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Broad-band multispectral microscope for imaging transmission spectroscopy employing an array of light-emitting diodes

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Optical spectral analysis and multispectral imaging provide powerful means for characterizing samples in a wide variety of applications and on many spatial scales. We present a simple implementation of these techniques in the context of microscopy. A modified commercial microscope equipped with a CMOS imaging detector, combined with an array of light emitting diodes with emission ranging from ultraviolet to near-infrared wavelengths, is described, and examples of information enhancement using multivariate analysis are presented. © 2009 American Association of Physics Teachers.
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I. INTRODUCTION

The response to different wavelengths is characteristic of an object, and its optical reflectance spectrum (related to color) is important in identifying it. Color discrimination varies widely in biological and technological vision systems. Vision and color registration are illustrated in Fig. 1, as well as the corresponding interpretation methods. Humans have three broad color channels, while birds frequently have four, including ultra-violet (UV) sensitivity. The Mantis shrimp has 12 color channels. Clearly, the human visual perception makes quantification of the true spectral composition of an object difficult. In contrast, artificial vision systems (imagers) such as RGB (red-green-blue) cameras, satellite sensors, and spectrometers allow a much more precise quantification of the spectral properties, which are directly linked to the chemical composition of the object of interest. In spectroscopy the exact spatial origin of the emission is irrelevant; we only collect enough photons to record the spectral distribution (“fingerprint” or “signature”) of the material. If spectra are recorded in all spatial locations over the area studied, we obtain an image with distributed spectral fingerprints, a *multispectral image*. Such an image can be formed by sequentially recording the full spectra at adjacent points or by observing the entire object in a number of selected spectral bands. The number of independent spectral bands may vary from 1 in a black-and-white camera to 1024 or more in a modern spectrometer. Because digital data are provided by technological sensors, computer processing is used to interpret the data, frequently using multivariate analysis.

The purpose of this paper is to provide insight into the nature of digital multispectral imaging and to discuss an instrument suitable for projects in imaging spectroscopy. The power of multivariate analysis is demonstrated in the process. A simple and affordable implementation in terms of a microscope equipped with 13 quasi-monochromatic light-emitting diodes (LEDs) is presented.

Multispectral imaging allows us to extract physical and chemical information about an image scene. In contrast, normal image processing mostly focuses on the spatial properties of the image,¹ that is, on shapes. The applications of multispectral imaging range from astronomy to microscopy and cover the electromagnetic spectrum from gamma rays to radio waves.² Examples are the identification of possible cancer regions in medical imaging, delineating areas of for-

est decline in satellite images, and mapping areas containing a certain concentration of calcium ions in a fluorescence microscopy image.³⁻⁹

A relevant question for understanding our natural color perception and the potential for multispectral imaging is “What would the world look like and what information would we be able to see if we could manipulate the spectral sensitivity of our vision?” We are able to do so using various pieces of technological equipment as indicated in Fig. 1. The microscope discussed in this paper is one of many instruments providing imaging spectroscopy. Although the equipment used may vary widely, the general principles of multispectral imaging for extracting useful information are the same.²

Multivariate techniques^{10,11} are well known in chemistry and are frequently referred to as chemometry.¹² In our case a given spectral distribution of a particular point in an object is approximated as a linear combination of a few base spectra (called principal components), in a way similar to the expansion of a wavefunction in a set of eigenfunctions. The base functions can be automatically generated by analyzing the variance in the ensemble of spectra from every pixel in the image. A pedagogical example of the use of the techniques in imaging spectroscopy can be found in Ref. 13. Multivariate techniques are provided in a MATLAB package,¹⁴ and more specialized commercial programs are available.

In our demonstration of multispectral transmission imaging the spectral resolution arises from data acquired by multiplexing several LEDs,¹⁵ which cover the visible range (400–700 nm) and extend into the UV and near infrared (NIR) regions. A commercial low-cost microscope equipped with a CMOS (complementary metal oxide semiconductor) imaging chip¹⁶ was modified for multiple LED illumination, and the sensitivity spectrum of the CMOS device, which is well outside that of the human eye, is critically utilized.

