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COMMENTARY

How to measure color using spectrometers and calibrated photographs

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ABSTRACT

The measurement of color in biology has become increasingly common. These measurements are not limited to color vision research, but are also found in studies of communication, signaling, camouflage, evolution and behavior, and in the examination of environmental, artificial and biogenic light. Although the recent availability of portable spectrometers has made it simpler to measure color, guidance on how to make these measurements has not kept pace. Because most biologists receive little training in optics, many measure the wrong thing, or measure the right thing in the wrong way. This Commentary attempts to give biologists a brief overview of how to measure light and color using spectrometers and calibrated photographs. It focuses in particular on the inherent ambiguities of many optical measurements, and how these can be addressed.

KEY WORDS: Light, Irradiance, Radiance, Reflectance, Spectrum, Vision

Introduction

To begin at the beginning, one does not measure color. Color is a perceptual quality, and measuring it makes no more sense than measuring poetry. So, when we discuss the measurement of color, we are in fact talking about measuring variation in optical properties such as intensity, reflectance and transmittance (see Glossary) over a spectral range – usually the ultraviolet and human-visible portions of the electromagnetic spectrum. Typically, this measurement is performed at a fine wavelength resolution and is called a spectrum (e.g. spectral reflectance, spectral radiance; see Glossary), but there are cases where it is sufficient to measure the variation between a few broad spectral regions. In either situation, the resulting data are often integrated into one of the various models of color vision to create an estimate of color as perceived by humans or other animals (Endler, 1990; Kelber et al., 2003; Endler and Mielke, 2005; Kemp et al., 2015). This Commentary addresses the first part of this process, focusing on the physical act of measuring spectral variation using spectrometers or calibrated photographs.

There are two primary classes of spectra. The first class compares what an object does to light relative to what some standard does to it and falls into two types, reflectance spectra and transmittance spectra. In the former, the amount of light reflected from an object is compared – wavelength by wavelength – with the amount of light reflected from a standard object, usually a flat white surface. In the latter, the amount of light transmitted by an object is compared – again wavelength by wavelength – with the amount of light transmitted by a standard medium, usually air or water. The second class of spectra measures light itself, for example, photon flux as a function of wavelength. These spectra are again of two different types, radiance and irradiance (see Glossary). Radiance measures the amount of light arriving from a certain small region, and irradiance measures the amount of light arriving at a certain small region.

Although these two classes of spectra appear similar, they are fundamentally different. Most importantly, reflectance and transmittance – because they are normalized by standards – are unitless ratios, whereas radiance and irradiance measure power or photon flux. This means that measurements of the latter have to be done using calibrated instrumentation. It also means that the measured values depend on the units (e.g. Watts versus photons), including whether you think of light in terms of wavelength or frequency. On the one hand, this can make measuring radiance and irradiance complicated. On the other hand, radiance and irradiance have well-defined meanings, whereas reflectance and transmittance do not. For example, a leaf does not have a single reflectance spectrum, even at just one spot. Instead, it has many reflectance spectra, which depend on the angle of the light hitting the leaf and the angle of the detector viewing the leaf. Similarly, a single spot on a leaf does not have one transmittance spectrum, but an infinite number, depending on how you measure it.

Depending on the property that one wants to characterize, measurement of any of these spectra may be a valid approach, but choosing one requires clear-mindedness about what any given measurement means and what properties are important to your system. Therefore, although this Commentary does give a brief tutorial on how to measure spectra, it mostly focuses on how to be more thoughtful. As with all instrumentation, a spectrometer will happily spit out numbers. The trick is knowing what those numbers mean.

Measuring reflectance spectra

As mentioned above, there is no such thing as the 'reflectance spectrum' of a surface, even a surface as uniform as blank paper. For nearly all surfaces, the measured reflectance depends on the angle of the incident light and the angle of the detector viewing the reflected light. Because both of these can vary over the entire hemisphere of angles above the surface, the true reflectance of an object (even at just one wavelength) is a four-dimensional object known as the bidirectional reflectance distribution function (BRDF), the four dimensions being the angle and azimuth (see Glossary) of both the incident light and the detector. The variation in reflectance over the BRDF can be large, even for simple surfaces at just one angle of incidence (Fig. 1) – a fact that is ignored by virtually all biology studies that measure reflectance, including many of the author's own.

Experimental set-ups for measuring reflectance

Unfortunately, measuring the BRDF for even a sheet of paper is difficult, and for biological samples it is impractical at best. So what

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Glossary

Azimuth
In a polar coordinate system, the azimuth is the horizontal angle, as opposed to elevation, which is the vertical angle. For example, the azimuth of the rising sun in an equatorial region is East, whereas the elevation of the sun is close to zero.

Collimation
The shaping of light into a parallel beam. This is usually done by putting a convex lens one focal distance away from a light source.

Lambertian surface
A surface that reflects light evenly in all directions. The radiance of the surface is equal to the irradiance striking it multiplied by the reflectance of the surface, divided by π. Good examples of approximately Lambertian surfaces are cotton cloth and matte paint.

Irradiance
The amount of light striking a surface or intersecting a small volume of space. The first, most commonly used by biologists, is termed vector irradiance, the second is termed scalar irradiance. Irradiance measurements are most often used to quantify the overall illumination level.

