

Because the CIELAB color space permits encoding two dimensions of information within two, independent and uniform perceptual quantities, a CIELAB-based color display necessarily conveys to a viewer more and more accurate information about two grayscale images than any RGB-color-space-based display (where the R, G, and B values are not perceptually uniform or independent as mentioned above).

Although perceptual impressions are never a substitute for quantification, and statistically significant but subtle effects can be imperceptible, many areas of biology remain exploratory, relying on human perception to recognize unexpected outcomes. Only after an outcome is suspected can the relevant quantitative tools be applied to verify the putatively perceived result. By conveying more and more accurate information about two grayscale images within a single color image, CIELAB-based displays increase the chance that an unexpected outcome will be recognized. Thus, perceptually accurate image display is an important component of the discovery process.

Here, we use hue and luminosity in the CIELAB color space to encode two dimensions of information about two grayscale images within a single color image. Our methodology encodes the ratio of the pixel intensities as hue and then uses the luminosity to display some other dimension of information about the images. Although the luminosity could be used to encode any information, here we use luminosity to encode representative quantities (information) commonly encountered in cell biology: (i) a Merge display, where for a given pixel in the color image the luminosity represents the brightest intensity in either grayscale image; (ii) a Ratio display, where the luminosity is a function of only the intensity in the denominator image; and (iii) a Colocalization display, where the luminosity is a function of each intensity pair's perpendicular distance from $y=x$. We term these displays Perceptually Uniform Projection (PUP) displays, due to the formulas that describe the luminosity functions. To demonstrate the utility of PUP displays for cell biology applications, we then

apply each of them to cell biology images. An implementation of the method is provided in the supplementary material as an ImageJ plugin.

Selection of Perceptually Uniform Hues

RGB color space displays, such conventional merge image displays, employ some subset of the colors shown in Figure 1A. Unfortunately, as shown in the lower panel of Figure 1A, these colors have a wide-range of different luminosities (perceived brightness (Schanda & János, 2007)), demonstrating that color and luminosity are not independent in the RGB color space. Furthermore, these colors are not perceptually uniform, as suggested by the (perceptually) broad swath of green compared to red or blue.

Our method works with the CIELAB color space, where hue (A,B) and luminosity (L) are perceptually (and mathematically) independent and uniform. Using the color conversion tools of the Image J Plugins Toolkit (Sacha, 2014), we selected for use 33 CIELAB hues that are perceptually uniform (each separated from their neighbor by an equivalent distance in the A,B plane). These hues are shown in the upper panel of Figure 1B and span 78% of the visible spectrum, while a traditional merge image display (based on mixing primary colors) spans 33% of the spectrum. As shown in the lower panels of Figure 1B, these hues' luminosity is constant (and thus independent).

Although each hue interval is uniform, notice that five general color classes are distinguishable from left to right: magenta RGB=(213,0,246), red (250,0,20), yellowish (156,119,0), green (0,150,5), and azure (0,124,239). (The color words used here are merely descriptive). These five color classes can be mapped conveniently onto the five classes of pixels present in a merge image display: Image 1 (Img1) signal alone: azure (a greenish color), Image 1 signal predominates: green, Image 1 and Image 2 (Img2) signals equal: yellowish, Image 2 signal predominates: red, and Image 2 alone: magenta (a reddish color).

Creation of Perceptually Uniform Projection Displays

In a conventional merge image display, all pixel intensity combinations between two grayscale images can be represented on a two-dimensional Cartesian graph, where each axis represents the intensity of each grayscale signal at a given pixel location in the images. To define a merge display, each grayscale intensity is then assigned to one (or two) channels of an RGB image. Since there are two images, but three RGB channels, this assignment can be performed in several ways: First, each image can be assigned to a single channel (e.g. a red-green display as shown in Figure 2A). Second, one image can be assigned to a single channel and the other to the remaining two channels (e.g. a green-magenta display). Third, each image can be assigned to a single channel, and the third channel is set based on the sum of the intensities of the other two channels (e.g. a cyan-yellow display as shown in Figure 2B).

In contrast, the PUP display technique uses a polar coordinate system to map pixel intensity combinations onto the set of perceptually uniform hues and independent luminosities described above. When polar coordinates are used, the angle (θ) corresponds to the ratio of

the signals' intensities at a given image location, while the length of the radius (r) corresponds to their distance from the origin.