II. MULTISPECTRAL IMAGING AND MICROSCOPY

When light impinges on a sample as in transmission microscopy, the incident light intensity is split into a few parts. One part is reflected back toward the source, some light is refracted or scattered and results in a change in the angle of propagation, some light is absorbed by the molecules in the sample, and some light is transmitted. Also, part of the absorbed light might be reemitted as fluorescence light. These

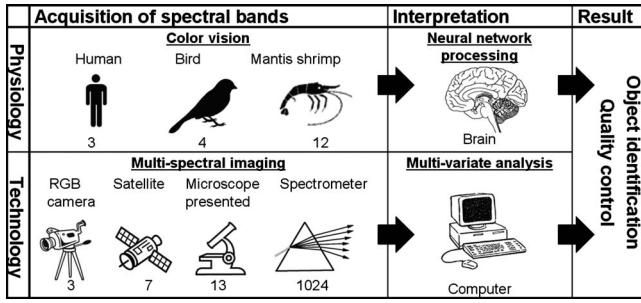


Fig. 1. Relation between color vision, multispectral imaging, multivariate analysis, and spectral bands of various species or spectral instruments (the digits refer to the number of spectral bands). The spatial resolution varies from megapixels in the RGB imager to a single pixel in a nonimaging point spectrometer.

phenomena are governed by Snel's law, the Fresnel equations, and the Beer-Lambert law (see, for example, Ref. 17). The transmittance T at a point is defined as the ratio between the transmitted intensity and the incident intensity on the illuminated side of the sample. When values for the transmittance are obtained at several wavelengths, $T(\lambda)$, we do transmission spectroscopy. When spectroscopy is done at several spatial locations, we obtain $T(x, y, \lambda)$ and refer to multispectral transmission imaging. Similarly, absorption and reflection spectroscopy can be performed.

In normal transmission microscopy contrast arises partly from differences in the absorption properties of different parts of the sample and partly from spatial gradients in the refractive index. Absorption spectra and the influence of the refractive index are more likely to vary when a higher spectral resolution is available. Thus, increasing the number of spectral channels usually makes object identification easier. However, very high spectral resolution of absorption in the optical regime seldom leads to any additional information for solids and liquids, because the energy level structure is broad. The sharpest features are typically on the order of tens or even hundreds of nanometers in optical absorption spectra. In unaided transmission microscopy the naked eye perceives the three spectral bands of the corresponding cones of the human retina (in comparison to the thousands of spectral bands that commercial point spectrometers provide). Certain scenes appear without color, but clear spectral characteristics might be present in the UV or IR regions. UV vision is utilized by bees who can see color encodings of flower petals; these encodings are visible to humans. Neither can we see the strong reflectance increase of green leaves in the near IR. We are unable to distinguish spectra even in the visible range. For example, filament and fluorescent light bulbs have different spectra, but both appear to be white to the human eye.

The contribution to the intensity u at a pixel for the color channel ch within a given spectral band is given by

$$u_{ch} = \int_0^{\infty} E(\lambda) T(\lambda) S_{ch}(\lambda) d\lambda, \quad (1)$$

with E the emission spectrum of the illumination, T the transmittance, S_{ch} the sensitivity spectrum or spectral band of the recording system in color channel ch , and λ the wavelength. Equation (1) expresses the fact that a response is

obtained only if the spectra of the illumination, transmittance, and detector sensitivity overlap.^{3,4}

The same approach can be applied to all spectral instruments including color cameras and spectrometers; the main difference is that with a spectrometer the sensitivity bands are preferably narrow and ordered according to the wavelength, whereas in commercial color cameras the spectral channels are broad and overlapping in order to emulate the human eye. Natural vision channels are usually overlapping and can even have secondary sensitivity regions that require a brain to learn how to interpret the input from the visual channels. When constructing a spectral instrument we can change E and S , whereas T is unchanging and characterizes the sample. Because the order of multiplication is irrelevant, E and S are mathematically equivalent, which implies that we can either filter out certain wavelengths in the detector or equally well change the illumination spectrum. (In the time domain temporal resolution can be achieved either by using a short shutter time for continuous illumination, or by a short flash combined with an open shutter. In the latter case, as in the one considered by us, it is important that the background light be at a low level.) We can thus create a color picture by taking three black-and-white pictures of the same scene through three different color filters and combining the pictures. As we will see in the following, it makes no difference if we take three black-and-white pictures without color filters and sequentially use three differently color light sources illuminating the scene. In the first case we have a passive sensing method, and in the second case we use an active sensing method, where we need to be able to control the illumination spectrum. This approach is valid as long as fluorescence processes can be neglected.