Radiance
The amount of light reaching a viewer (or a detector) divided by the (typically small) angular area of the sampled region. For example, to measure the radiance of the sun using a light detector, one would first measure the amount of light entering the detector and then divide it by the angular area of the sun in steradians (~0.00006 sr).

Reflectance
The amount of light leaving a surface divided by the amount of light that strikes it. There is no single value for this, because it depends on the angle of the incident light and the angle of the reflected light.

Spectrum
The amount of light (or reflectance or transmittance) as a high-resolution function of the wavelength or frequency of the light.

Specular surface
A surface that reflects light like a mirror.

Transmittance
The amount of light that passes through a substance divided by the amount of light that entered it. As with reflectance, the value depends on the geometry of the entering light and how much of the exiting light is measured.

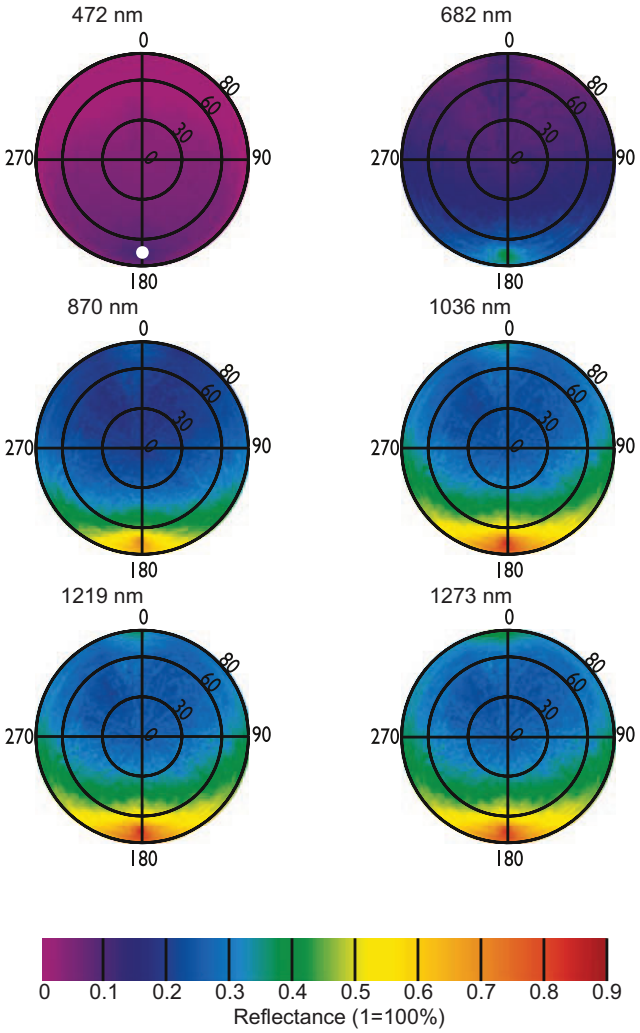

Fig. 1. Sample bidirectional reflectance distribution function (BRDF) of one location on the savannah near Skukuza, South Africa (25.0°S, 31.5°E), for six visible and near-infrared wavelengths. In each polar plot, the distance from the center represents the angle of the reflected light relative to the perpendicular to the ground. The angle and azimuth of the incident sunlight is constant and roughly at the location of the brightest spot on nearly all six plots (zenith angle 67 deg, azimuth 180 deg, marked by a small white circle in the upper left plot only). Note that the savannah is strongly backscattering at near-infrared wavelengths. Reprinted from Gatebe et al. (2003), with permission.

can be done? Certain surfaces, termed Lambertian (see Glossary), reflect light equally in all directions and also have a reflectance that does not depend on the angle of incidence. Commercial reflectance standards approach this ideal and cotton cloth is not a bad approximation to it, but biological surfaces tend to be more complicated. So the researcher is left with three options, none of which are ideal: (1) measuring reflectance using an established geometry (i.e. an established experimental set-up); (2) using a measurement geometry that mimics the biological situation of interest; or (3) studying only Lambertian or mirrored surfaces.

First and most commonly, one can measure reflectance using a geometry that has been used many times before. Although this does not measure the one true reflectance, it does allow one to compare new data with previous work. Two common geometries are: (1) illuminate the surface from directly above and place the detector at a 45 deg angle (Fig. 2A) and (2) illuminate the surface at 45 deg and use an integrating sphere to collect all the reflected light (Fig. 2B). The integrating sphere is coated with a highly reflective inner surface that scrambles the light to the point where its original direction of reflection is lost. Certain companies sell integrating spheres with built-in light sources and a switchable light trap at just the right angle so that specularly reflected light (as from a mirror or wet surface) can be included or excluded with the flip of a lever. A third common geometry is to illuminate the surface and measure it at the same angle, using a reflection probe (Fig. 2C). This generally consists of a parallel bundle of fiber optic cables, some of which carry light from a source that illuminates the surface (usually at 45 deg), and some of which carry the reflected light back to a spectrometer. These three measurement methods may give different answers for many natural surfaces. For instance, in the example shown in Fig. 1, a reflectance probe at 67 deg from the perpendicular would measure the reflectance of the savannah ground at 870 nm as approximately 75%, whereas an integrating sphere measurement of the same location (with the incident light also at 67 deg) would instead measure a value averaged over all reflected angles (~40%).