For all PUP displays, we map each angle (θ) onto a perceptually uniform hue, similar to how a traditional merged display implicitly uses distinct colors to represent the ratio of the intensities in each image. The luminosity coordinate is then available to encode another dimension of information about the relationship between the pixel intensities in the two grayscale images. The ability to encode a second dimension of information within a second, perceptually independent quantity is a unique and useful feature of the PUP display.

The additional information encoded within the luminosity depends on the use case. Here, we consider cases where the desired information pertains to the relationship between the intensities of corresponding pixels in the grayscale images. As representative examples, we next use three different luminosity functions to define three different types of PUP displays:

- 1 The PUP Merge display: In an ideal merge image, both of the grayscale signals should be treated equally so that the contrast in the final color image is not dominated by one signal or the other. First, at pixel locations where one of the signals is absent (has zero intensity), the luminosity of the other signal can be set proportional to its luminosity in the grayscale image. Within a polar representation, this effect is achieved by setting the luminosity of the signal proportional to the length of the radius (r) when $\theta = 0^\circ$ or 90° . Second, in pixels where both signals are present ($0^\circ < \theta < 90^\circ$), it is not possible to represent both intensities by a single luminosity value. In these cases we have chosen to set the luminosity proportional to the maximum intensity between the grayscale images. (The maximum intensity is found by calculating the projection of the radius onto each intensity axis and then picking the largest value). These choices ensure that the overall intensity distribution ('contrast') of the grayscale images is preserved and equally represented in the color image.

The color 2D-LUTs produced by the PUP Merge display are shown in the first panels of Figure 2C and 2D, when mapping θ onto either a broad or narrow range of uniform hues, respectively. A broader range of hues can be beneficial for distinguishing small, relative intensity differences between two signals (Figure 2C). However, when displaying features comprised of large groups of pixels, we found that using a broad range of hues can result in a single feature being displayed in two (or more) very different colors. As a result, the feature became camouflaged, i.e. it was perceived as multiple features, each of a different hue. In cases where this effect should be avoided, a narrower subset of hues can be used, spanning from orange (255,72,0) to teal (0,163,130), and covering 42% of the visible spectrum (see Figure 2D). While this spectral range is about the same as that used in a conventional merge display, the CIELAB colors still have the advantages of being perceptually uniform and of independent of luminosity.

To prove that our method is working as designed, we then quantified the luminosity profiles across the PUP Merge 2D-LUTs. The upper graphs in Figure 2C and 2D show that the luminosity along the main diagonal is a linear function of the brightest signal in either grayscale image. The lower graphs show that the luminosity of either signal is equal when

the signals are alone, and then changes in proportion to the maximum intensity of either signal where the signals overlap. Thus, the PUP Merge display creates color images that are ideal merge displays and, we recommend using it in all cases where a conventional merge image display would usually be used, when the intent is to display both signals in an equivalent way.

For comparison, the left panels of Figure 2A and 2B illustrate the two-dimensional color look-up tables (2D-LUTs) that result from red-green and cyan-yellow conventional merge image displays. The luminosity profiles of these look-up tables are quantified in the graphs to the right. The upper graphs plot the luminosity along the main ($y=x$) diagonal of each 2D-LUT, while the lower graphs plot the luminosity along the perpendicular ($y=-x+255$) diagonal. The graphs in Figure 2A for the red-green 2D-LUT show that although the luminosity along the main diagonal is ~linear (upper graph), the luminosity along the perpendicular diagonal is not symmetric, indicating that the signal assigned to the red channel is perceived as much less (55%) bright than its green counterpart, both when the signal is alone as well as when an equal or lessor amount of the signal displayed as green is also present. The graphs in Figure 2B show that the cyan-yellow 2D-LUT has the converse problem: The lower graph is ~symmetrical, indicating that cyan and yellow are ~equally bright when they are alone, but when both are together the perceived brightness does not correspond to either component in a linear way: The luminosity is essentially saturated for intensity combinations falling to the right of the perpendicular diagonal. Such perceptual saturation is as damaging to data interpretation as sensor saturation. Thus, neither of these displays create ideal merge images. The PUP Merge display is further quantitatively compared to traditional merge image displays in Figures 3 & 4 below.