The light intensity in multispectral images typically needs two spatial dimensions and one spectral dimension. For a color movie we would need four dimensions, with time the fourth dimension. Thus, the light intensity in a color movie is a function of four parameters: $I(x, y, \lambda, t)$. In contrast, the signal from a black-and-white CCD (charged coupled device) or a CMOS imaging chip is a function of only three parameters: $I(x, y, t)$.

Light levels are discretized in the detector by bits, space discretized by pixels, time by frames, and wavelength by color channels. The corresponding resolutions are dynamic resolution, spatial resolution, temporal resolution, and spectral resolution. When acquiring more dimensions than the imager possesses, sacrifices must be made in the resolution and/or the orthogonality of the dimensions. In commercial color cameras microscopic colored filters are placed in a regular pattern on the imaging chip so that some pixels become sensitive to one wavelength band and the neighboring pixels to another wavelength band. Spatial resolution is decreased because one color pixel consists of several original pixels. It is also clear that the pixel positions in space are not exactly the same for the different color channels, and thus the spectral dimension is not orthogonal to the spatial dimensions. In the example where a color picture was made by illuminating the scene with light from three differently colored light bulbs, it is understood that the pictures are taken at different times, and thus the spectral dimension is not orthogonal to the temporal dimension. In other acquisition systems such as the push-broom imager often used in hyperspectral satellites,^{3-5,18} a spatial line is scanned where the colors in the individual elements on the line are dispersed by

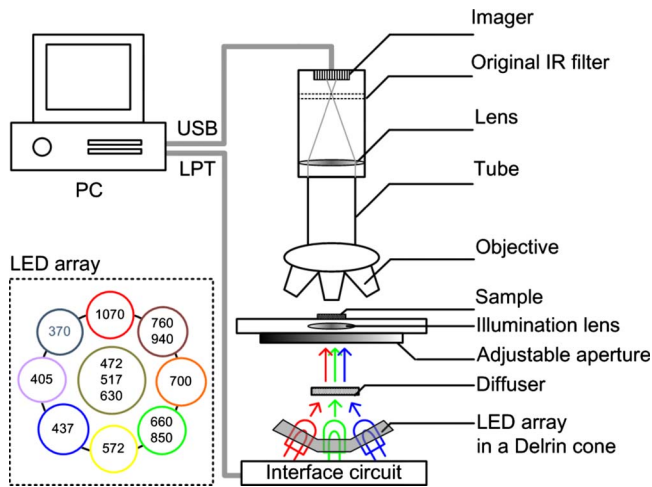


Fig. 2. Arrangement for multispectral transmission microscopy employing multiple LED illumination. Different wavelengths are emitted from a computer controlled LED array and the rays are combined by a diffuser. The light interacts with the sample differently for each wavelength, resulting in a unique picture for each wavelength.

a grating and imaged onto a matrix detector. In this case the spectral dimension is orthogonal to one of the spatial dimensions, but the remaining spatial dimension is not orthogonal to any of the others, including the temporal dimension. In this paper we will present a simple multispectral imaging system, based on the sequential multiplexing of illumination spectra.

III. EQUIPMENT

We used a low-cost USB digital microscope available from www.discoverthis.com. An overview of the system is shown in Fig. 2. It is a transmission microscope, providing magnifications of $4\times$, $10\times$, and $40\times$, and is equipped with a CMOS imager with 640×480 resolution. The imager is used in the black-and-white (gray-scale) mode. The microscope in its standard version is equipped with white-light LED illumination. We adapted this microscope for multispectral imaging by performing the steps discussed in the following. In principle, any conventional microscope can be used for the modifications described.