A second option for dealing with the unavoidable ambiguity of reflectance spectra is to use a measurement geometry that mimics the ecological situation being studied. For example, if one is interested in how a ladybug looks to another ladybug crawling behind it on a sunny day, one can illuminate from above and measure from the angle of the potential viewer. However,

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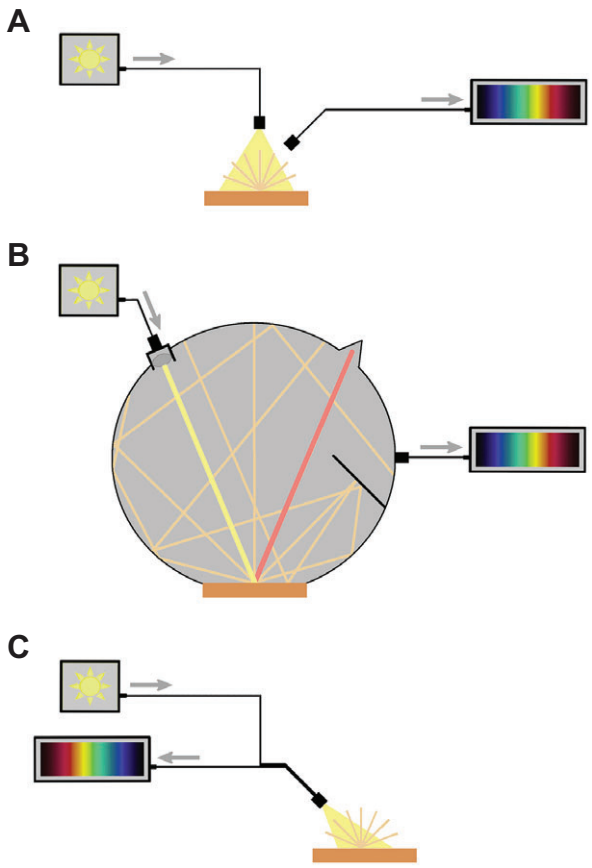

Fig. 2. Three common geometries for measuring reflectance. The direction of the light is indicated using gray arrows. (A) Light is passed from the source (box with sun) through a fiber optic cable and then illuminates a sample (orange rectangle). A fraction of the reflected light (orange lines) is collected by a second fiber optic cable that is coupled to a spectrometer (box with spectrum). (B) Light from the source is collimated (see Glossary) into a beam (shown in yellow) that then strikes the sample, which covers the opening of an inverted integrating sphere. The black line inside the sphere denotes a baffle that prevents directly reflected light from reaching the exit port of the sphere. A fraction of the reflected light, which is scattered about the inside of the sphere, is collected by a second fiber optic cable that again is coupled to a spectrometer. The light that is reflected in a mirror-like fashion (shown in red) is often caught in a light trap so that it can be excluded. (C) Light from the source runs through a fiber optic cable that is parallel to and bundled with a second fiber optic cable that is connected to the spectrometer, so that the angle of incidence equals the angle of detection.

unfortunately, illumination and viewing geometries in nature are seldom constant. A third option is restricting oneself to studying nearly Lambertian or highly mirrored surfaces, both of which are easily characterized. Unfortunately again, these are uncommon. So, in general, one must accept that reflectance is in the eye of the beholder and be clear-minded and explicit about what is being measured.

Instrumentation and standards

Aside from the critical issue of reflectance geometry, the measurement of spectral reflectance is not difficult, but it does require attention to detail. The illumination source can be anything, so long as it provides adequate intensity over the required spectral range. The light source also needs to provide even and stable illumination over the region seen by the detector and not move during the actual measurement. The detector is typically a fiber optic cable coupled to a portable spectrometer. The fiber optic cable allows the user to easily choose the viewing angle and region to be viewed, and the spectrometer measures the entire spectrum at once, usually in a small fraction of a second and to a resolution of ~1 nm. The issues of purchasing and using a spectrometer are given in detail in Johnsen (2012), but most spectrometers are simple to use in reflectance mode. The best way to know whether the fiber optic cable is truly viewing the correct region of your sample is to temporarily unplug it from your spectrometer and plug it into a light source. Because light travels the same path forward and backward, what is now illuminated on your sample is what is being viewed by the spectrometer.

The spectrum of the reflected light is not reflectance. Instead it is reflected radiance, which is discussed in the penultimate section of this Commentary. In order to convert it into spectral reflectance – thus making it independent of the spectrum of the illumination – it must be normalized wavelength by wavelength by the reflected radiance from a standard. As mentioned above, this standard is usually a white Lambertian surface with a reflectance that is essentially 100% in the ultraviolet and visible portions of the spectrum. This surface is typically made of an expensive, proprietary plastic known as Spectralon that is formed into a disk, although barium sulfate and magnesium oxide coatings are sometimes used. One can use a sheet of white paper for a standard, but at some point the reflectance of the paper itself will need to be measured, which requires a genuine standard. In addition, most paper strongly absorbs ultraviolet radiation (UVR) and often fluoresces as well. White Teflon plumber's tape also works and typically does not absorb UVR or fluoresce, but at some point still needs to be calibrated.