- 2 The PUP Ratio display: In its simplest form, a ratio display shows the quotient of corresponding pixel intensities between two images. However, the raw quotient may be biologically meaningless when the magnitude of the denominator is very small (and so could be due mostly to noise or non-specific staining). A better ratio display should convey both the quotient as well as the absolute intensity of the denominator. To display both of these dimensions of information with a single color image, as for a PUP Merge display, the value of the quotient is encoded as hue. We then set the luminosity proportional to the absolute intensity of the denominator image (by calculating the projection of the radius onto the axis corresponding to the denominator). The resulting 2D-LUT using a broad range of hues is shown in Figure 2E. (The order of these hues was chosen to maximize the total luminosity in the final display). The accompanying graphs show that luminosity is a linear function of intensity when both signals are together (upper) and decreases in proportion to the intensity in the denominator (lower). The PUP Ratio display is quantitatively compared to representative examples of existing 1D- and 2D-LUT ratiometric displays in Figure 5 below.
- 3 The PUP Colocalization display: When visualizing (linear) colocalization, it can be useful to highlight those pixels whose intensity combinations fall near the line $y=x$, while for off-diagonal combinations it is useful to know which signal

predominates. A conventional red-green merge display assigns colors according to the amount of each signal present in a pixel so shades of yellow indicate pixel intensity combinations that fall along the main diagonal ($y=x$) in a scatter plot. However, these yellows are difficult to distinguish from colors that represent non-colocalized intensity combinations (falling off the main diagonal) and each yellow's luminosity varies, depending on where it falls along $y=x$. The PUP Colocalization display produces a color image where pixel intensity combinations near the main diagonal have a unique color (yellow) and a constant, strong luminosity. As always, the PUP Colocalization display uses hue to indicate the signals' relative intensities. We have used a broad range of hues in this case, to increase discrimination of small differences in relative intensity between the signals. Luminosity is then set according to a function of each intensity combination's perpendicular distance from the $y=x$ diagonal. (Here, we have set the luminosity according to a logistic function of this distance, though other choices are possible). The resulting PUP Colocalization 2D-LUT is shown in Figure 2F. The upper luminosity graph shows that all intensity combinations falling along the main diagonal are assigned the same, high luminosity, while the lower plot shows how the luminosity decreases as a logistic function of the distance from $y=x$. The PUP Colocalization display is further compared to other colocalization displays in Figure 6.

Beyond the displays just considered, other biologically relevant quantities in or between two images could also be encoded as luminosity in the color image. For example, it may be useful in some cases to set the luminosity according to the intensity values in a third image or other 2D matrix of numbers. We do not consider additional cases here, but our code's luminosity mapping function can be easily modified to facilitate novel uses.

Application of the PUP Merge display to cell biology images

Cell biology signals commonly consist of numerous, small ($<1\text{ }\mu\text{m}$) objects (organelles, vesicles, synapses, etc.), where the experimental goal is to visualize the spatial relationship and relative levels between the two signals. As an example of this application, Figure 3A shows grayscale images of vesicle associated membrane protein 2 (VAMP2) and adaptor protein 2 (AP2) in a primary neuron (Wang & Tang, 2006). Grayscale 1D-LUTs are the most informative way to display single channel images, (since white is the most luminous mixture of colors), so we used these images as a basis for comparison to quantify how assigning other colors to each signal varies their perceived luminosity. Measuring the total luminosity of each grayscale signal shows that the total VAMP2 signal is 1.19x more luminous overall than the AP2 signal. When these signals are displayed as green and red respectively, (Figure 3B) the total VAMP2 signal becomes 2.8x more luminous, showing that a major perceptual imbalance now exists between the signals. When the grayscale signals are displayed as cyan and yellow (Figure 3C) the total VAMP2 signal is 1.30x brighter than the AP2 signal, a good match to the grayscale displays. When the grayscale signals are displayed using either the narrow range (Figure 3D) or broad range (Figure 3E) PUP display, the total VAMP2 signal is 1.21x more luminous than the AP2, a nearly perfect match to the relationship between the grayscale signals. Although we do not recommend displaying single channel images in

color, these results give some indication of how the signals will be impacted when both are displayed together within a single color image.