Most commercial CCD and CMOS imagers are used for visual applications although they also have sensitivity in the NIR spectral range. To suppress the influence from NIR light, visual digital imaging systems are equipped with an IR filter, cutting off the region outside human perception. In our application the NIR range is of great importance and is available more or less for free. Hence, it is important to remove the IR filter, which is located in the optical path in front of the imaging chip.

To achieve multispectral imaging we use multiple LEDs, which can be activated sequentially. We use nine LEDs, three of which have more than one emission wavelength (common anode, multiple cathode pins allowing sequential activation of individual diodes) to provide quasi-monochromatic illumination in 13 wavelength bands ranging from 370 to 1070 nm. The LEDs are Roithner LaserTechnik Models NS370L5RLO, $\lambda=370$ nm; 3P4FCA, $\lambda=405$ nm; B5-4RGB-CBA, $\lambda=472, 517, 630$ nm; B5-433-20D, $\lambda=572$ nm; LED 660/760-04A, $\lambda=660, 850$ nm; ELD-700-

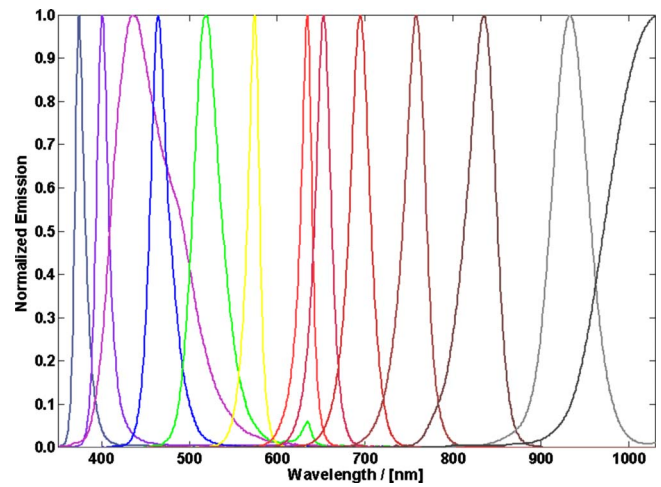


Fig. 3. Normalized spectral emissions of the different LED sources used in the multispectral microscope. Because illumination and sensitivity can be interchanged in spectroscopic instruments, we can consider the emissions as the spectral bands of the system.

524, $\lambda=700$ nm; LED850/940-04A, $\lambda=760, 940$ nm; LED1070-03, $\lambda=1070$ nm; and Farnell TLHB5800, $\lambda=437$ nm. The normalized spectral emission characteristics are given in Fig. 3, as measured separately with a compact Ocean Optics Model USB4000 spectrometer. The nine LEDs are mounted in a machined compact circular arrangement (diameter 32 mm) made of Delrin with eight LEDs on a circle (outer diameter 16.3 mm, inner diameter 13.3 mm); the ninth LED is placed in the center. They are arranged to centrally illuminate the same spot on the light diffuser plate of the microscope. The nine LEDs all have a 5.0 mm diameter epoxy casing and a divergence angle of 20° . The outer eight LEDs are tilted inward 16° in such a way that their optical axes meet 22 mm in front of each LED, where the optical diffuser is situated. The glass components in the microscope are fully transparent in the wavelength range covered by the LEDs. Detailed CAD drawings can be obtained upon request.

The LEDs are powered by an LM317 regulator with constant current of 30 mA. The LEDs are activated from four TTL signals from the parallel port of a computer used to control the equipment and process the images. The TTL signals are converted to VCC voltages by a quadruple LM324 operational amplifier. The binary addressing for the LEDs is decoded by a 4514 integrated circuit, which activates one output at a time as directed by the binary address. The decoded output opens individual BC547 transistors, which in turn activate the specified LED. The electronics fit on a board of dimensions ≈ 5 cm by ≈ 10 cm, which can be incorporated in the foot stand of the microscope. The power is supplied by a 12 V wall plug adapter. A detailed circuit schematic is available.