The operating software that is packaged with most spectrometers automatically divides the reflected radiance from the sample by the reflected radiance from the standard to create a reflectance spectrum. However, it is essential that the geometry of the standard measurement exactly matches that of the sample measurement. In other words, the standard's surface must be in the same spot (relative to the light source and the detector) as the sample surface to within a millimeter. The relative angles must also match to within a degree or so, which is difficult because few biological surfaces are flat. This means clamping everything down, which can be tricky because fiber optic cables and biological samples do not clamp well. This cannot be overstressed, and it is instructive to measure a sample (or the standard) multiple times to see how much variation can result from even small changes in geometry (or from drift in the output of the illuminating light source). This requirement for technical rigidity in the face of biological fluidity is the biggest challenge of reflectance measurement. Often, a person measuring reflectance of biological samples spends 95% of the time designing and building an adequate set-up and 5% of the time performing the actual measurements (see Meadows et al., 2011).

You must ensure that your light source emits light at all the wavelengths you require, otherwise both your sample measurement and your standard measurement will be approximately equal because both are simply the electrical noise of the spectrometer, and your calculated reflectance (which is a quotient of the two numbers) will be near 100%. There are many papers that erroneously report high reflectance in the ultraviolet because the illuminating tungsten bulb did not emit enough ultraviolet light. Xenon sources are better for this. Also, if you are measuring something that is not opaque, be sure to place it on a matte black surface, otherwise the reflectance of the material under your sample will artificially inflate the values. Environmental light may do the

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same, so shielding the set-up is sometimes necessary. Finally, because the refractive index of the medium relative to the sample affects reflectance (see Johnsen, 2012), as does simply being wet (consider wet sand relative to dry sand; Bohren and Clothiaux, 2006), aquatic samples are best measured within water, ideally with the source and detector probes within the water as well to avoid refraction and reflection at the water surface. This means that the reflectance standard must also be measured in water. Spectralon is hydrophobic, so one must wipe off any bubbles to get an accurate reading of its reflectance. After this is done, the reflectance of the underwater standard is not too different from the reflectance in air (Voss and Zhang, 2006).

Measuring transmittance spectra

As with reflectance, there is no single transmittance spectrum for an object. Transmittance of course depends on the thickness of the sample, but even for a uniform material of one thickness, there are an infinite number of transmittances. In this case, the variation does not arise from the angles of the light source and the detector, but instead from the angular spread of the light that is collected by the detector. This is because the transmittance of light through an object depends on both light absorption and light scattering, the former not changing the direction of the incident light, the latter changing it substantially (see Johnsen, 2012 for further details on the absorption and scattering of light). In certain cases, one wants to know how much light has passed through an object, regardless of whether the direction of that light has been hopelessly scrambled by scattering within the object. This is often known as diffuse transmittance and is useful when one only cares about how much light is transmitted (e.g. studies of light under forest canopies or light deep within tissue). In other cases, you may want to know how much of a parallel beam of light has made it through the object without being absorbed or scattered. This is sometimes referred to as direct transmittance. For example, frosted glass and clear glass may ultimately let through the same amount of light, but nearly all the light that has passed through the frosted glass has lost its original direction, whereas the directionality of the light passing through the clear glass is preserved. Direct transmittance is especially important for studies involving vision (e.g. image propagation through water or fog, or investigations of the clarity of the cornea and ocular lens).

However, there is no clear way to distinguish between light that has gone straight through a substance and light that has been scattered over small angles. The platonic ideal for measuring direct transmittance involves a perfectly parallel and infinitesimally thin beam of light that goes through the material and then is detected by an infinitesimal (or infinitely far away) detector that thus only collects that light that has not scattered at all, even over the tiniest of angles. In reality, however, detectors have finite sizes and are not infinitely far away, so they collect both the unaffected beam and some of the forward-scattered light. The ratio of the diameter of the detector to its distance from the sample determines how much of this scattered light is collected, so a bigger or a closer detector collects scattered light over larger angles, making the transmittance larger. For many important biological materials, much of the light is scattered over these small angles, so this is a relevant and unavoidable problem.

For better or worse, there is no standard working definition of direct transmittance. So, as with reflectance, this again leaves one making choices. In the case of diffuse transmittance, the best method is to use an integrating sphere that collects all the light that has passed through the sample, whether it has been scattered or not (Fig. 3A). In the case of direct transmittance, choosing a detector size and distance so that all light scattered by <1 deg is collected seems a reasonable, although arbitrary, choice (Fig. 3B). Just remember that it is a choice; there is no official direct transmittance, but instead one that depends on the geometry of the measurement system. As with reflectance, in certain cases one can make an ecologically informed choice.