Figure 3F–3I shows the result when the individual signals in Figure 3A are merged together into a single color image, according to 2D-LUTs shown previously in Figure 2. Figure 3F is displayed using the red-green 2D-LUT shown in Figure 2A. Figure 3G is displayed using the cyan-yellow 2D-LUT shown in Figure 2B. Figure 3H is displayed using the narrow range PUP 2D-LUT shown in Figure 2D. Figure 3I is displayed using the broad range PUP 2D-LUT shown in Figure 2C. Figure 3J–3M shows insets from regions of the merge displays (boxed) where the signals are overlapping. Each color inset is also paired with a gray scale image that is the maximum intensity projection of the grayscale signals.

Most visually evident in the conventional merge color images is that both perception of contrast (vesicles appear saturated with poorly defined boundaries) as well as relative levels (compare vesicles marked with asterisks across techniques) are lacking in the yellow-cyan merge (Figure 3G & 3K). These effects are due to the perceptual saturation of bright yellows, cyans, and whites, as measured in Figure 2B. The other three displays appear to have a similar contrast that roughly follows the signals' maximum intensity projection (but see Figure 4). Relative signal levels are most evident in the broad range PUP merge display (compare vesicles marked with asterisks across techniques), as expected since a larger range of colors is used.

Taken together, the red-green display causes the signal displayed as red to be less perceptible than its green counterpart, when the signals are alone, although the performance is reasonable, where the signals are overlapping. (A green-magenta merge is perceptually similar to the green-red merge, although magenta is ~20% brighter than red – not shown). The cyan-yellow display is bright and displays each signal in a perceptually similar way when the signals are alone, but it fails to preserve contrast or convey relative levels when the signals are together. Only the PUP Merge displays exactly preserve the relative brightness of the signals when they are alone as well as when they are together (Figure 3D–3E, 3H–3I, 3L–3M). The PUP Merge display also conveys slightly better (narrow range hues) or significantly better (broad range hues) the relative levels of each signal where they are overlapping.

An additional advantage of the PUP Merge display is that, since it treats both grayscale signals in a perceptually equivalent way, the order in which the signals are assigned to colors has no perceptual impact. This parity is not true of conventional merge displays. The upper panels of Figure 4A and 4B both use the red-green 2D-LUT described in Figure 2A, except that Figure 4A assigns the VAMP2 signal to green and the AP2 signal to red, while in Figure 4B this assignment is reversed. The images look very different even though the same signals and same colors have been used. The upper panels of Figure 4C and 4D both use the narrow range PUP 2D-LUT as described in Figure 2D. In this case, the appearance of the displays is the same in both cases, due to the fact that hue and luminosity are independent in a PUP display. The lower panels confirm this effect based on the luminosity of each merge display (all images shown using the same luminosity scale).

To quantify how and where the luminosities differ on a pixel-by-pixel basis, Figure 4E and Figure 4F show the difference in luminosity between each pair of either the green-red or narrow range PUP merge displays, respectively. Indeed, the luminosity between the green-red displays can vary by up to $\pm 30\%$ on a pixel by pixel basis, while the luminosity profile between the PUP Merge displays is identical (there is no difference).

Application of the PUP Ratio display to cell biology images

Another common reason to display two grayscale images as a single color image is to illustrate the concentration of one protein relative to another, but only where the denominator signal is present, termed a ratio display (Chew *et al.*, 2002). (Unlike a merge display, which attempts to display both signals in an equivalent way). Figure 5A shows two grayscale images of a Hela cell expressing EGFP-Actinin (the denominator) and mCherry-Paxillin (the numerator). In a conventional ratiometric display, corresponding pixel intensities in each image are divided, and only the value of the quotient is shown, often using a 1D-, pseudo-color LUT, such as the ImageJ 'Jet' LUT illustrated in Figure 5B. In the Jet LUT, green indicates a ratio of 1, while red and blue indicate large and small ratios, respectively. However, this display method provides no information about the absolute value of the denominator, which is also important, since a large quotient could be meaningless if the magnitude of the denominator is small (and thus possibly contaminated with noise or due to non-specific staining). Asterisks in Figure 5B indicate regions where the ratio is large, because the denominator is very small. In order to also display the absolute value of the denominator (a second dimension of information), a ratio display based on a 2D-LUT termed Intensity Modulated Display (IMD) was previously developed (Hinman & Sammak, 1998). IMD's 2D-LUT is based on the hue-saturation-brightness (HSB) color space (Joblove & Greenberg, 1978). IMD encodes the quotient as a color (H) and then sets the brightness (B) according to the absolute intensity of the denominator. Large ratios are shown as a shade of red, ratios near 1 as a shade of yellow, and small ratios as cyan. While mathematically accurate, as shown in Figure 5C, IMD fails to perceptually recapitulate the intensity profile of the grayscale denominator image because, like the RGB color space, the H and B in the HSB color space are not perceptually independent or uniform. Thus, as shown in Figure 5E, significant differences (up to 25%) exist between the luminosity of the IMD display and the luminosity of the grayscale denominator image.