IV. MEASUREMENTS AND IMAGE ANALYSIS

The objects to be studied are placed on the microscope table. For the lowest magnification setting, $4\times$, an object size of $1260 \mu\text{m}$ times $950 \mu\text{m}$ can be accommodated on the CMOS imaging chip. To illustrate the concepts of multispec-

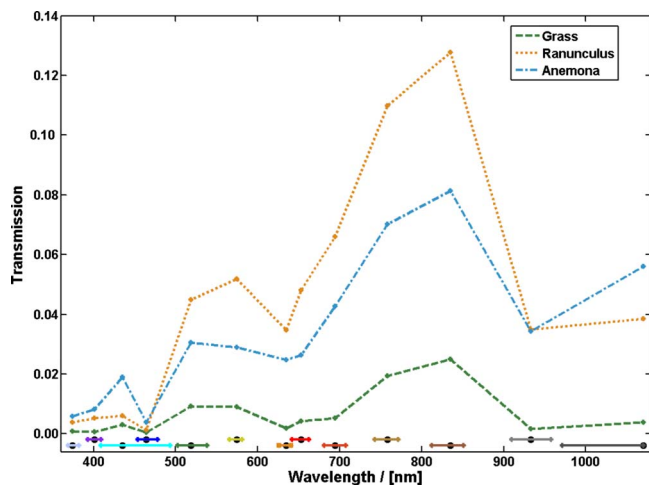


Fig. 4. Transmission spectra of a fresh leaf of grass, and for petals from *Ranunculus* and *Anemone*. Vegetation transmits considerably in the near infrared. The symbols in the lower part indicate $\lambda_{\max}$ and FWHM for each spectral band.

tral imaging we have imaged a number of objects as described in the following. Biological samples were chosen, but the techniques are very general.

Chlorophyll in the chloroplasts of green vegetation has a strong absorption in the blue and red parts of the spectrum, with a somewhat weaker absorption in the green spectral range, causing vegetation to look green. What is not observed by the human eye is the strong transmission for NIR wavelengths beyond 700 nm. A leaf of *Poaceae* (grass) and petals from *Ranunculus* and *Anemone* (Nordic spring flowers) were studied. Pieces of the samples were placed in the same field of view with $4\times$ magnification. A small piece of aluminum foil was also added. Two reference fields were selected in the image; one with no object, which was assumed to have 100% transmission, and one in the aluminum foil area with the assumption of 0% transmission. The transmission spectra were calculated and are shown in Fig. 4. The transmission increases dramatically for the invisible wavelengths from 700 to 760 nm. The purple *Anemone* has a transmission peak in the blue spectral region as well as substantial transmission at longer wavelengths including red. The *Ranunculus* features a high level of longer visible wavelengths, including yellow. The three fresh species also exhibit a strong absorption band due to water, centered around

980 nm. The fact that we acquire an entire spectrum for each of the spatial pixels by sequentially multiplexing the LEDs means that we can perform spectral identification of objects according to their transmission properties. For illustration we need an object with considerable spectral variance within the small field of view of the microscope.

A major difficulty for ecologists is to understand the visual perception of the environment by certain species, particularly because their spectral bands do not always correlate with our own. Physiological (natural) vision systems include complex photonics, such as polarization dependent multimode waveguides, interference filters, and spectral dispersing techniques.¹⁹ In most cases spectral sensitivity is mainly due to a variety of receptors. For certain species receptors are combined with superimposed oil droplet color filters.^{20,21} Spectral resolution varies in number from monochromatic to a dozen bands. Sensitivity ranges from 300 to 800 nm. Because the receptors and their accompanying droplets are spatially separated, it is expected that the effective sensitivity spectra can be retrieved with multispectral transmission microscopy.