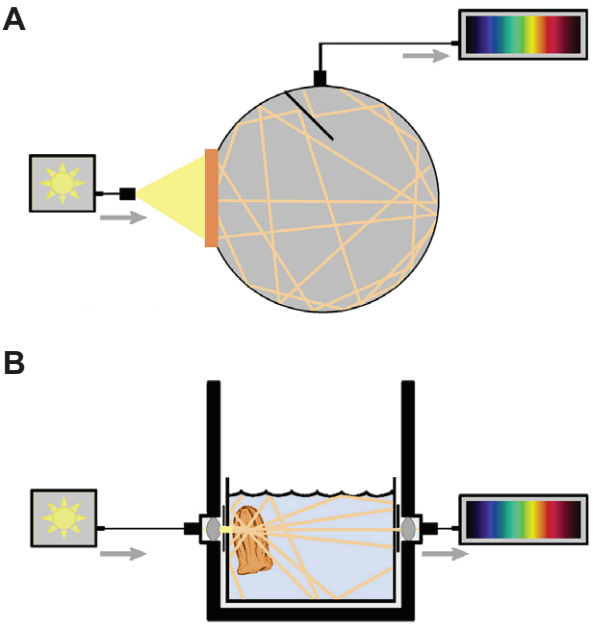

Fig. 3. Possible geometries for measuring diffuse and direct transmittance. (A) A common geometry for measuring diffuse transmittance. Light from the source that has passed through the orange sample is then reflected many times within an integrating sphere. A fraction of this light is collected by a fiber optic cable that is coupled to a spectrometer. (B) A common geometry for measuring direct transmittance. Light from the source is collimated into a beam by a small lens. This then passes through the sample (in this case a beroid ctenophore), which both scatters and absorbs it. The light that is neither absorbed nor scattered over large angles passes through a small hole, is focused onto a fiber optic cable and sent to a spectrometer. Adapted from Johnsen (2012).

The mechanics of measuring transmittance are much like those of measuring reflectance, with the standards for measures of transmittance being air for terrestrial samples and water for aquatic samples. One must still worry about the mechanical stability of the set-up, the spectral range of the light source and whether the sample should be in water or not. Designing the set-up can be especially challenging for measuring direct transmittance because the shape of a transparent object can deflect and even focus the light beam. For example, measuring the direct transmittance of an ocular lens can be nearly impossible, because, despite its exquisite clarity, it completely reshapes the light beam.

Measuring spectral variation from calibrated photographs

Although reflectance and transmittance measurements of artificial substances such as paper and glass can be precise, measuring the optical properties of biological samples is often frustrating. Animals and plants can be squishy, flimsy, small, morphologically complex or all of the above. They can also move. It does not help that so many organisms and their associated parts are either spheroids or cylinders, both of which can focus transmitted light and send reflected light in all directions. Repeated measurements of the spectral reflectance of even a dead spider leg, for example, can be extremely variable. Finally, individual spectral measurements are a

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slow and inefficient way to understand the spatial variation of an optically complex tissue, such as a butterfly wing.

Spectral imaging is one potential solution to many of these problems. An entire tissue or organism illuminated by a diffuse flash can be recorded at once, eliminating the need for individual measurements. The downside of this approach is that it sacrifices spectral resolution. Instead of obtaining a measurement for every nanometer, all the information is dumped into a few bins, usually the red, green and blue channels of the camera. There are multispectral imaging systems with high spectral resolution, but they tend to be either custom built or extremely expensive (e.g. Chiao et al., 2011), so the rest of this section focuses on what can be done with a standard camera.

Although a camera only provides three channels, this spectral resolution can be sufficient for many purposes. For example, although color vision models typically demand fine-resolution spectra, many measurements of color are motivated by other factors, such as the desire to classify organisms or tissues [e.g. comparing the colors of flowers (Muchhala et al., 2014) or assessing the saturation of body coloration in guppies relative to immunocompetence (Martin and Johnsen, 2007)]. It is also well known that most variation in natural spectra can be captured using only three or four principal components (Buchsbaum and Gottschalk, 1983; Chiao et al., 2000). Also, with filters and some cleverness, it is sometimes possible to alter the spectral sensitivities of the camera channels so that they approximate those of various photoreceptor classes (e.g. Troscianko and Stevens, 2015). Thus, the three channels of a commercial digital camera, although designed for human vision, are useful for capturing spectral variation in general, though only in the human-visible portion of the spectrum. Important exceptions to this rule are the few narrow-band spectra in nature, such as bioluminescent emissions or reflections from iridescent tissues. For example, a camera without specialized filters cannot distinguish violet from blue bioluminescence – both will appear blue.

Assuming that three channels do suffice for the project at hand, the main issue is calibration. Unlike a spectrometer, a camera is not designed to deliver calibrated numbers. Before one even sees the image on the monitor on the back of the camera, it has been tweaked in many unseen and proprietary ways, mostly to make human faces more appealing. The image is also not linear, meaning that a grayscale value of 200 is not twice as bright as a grayscale value of 100. In addition, lighting tends not to be constant. Tungsten and fluorescent bulbs, and even the on-camera flash, can vary erratically, and some studies demand outdoor light, which varies even more. Finally, most cameras also have automated white balance settings, which further affect the image. The color constancy of our own visual system (Cronin et al., 2014) minimizes the perceptual effects of these changes, but they occur nevertheless and affect any attempt to measure color.