Conceptually similar to IMD, the PUP Ratio display also employs a 2D-LUT, but it encodes the quotient as a perceptually uniform hue (A,B) and the absolute value of the denominator as a perceptually independent luminosity (L). As shown in Figure 2E, large ratios are shown as a shade of red, ratios near 1 as a shade of yellow, and small ratios as a shade of azure. As shown in Figure 5D and quantified in Figure 5F, the luminosity profile of the PUP Ratio display now exactly matches the luminosity distribution of the signal in the grayscale denominator image.

Application of the PUP Colocalization display to cell biology images

Finally, cell biologists may be interested in visualizing where two signals (Figure 6A) occur together at roughly equal levels (after appropriate scaling), a distribution termed

colocalization. Although colocalization can be quantified in several different ways and only at the population level (Bolte & Cordelières, 2006), here we follow previously work (Jaskolski *et al.*, 2005) and define the colocalization within a single pixel as the distance of the corresponding signal intensities from $y=x$ in a scatter plot. Other measures could also be used.

One approach to colocalization display is to create a single channel image with a 1D-LUT that represents only the value of the colocalization measure. An example of this approach, termed normalized Mean Deviation Product (nMDP) display, is shown in Figure 6B. nMDP displays the distance of each pixel intensity combination from $y=x$, using a set of perceptually arbitrary RGB space colors, where green indicates intensity combinations nearest to $y=x$ (Jaskolski *et al.*, 2005). Mathematically, the 2D-LUT used in a conventional merge image display also show each intensity combination's distance from $y=x$ as some color (shades of yellow represent intensity combinations falling along $y=x$ in this case; see Figure 2A). However, as discussed above, many other colors in a conventional merge image (not falling along $y=x$) are perceived to be highly similar to these yellows. Adding further confusion, intensity combinations along $y=x$ will be represented by both brighter and dimmer yellows, depending on their position along the line. Such a red-green merge image display is shown in Figure 6C.

To provide greater perceptual discrimination based on the pixel-wise level of colocalization between two images, we produced a dedicated PUP Colocalization display that assigns intensity ratio to hue and then varies the luminosity according to a function of each intensity combination's distance from, but not along, $y=x$ (see Figure 2F for details). Using this procedure, intensity combinations falling along $y=x$ are highlighted as a bright yellow, while regions of lesser colocalization are displayed in a less luminous hue, where the color indicates which grayscale image's intensity predominates. As shown in Figure 6D, bright yellow pixels show that paxillin and actinin are most colocalized at the trailing edge of focal adhesions (Morimatsu *et al.*, 2015) (and regions of overexpression). Where a lesser, but still modest, degree of colocalization is present, the modestly bright red (paxillin) or green (actinin) color indicates which of the signals predominate, while the regions shown as a very low luminosity magenta (paxillin alone) or blue (actinin alone) indicate low colocalization. For comparison, Figure 6E shows in green (overlaid on one of the grayscale images for context) a binary mask of all pixels whose intensity combinations fall within a fixed angle of $y=x$ (produced using the Image J Colocalization Finder plugin by Christophe Laummonerie).

Discussion

Life scientists often desire to display two dimensions of information about the signal from two different molecular probes within a single color image. Existing techniques work in the RGB (or equivalently, HSB) color space, resulting in color images that are radiometrically accurate but do not account for human color perception. As a result, crucial biological information is lost at the perceptual level. Based on the CIELAB color space, we described how to encode two dimensions of information within two perceptually independent and uniform quantities of a single color image. We then presented three types of representative

PUP displays: Merge, Ratio, and Colocalization, each of which conveys information about the grayscale inputs more accurately than comparable existing displays. Moreover, the mathematical as well as perceptual independence of hue and luminosity in the CIELAB color space allow the PUP display to be flexibly extended to other use cases through modification of the luminosity function.