A $2\text{ }\mu\text{m}$ thick cross-section of a budgerigar (*Melopsittacus undulatus*) retina was provided by the Vision Group in the Department of Cell and Organism Biology at Lund University. The sample was prepared with stabilizers and conserving agents and molded in epoxy. For other purposes the sample was contrast enhanced with methylene blue dye. The retinal sample was observed at $40\times$ magnification as shown in Fig. 5(a). The multispectral images were acquired with refocusing in between changing the wavelength by sequentially activating the individual LEDs. The background was subtracted and a two-dimensional median filter was applied to remove “dead” pixels. Due to the large numerical aperture and the refocusing, the magnification changes considerably between the spectral acquisitions. This change was compensated by identifying “land marks” and performing spatial rectification transforms.^{1,22} This procedure is time consuming whether the operation is done manually or by correlation techniques. It was observed that the methylene blue dye absorbs strongly between 550 and 700 nm [see Fig. 5(b)], which gives rise to a bluish tint, which is consistent with the well known spectrum for methylene blue. The larger brownish cells are rod cells used for monochromatic low-light level vision. The cones providing the budgerigar four spectral channels are considerably smaller in Fig. 5(a). The molecules responsible for the photoreception are highly unstable and

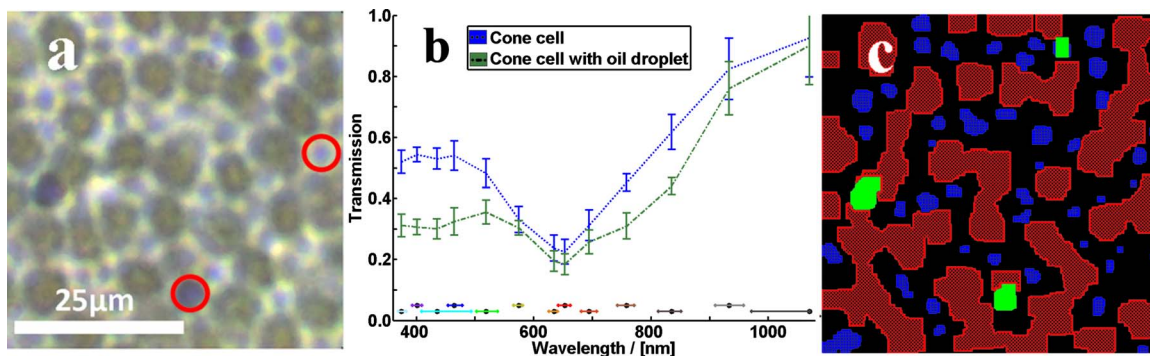


Fig. 5. (a) True color RGB picture of a cross section of budgerigar retina. (b) Spectra from two selected cone cells with and without oil droplet, such as those marked with small circles in (a). (c) Patterned answers from the three contrast functions; each pixel has been identified individually based on its spectral properties. Checker pattern (red) corresponds to rods, bright (green) to cones with oil droplet, and meshed patterns (blue) to plain cones.

photosensitive. Thus the procedure for analyzing these compounds requires darkroom dissection and immediate picture acquisition, which was not done for this paper. Also, the transmission through such samples is influenced by exposure time and light intensity, which makes analysis slightly more difficult. Nevertheless, the oil droplet filters are stable and can be analyzed. Two cones, with and without color filters, were spectrally probed as shown in Fig. 5(b).

The filter effect suppresses wavelengths below 500 nm, which suggests that this particular oil droplet is yellow.^{20,21} As mentioned, budgerigars have four visual pigments, each with a superimposed oil droplet consisting of one out of four types. The purpose of these filters is believed to be to sharpen the sensitivity bands of the cone cells, although the spectral sensitivity is still limited to four spectral channels. It is well known that even very thin oil layers can absorb short wavelengths efficiently. Thus it is surprising that transmission only decreases by a factor of 2 in the short-wavelength range. The reason is that the simple optics of the microscope leads to substantial stray light, which gives rise to an offset in the transmission.

We will now demonstrate how the transmittance coefficients can be combined to answer a specific question of interest. As mentioned in Sec. I, we can approximate a spectrum as a linear combination of a few base spectra (principal components), thus representing the entire spectrum for each pixel (that is, the transmission coefficients for the 13 bands) by a reduced number of terms describing the projection of the pixel spectrum on the dominant principal components. We retain only the dominant projections necessary to describe the detectable independent spectra within the limitations of the noise present. The situation is similar to the case when the true spectral contents of a photographed object are projected onto the sensitivity curves in a commercial RGB digital camera [see Eq. (1)]. The only difference is that the principal components are sensitivity bands, perfectly matched to cover a complete spectral reconstruction in the picture (in contrast, the fixed RGB channels of the camera are present, even if there is no information, for example, in the red color channel for a particular scenario).