It is possible to create a highly controlled lighting environment and to develop a repeatable photographing procedure, allowing one to calibrate everything ahead of time (see Troscianko and Stevens, 2015), but a safer solution is to place a calibration standard within each image so that it can never be lost (Tedore and Johnsen, 2012), much like geologists place a hammer or a coin for scale. For example, the calibration standard can be a set of gray regions that vary from dark to light gray (Fig. 4A). Grayscale standards can be purchased, but they are also easy to create on a computer for printing out or can be assembled using paint swatches. The paint swatch approach is especially useful for small standards where it becomes obvious that computer-printed grays are just a collection of small

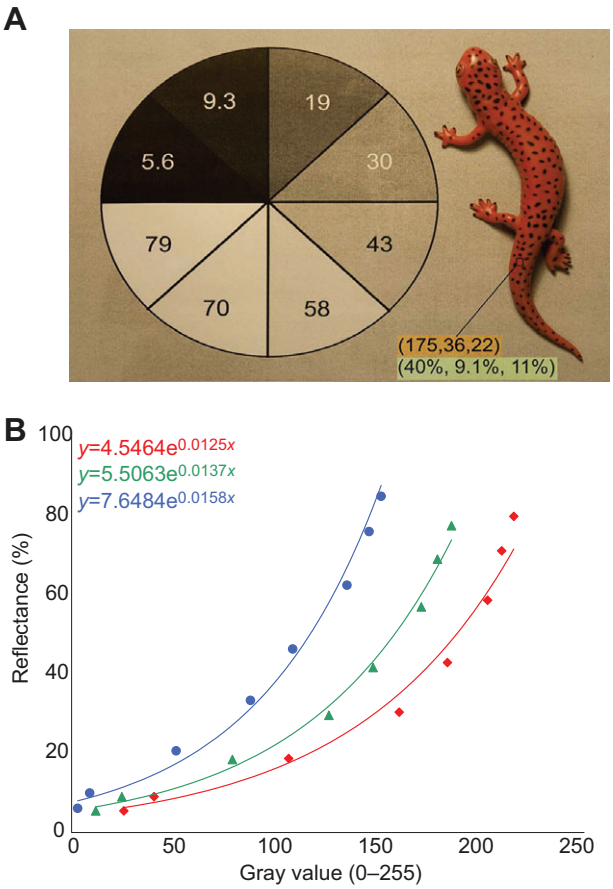

Fig. 4. A method of obtaining reflectance from a photograph. (A) A gray standard set with known reflectances (numbers in pie chart, given as percentage reflectance averaged over 400–700 nm) is placed next to the sample and photographed. The average RGB values for a small square on the toy salamander are shown in the orange box. The image was deliberately left with a poor white balance to show that this does not affect the success of this process. (B) The RGB values of each gray shade in the pie chart are recorded and then plotted against the actual reflectances of the shades in each color channel to create three fitted curves, one for each color. The equations of these curves can then be used to convert the RGB values of any part of the sample into calibrated reflectance values (shown in the green box in A for the sampled region on the salamander).

black dots. The exact shades are unimportant, so long as they span the range that might appear in the sample. The reflectances of the shades must be known and are best measured using the integrating sphere process described above (Fig. 2B). The exposure of the photo that includes the sample and the standard needs to be such that no color channel of the darkest square of the standard in the image reaches zero, and no channel of the lightest square reaches 255. If this does occur, then it is impossible to determine how light or dark that region is. For example, one cannot determine whether the white region is just a little brighter than a grayscale value of 255 or ten times brighter. So long as the exposure does not saturate the standard in either direction, then it does not matter what the camera software has done to the image or whether the lighting changed from a previous photo, because the standard has been affected in the same way as the sample. The one exception to this is the saturation control of the camera software, which can leave grays as gray but make colors more saturated. For this reason, it is best to use RAW images, which are the data from the sensors prior to any white-point balancing or other non-linear transformation. Another solution is to

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substitute the grayscale standard series with three series, with increasing intensities of red, green and blue.

To measure the reflectances (averaged over the three color channels of the camera) of a region of the sample, one first obtains the R, G and B values of the region using image processing software – generally averaged over a set of adjacent pixels. The R, G and B values of each of the gray standards is then plotted and fitted to a curve for each channel, typically an exponential function for most color spaces (Fig. 4B). Then, the actual reflectance corresponding to the RGB values obtained from the sample can be obtained from the equations of the three fitted curves. This process can be automated in a number of different ways and gives accurate results regardless of camera software, exposure settings and variation in the illumination – so long as the illumination of the sample is the same as that of the standard.

Measuring spectral radiance and irradiance

Radiance and irradiance

Although most measurements of 'color' are of reflectance (and occasionally transmittance), there are times when one wants to measure the actual spectrum – i.e. the power or photon flux as a function of spectral region. These cases usually involve the measurement of environmental light and bioluminescent emissions, but can also include measurements of reflected or transmitted light. For example, although many researchers model the appearance of an object by multiplying the spectral reflectance by the spectrum of the light illuminating the object, for all the reasons discussed in this Commentary it is more accurate to measure the actual reflected light.

However, there are a number of issues to consider. First, as with reflectance and transmittance, one must make a decision about measurement geometry, in this case the angular region over which the light is collected. Although one can collect light from regions of an infinite variety of shapes and sizes, two limiting cases are most commonly used – termed radiance and irradiance. Radiance measurements collect light from a region in space, usually a small one, and are divided by the solid angle of the region viewed. They are used when one wants to measure the 'brightness' or 'color' of an object. Irradiance measurements, by contrast, collect light from a large region of space, usually an entire hemisphere and sometimes over the complete sphere. The most common form of irradiance in biology is termed 'vector irradiance', and involves the collection of light over the entire hemisphere above the surface of the detector. This light is weighted so that light perpendicular to the surface counts the most, and light parallel to the surface does not count at all. In between these two angles, the weight of light depends on the cosine of the angle. Although this seems arbitrary, the integrated end result is an accurate measure of the amount of light energy imparted to a surface. Irradiance measurements are used when one wants to measure the overall illumination spectrum in a habitat or the amount of light entering an eye.