A limitation of the PUP display is that the CIELAB color coordinates must still ultimately be displayed within the gamut of an sRGB monitor (or CMYK printer). This conversion can require approximations and excludes the use of some CIELAB colors. To address these issues, we have limited the spectral range of the CIELAB colors used from magenta through azure to avoid the blue region of the spectrum that cannot be displayed at higher luminosities within the sRGB gamut. Within the magenta to azure range, some hues of very high or very low luminosity still fall outside the sRGB gamut, which modestly limits the range of luminosities available for some hues. For example, in PUP Merge displays, the luminosity had to be restricted to a range from 0–75 (out of 100), causing the perceptually brightest values in a PUP Merge display to be modestly less bright than the brightest green (luminosity ~85) in a conventional merge displays. To display CIELAB values that still fell beyond the sRGB gamut, we used an iterative procedure to pick the closest RGB value whose luminosity exactly matched the desired value (while leaving the hue alone free to vary). This procedure has no practical impact but implies that for some luminosities, some hues are not exactly proportional the ratio of the input images' intensities as otherwise described. Future monitor technologies that display a larger range of visible colors will remove these limitations and further enhance the performance of the PUP display.

Like any color display, the PUP displays are also subject to higher-order color perception phenomena. The CIELAB system is calibrated to human perception when broad swaths of colors are presented, but human visual perception is complex and neither the CIELAB system nor our method accounts for these higher-order influences such as color constancy, simultaneous brightness contrast, color assimilation, or lateral inhibition (Backhaus *et al.*, 1998). Relatedly, standardized lighting and display conditions are assumed when the CIELAB colors are viewed. Thus, PUP displays are best viewed in a dimly lit room with white ambient lighting, and the white balance of the display device should be set to factory defaults.

Perception is never a substitute for quantification, but many areas of biology are exploratory, relying on human perception to recognize unforeseen or unintended outcomes. In many cases, displaying two grayscale images as a single color image in the RGB color space causes biological information present within and between the signals to be lost, obscured, or misinterpreted at the perceptual level. The PUP display renders two dimensions of information about two grayscale images as a single color image in a way that most accurately and effectively conveys this information for human perception. Thus, PUP displays will enhance recognition of unexpected outcomes in areas of the life sciences that rely on visual interpretation of images.

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Figure 1.

A) Upper: The RGB color space spectrum. A subset of these colors is generated in conventional merge image displays. Lower: The luminosity (perceived brightness) of these colors is highly variable, as shown by a luminosity profile plot. B) Upper: The set of perceptually uniform hues derived from the CIELAB color space that is used by the PUP display. Lower: By design, these hues have a constant luminosity.

Figure 2.

A) Left: The color look-up table (LUT) produced by a red-green merge image display. The intensity on the green channel is set according to the grayscale intensity in Image 1 ($G(x)$), while the intensity on the red channel is set according to the grayscale intensity in Image 2 ($R(y)$). Right: Graphs of the luminosity (perceived brightness) along each diagonal of the LUT show that the signal assigned to the red channel will be much less perceptible. B) Left: The color LUT produced by a cyan-yellow merge image display. The intensity on the yellow channel is set according to the grayscale intensity in Image 1 ($Y(x)$), while the intensity on the cyan channel is set according to the grayscale intensity in Image 2 ($C(y)$). Right: Graphs of the luminosity along each diagonal of the LUT show that color pixels where both signals are present become perceptually saturated, leading to a loss of contrast. C) Left: The color LUT produced by the PUP Merge image display using a broad range of hues. Hue is set according to the signal's ratio, while luminosity is set according to the most intensity signal between the grayscale images. Right: Graphs of the luminosity along each diagonal of the LUT show that the color pixel's relative luminosity always matches the relative intensities in the grayscale images. D) Left: The color LUT produced by the PUP Merge image display using a narrow range of hues has the same luminosity properties as the broad range of hues, since hue and luminosity are independent. E) Left: The color LUT produced the PUP Ratio image display. Hue is set according to the signal's ratio, while luminosity is set according to the intensity of the denominator image, as shown in the graphs to the right. F) Left: The color LUT produced by the PUP Colocalization image display. Hue is set according to the

signal's ratio, while luminosity is set according to a logistic function of the perpendicular distance from $y=x$. Right: Graphs showing that pixels falling along $y=x$ have a high, constant luminosity, while other pixel's luminosity is set according to their perpendicular distance from $y=x$.