We now construct a *discrimination* or *contrast function* F by a multidimensional polynomial expansion in the principal components: $F(PC1, PC2) = k_0 + k_1 PC1 + k_2 PC2 + k_3 (PC1 \times PC2)$, which gives a reduced spectral representation (describing the amount of $PC1, PC2, \dots$ in every measured pixel). We can find k 's such that F is close to one for pixels of the object of interest and close to zero for all others. A matrix formulation is used and solved by minimizing the least square residuals for a group of training pixels where the true answer is provided by an expert in the subject matter (in our case an animal physiologist marking the different cell types). The solution of such overdetermined systems of equations is well known from linear algebra. Setting a threshold in-between (for example, $F > 0.7$) determines if the property is fulfilled (for example, a rod in our case). These procedures form the core of multivariate analysis as discussed in Refs. 10–14.

This approach is now applied to identify the specific oil types and to determine the spatial distribution of the oil. Because we are studying a slice that is only 2 μm thick, droplets are not necessarily incorporated in the specimen slice used. Thus, only some of the cones will be associated with droplets. Three contrast functions were used for a spe-

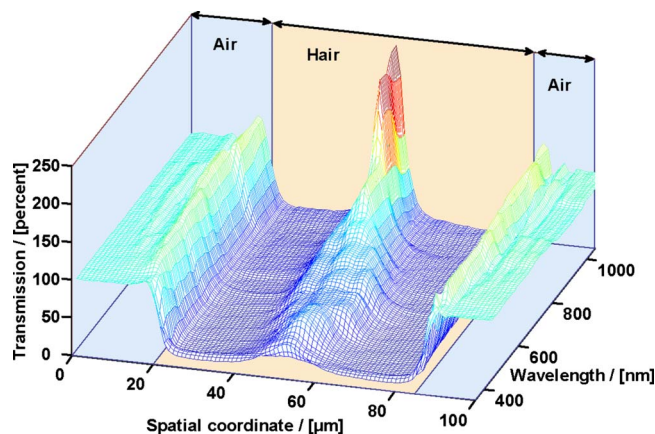


Fig. 6. Transmission cross section for a blond hair, represented in terms of one spatial and one spectral dimension. The hair acts as a cylindrical lens and focuses the light, causing the transmittance to pass 100% in the near infrared region, where absorption is low.

cific type of oil. As described, they are generated by reduction of data by a principal component analysis, followed by a multivariate polynomial model,²³ and eventually employing the binary morphological operations, erosion and dilation.^{1,22} The spectrum for the rods [not shown in Fig. 5(b)] is useful for distinguishing them against the cones. The contrast functions answer the three questions: Is the pixel a rod? If not, is it a cone and, if so, does it carry an oil droplet superimposed? For these three cases we use the following representation: checker pattern (red) for rods, bright (green) for cones with oil droplet, and a meshed pattern (blue) for a plain cone, respectively. The result is shown in Fig. 5(c). We note that sometimes the droplet happen to be larger than the cone proper.

Human hairs have a typical diameter in the range 20–130 μm , with blond hair thinner and black hair thicker. The color of hair is caused by two melanin pigments giving rise to brownish, blackish, and reddish variation. The appearance and characteristics of human hairs is discussed in Ref. 24. Several hair strands were spectrally analyzed.²⁵ Black and gray hair showed constant transmission in the visible region although at different levels, and transmission of brown hair curved upward for red light. Blond hair showed a linear transmission increase from 400 to 800 nm. Of special interest is the symmetry of hairs, which makes them behave like cylindrical lenses, adding further aspects useful for strand characterization. For closer study the blond hair was accommodated in the microscope using 10 $\times$ magnification, and pictures were acquired sequentially for the different LEDs. The background signal was also recorded. Exposure times were adjusted for each spectral channel to exploit the dynamic range. Pictures were normalized by the exposure time, and the background was subtracted. The transmission as shown in Fig. 6 is calculated from the ratio between a region of interest on the object and a nonobscured reference field next to it. Note that refraction has a significant impact on the measurements. Transmission close to the borders of the hair is close to zero because of reflection losses due to the large incident angle. The defocusing caused by achromatic aberrations in the microscope lenses is observed and can be estimated from the steepness of the edges of the hair. We also note that the transmission in the IR region shown in Fig. 6 rises to more than 200% because of the focusing properties