A spectrometer can only measure how much energy enters it; it does not know what it is looking at. So setting a spectrometer to measure radiance or irradiance depends on what are called the 'sampling optics', in most cases something screwed onto the end of the fiber optic cable that determines the field of view. If the sampling optic is a Gershun tube (Fig. 5A), which is a device used to limit the field of view of the fiber to a small angular area, then the spectrometer is measuring radiance (so long as the measurement is divided by this area). If the sampling optic is a cosine corrector (a small, expensive piece of white plastic) or an integrating sphere (Fig. 5B), then the spectrometer is measuring vector irradiance. The 'vector' in vector irradiance is there to remind you that the irradiance depends on the orientation of the sampling optics. For example, pointing the cosine corrector up will measure downwelling irradiance and pointing it sideways will measure sidewelling irradiance.

Calibration

The second major issue related to measurements of spectra is calibration. Because reflectance and transmittance spectra are ratios relative to a standard and not measurements of light itself, they do not have units and thus do not require calibration. Radiance and irradiance, however, do require this. Most spectrometer software will guide you through the tedious calibration process, and some spectrometers even come pre-calibrated. This can be a time-saver, but it is important to realize that anything you attach to your spectrometer – for example, a fiber optic cable – will alter this calibration. Short lengths of these cables do not affect visible light

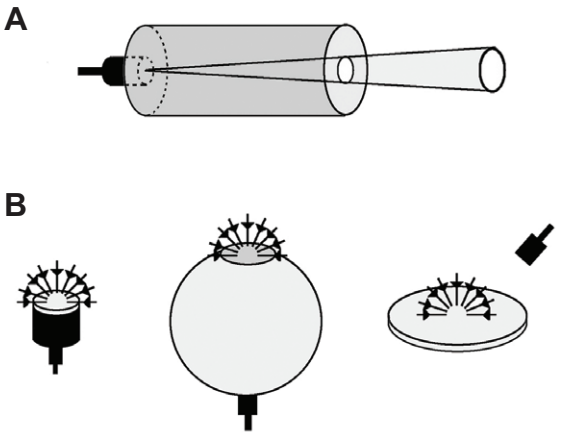

Fig. 5. Sampling optics for the measurement of radiance and irradiance. (A) A Gershun tube used to measure radiance. The normally large field of view of the fiber optic cable is reduced by a small opening at the end of the cylinder. (B) Three different, but functionally equivalent, sampling optics for measuring vector irradiance. The left panel shows a cosine corrector that diffuses light that passes through it. In the center is an integrating sphere that collects light from all directions. The right panel shows a Spectralon surface that is viewed by a fiber optic cable. The inward-pointing arrows in all three images depict the incident light. Adapted from Johnsen (2012).

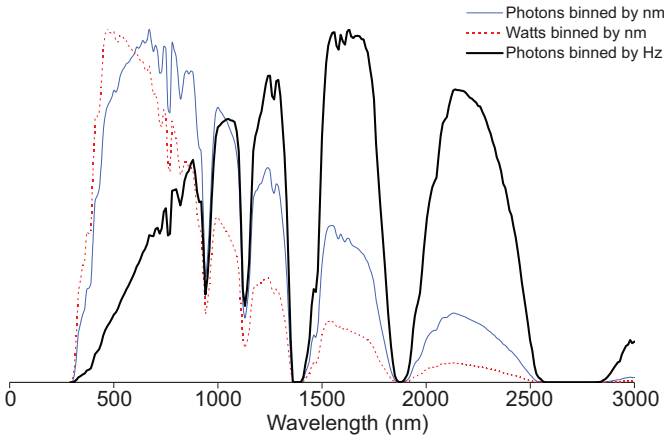

Fig. 6. The shape of a light spectrum depends on the units used. The same daylight spectrum is shown plotted as Watts nm^{-1}, photons nm^{-1} and photons Hz^{-1}. The spectra are all normalized to the same peak for clarity.

significantly, but long lengths (>5 m) will, and any length can affect calibration in the ultraviolet, because the glass in the cable significantly absorbs radiation in this region. These pre-set calibrations also drift over time, usually requiring you to send the spectrometer back to the company for re-calibration.