Figure 3.

A) Grayscale images showing the distribution of GFP-VAMP2 and AP2 labeled with AlexaFluor549 in a primary neuron. B) Assigning the AP2 signal to red makes it far less perceptible relative to the green VAMP2 signal than in the grayscale images. C) Assigning the AP2 signal to cyan and the VAMP2 signal to yellow results in the relative brightness of each signal remaining close to that of the grayscale images. Using the PUP Merge display with either D) a narrow range of hues, or E) a broad range of hues, shows that the relative brightness of each signal exactly matches that in the grayscale images. F) A green-red merge display shows that red is difficult to perceive. G) A yellow-cyan merge image display shows that perceptual saturation occurs where both signals are present together, leading to loss of contrast. H) A PUP display with a narrow range of hues shows that the perceived brightness of each signal matches the grayscale images both where the signals are alone as well as where the signals occur together. I) The PUP Merge display using a broad range of hues allows for increased discrimination of the relative signal levels based on color. J–M) Insets from the merge displays show that the relative levels of each signal are slightly more distinguishable in PUP Merge displays than in traditional merge displays (compare ability to distinguish vesicles marked with asterisks).

Figure 4.

A) Upper: A green-red merge display based on the grayscale images in Figure 3A. Lower: The image's luminosity. B) Upper: A red-green merge display shows that swapping the colors assigned to each signal results in a perceptually different result, as can be quantified by the different luminosity (lower). C) Upper: A narrow range PUP Merge display and its luminosity (lower). D) Upper: A narrow range PUP Merge display with the colors swapped and its luminosity (lower). E) The difference of the luminosity images in A and B show that the variations of traditional merge display are perceived very differently. F) The difference of the luminosity images in C and D shows that the variations of PUP traditional merge displays are perceived as identical. This property is desirable for a merge display, because the colors assigned should not influence the information perceived.

Figure 5.

A) Two grayscale images showing the distribution of mCherry-Paxillin (the numerator) and EGFP-Actinin (the denominator) in a HeLa cell. B) Upper: A ratio display using a 1D-LUT (ImageJ Jet LUT) where a perceptually arbitrary set of colors from the RGB color space are assigned to the quotient. Asterisks indicate regions displaying large ratios that are likely an artifact of the denominator being extremely small. These regions are not visible in the IMD and PUP Ratio images. Lower: These colors have different luminosities that are unrelated to the grayscale input images. C) Upper: An Intensity Modulated Display based on the HSB color space. Colors used cover the same region of the visible spectrum as originally published. Lower: Because the colors used (specified by the HSB H parameter) are not perceptually uniform or independent of the luminosity of the HSB B parameter, the resulting luminosity distribution in the IMD image does not match the intensity distribution of the grayscale denominator image. D) Upper: The PUP Ratio display encodes ratio as a perceptually uniform hue (spanning the same region of the visible spectrum as the IMD display) and the intensity of the denominator as luminosity. Lower: The resulting luminosity distribution exactly matches the intensity distribution of the denominator grayscale image. E) The difference in luminosity between the IMD image and the grayscale denominator. F) The difference in luminosity between the PUP Ratio image and the grayscale denominator shows that they are identical.

Figure 6.

A) Two grayscale images showing the distribution of mCherry-Paxillin and EGFP-Actinin in a HeLa cell. B) A one-dimensional colocalization display termed Normalized Mean Deviation Product display uses perceptually arbitrary colors to indicate the distance of each pixel intensity combination from $y=x$. Pixels colored green fall on $y=x$, but are very difficult to distinguish from the adjacent yellow and cyan pseudo-colors. C) A conventional red-green merge image display where pixel intensity combinations falling along $y=x$ are displayed as a shade of yellow. D) The PUP Colocalization display encodes the distance of each pixel intensity combination from $y=x$ using luminosity and their ratio as hue. This technique 'highlights' in bright yellow the most colocalized pixels and shows regions of lesser colocalization in a less luminous hue, where color indicates which channel's intensity predominates. E) A binary mask shown as green indicates the location of pixels whose intensity combinations fall within a fixed angle of $y=x$. These locations correspond closely to the 'highlighted' regions in the PUP Colocalization display.