Although calibration is straightforward, it is connected to the important concept that the shapes of light spectra are not universal, but instead depend on the units used (Fig. 6). First, because short-wavelength photons are more energetic than long-wavelength photons, a spectrum calibrated in photon flux (e.g. for a vision study) is shifted to longer wavelengths than a spectrum calibrated in power units (e.g. for a thermoregulation study). Second, all light spectra are histograms. Biologists nearly always calibrate them in bins of equal wavelength – i.e. the spectrum is 'per nanometer'. However, it is just as reasonable, arguably more so, to bin spectra in equal frequency units (Hertz), which dramatically changes the shape of the spectrum. This is important, because the spectral sensitivity curves of cameras and eyes do not suffer from this problem – they stay the same. Therefore, one cannot simply choose Watts versus photon flux and nanometers versus Hertz and assume that at least the relative shapes and positions of the relevant curves will stay the same – the radiance or irradiance will change, but the spectral sensitivity curve will not. This means that common statements such as 'human spectral sensitivity peaks near the peak wavelength of daylight' have no merit. See Soffer and Lynch (1999), Heald (2003), Bohren and Clothiaux (2006) and Johnsen (2012) for lively discussions of this subtle and far-reaching issue. Also, the oft-used concept of a 'neutral spectrum' as an experimental control similarly has no meaning. A flat spectrum in Watts nm^{-1} will not be flat in photons nm^{-1} and will be steeply sloped in Watts Hz^{-1} or photons Hz^{-1}. This is inescapable.

Conclusion

The recent improvements in spectrometers and cameras, combined with the growth of the field of visual ecology and the marvelous, slow-motion merger of biology and physics, mean that measurements of light and color in biology will become ever more common. As with all measurements, one must be careful that the property that is being measured is both the one that the measurer thinks is being measured and one that is relevant to the experimental system at hand. This is especially true for measuring spectra, because humans in general have little intuition for light levels. However, exercising some care, getting advice and explicitly reporting how a measurement was made (rather than, for example, stating 'reflectance was measured' in a methods section) will solve most problems, allowing us to explore the fascinating intersection of light and life in a meaningful and satisfying way.

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Competing interests

The author declares no competing or financial interests.

References

Bohren, C. F. and Clothiaux, E. E. (2006). *Fundamentals of Atmospheric Radiation*. Weinheim, Germany: Wiley-VCH.

Buchsbaum, G. and Gottschalk, A. (1983). Trichromacy, opponent colours coding and optimum colour information transmission in the retina. *Proc. R. Soc. B Biol. Sci.* **220**, 89-113.

Chiao, C.-C., Cronin, T. W. and Osorio, D. (2000). Color signals in natural scenes: characteristics of reflectance spectra and effects of natural illuminants. *J. Opt. Soc. Am. A* **17**, 218-224.

Chiao, C.-C., Wickiser, J. K., Allen, J. J., Genter, B. and Hanlon, R. T. (2011). Hyperspectral imaging of cuttlefish camouflage indicates good color match in the eyes of fish predators. *Proc. Natl. Acad. Sci. USA* **108**, 9148-9153.

Cronin, T. W., Johnsen, S., Marshall, N. J. and Warrant, E. J. (2014). *Visual Ecology*. Princeton, NJ: Princeton University Press.

Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biol. J. Linn. Soc.* **41**, 315-352.

Endler, J. A. and Mielke, P. W. (2005). Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc.* **86**, 405-431.

Gatebe, C. K., King, M. D., Platnick, S., Arnold, G. T., Vermote, E. F. and Schmid, B. (2003). Airborne spectral measurements of surface–atmosphere anisotropy for several surfaces and ecosystems over southern Africa. *J. Geophys. Res.* **108**, 8489.

Heald, M. (2003). Where is the "Wien peak"? *Am. J. Phys.* **71**, 1322.

Johnsen, S. (2012). *The Optics of Life: A Biologist's Guide to Light in Nature.* Princeton, NJ: Princeton University Press.

Kelber, A., Vorobyev, M. and Osorio, D. (2003). Animal colour vision – behavioural tests and physiological concepts. *Biol. Rev. Camb. Philos. Soc.* **78**, 81-118.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. A., Bennett, A. T. D., Dyer, A. G., Hart, N. S., Marshall, J. and Whiting, M. J. (2015). An integrative framework for the appraisal of coloration in nature. *Am. Nat.* **185**, 705-724.

Martin, C. H. and Johnsen, S. (2007). A field test of the Hamilton–Zuk hypothesis in the Trinidadian guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* **61**, 1897-1909.

Meadows, M. G., Morehouse, N. I., Rutowski, R. L., Douglas, J. M. and McGraw, K. J. (2011). Quantifying iridescent coloration in animals: a method for improving repeatability. *Behav. Ecol. Sociobiol.* **65**, 1317-1327.

Muchhala, N., Johnsen, S. and Smith, S. D. (2014). Competition for hummingbird pollination and the evolution of flower color diversity in Andean Solanaceae. *Evolution* **68**, 2275-2286.

Soffer, B. H. and Lynch, D. K. (1999). Some paradoxes, errors, and resolutions concerning the spectral optimization of human vision. *Am. J. Phys.* **67**, 946-953.

Tedore, C. A. and Johnsen, S. (2012). Weaponry, color, and contest success in the jumping spider *Lyssomanes viridis*. *Behav. Proc.* **89**, 203-211.

Troscianko, J. and Stevens, M. (2015). Image Calibration and Analysis Toolbox - a free software suite for objectively measuring reflectance, colour and pattern. *Methods Ecol. Evol.* **6**, 1320-1331.

Voss, K. J. and Zhang, H. (2006). Bidirectional reflectance of dry and submerged Labsphere Spectralon plaque. *Appl. Opt.* **45**, 7924-7927.

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