of the hair strand, which is particularly transparent in this spectral region, thus allowing light from further out in the periphery to pile up in the middle. This effect strongly depends on the spectral focal shift. For the long wavelengths we start to see fringes next to the straw edge due to diffraction. Several spatial peaks are seen in the middle of the spectrum. These phenomena can be explained by the combined action of the Snel and Fresnel laws, and the special conditions around the Brewster angle. By further modeling of our relatively simple measurement situation, absolute values for both absorption and refraction index can be found. Thus, the phenomena accessible with a simple setup are very rich.

A detailed analysis of the absorptive and refractive phenomena we have discussed could have considerable use in forensic sciences and animal biology. The method provides further parameters to compare hairs from a crime scene. Hairs are present in various groups, for example, mammals, birds and insects, and contribute significantly to the visual differences between animals. Visual information takes up the largest computational power in the brain²⁶ in most animal species and is a crucial source for decision making in the search for food, predator avoidance, and reproduction. Because the spectral channels of many animals do not match those of humans,^{19,20} a complete spectral analysis such as the one presented here is required for an accurate representation of hair appearance.

V. DISCUSSION

We have shown how multispectral imaging can be demonstrated with simple and readily available equipment. Spectroscopy without spatial resolution was first illustrated for vegetation, resulting in 1D data. For the example of hair the 2D transmission data are arranged in one spatial and one spectral dimension. Full multispectral imaging uses spectral data for all spatial points, in our case recorded simultaneously for each illumination color. The data obtained after recording all available bands constitute 3D data, with two spatial and one spectral dimension. The hair cross section study produces data similar to push-broom scanning, frequently employed on a much larger scale in, for example, satellite imaging.³⁻⁵ In our case one spatial dimension on the two-dimensional detector is used for line imaging, while the other one is used to analyze the dispersed light. The remaining spatial dimension is reconstructed by a sequential read out in the temporal dimension, taking advantage of the well defined movement of the satellite. For stationary objects sequential imaging through a set of pass-band filters can be made, producing spatially similar images which can then be co-processed. In such a setup the spectral dimension is created in the temporal domain. White-light illumination is normally used. Alternatively, as we have demonstrated, it is possible to illuminate small objects sequentially by narrow-band light sources, LEDs, which are commercially available in a range from 245 nm to 7 μm . Thus, the spectral range is frequently limited by the detector rather than the source. Also, in the LED case the full intensity of the light source is utilized, while filtering of the reflected light (or the illumination white light) results in poorer efficiency because most of the light is wasted.

The low price of LEDs makes it economically advantageous to use narrow-band illumination of small objects rather than expensive narrow-band interference or tunable liquid crystal filters on the detection side. The cost of our

entire system, including LEDs, microscope, imager, and interface electronics is less than \$200. For the epoxy LEDs in our setup the brightest LED provides 17 mW of continuous power in the NIR and 4 mW in the UV.²⁷ Significantly increased peak output can be achieved in intermittent operation, because LEDs are primarily limited by the average heat dissipation.

The proof of principle measurements of leaf and petal spectra was done using the original PixArt PAC207 USB CMOS imager, included in the eyepiece of the microscope. The images had to be taken manually with the microscope because of a bug in the drivers, which inhibited automation of the acquisition. For later measurements a Sony ICX205AL imager installed in a Firewire camera was used for convenience.

An important issue with the current microscope is the achromatic aberration in the system. Few systems have a constant focus from UV to NIR. Thus different wavelengths focus on different depths in the sample. We refocused the microscope manually for the different LEDs. To automate the focusing a computer-controlled stepper motor could be used. Better solutions can be found by treating the image data in the spatial Fourier domain.

Recently, several efforts have been made to optically multiplex LEDs using advanced ray tracing and molded optics.²⁸ The aim is to join rays from several spatially distributed sources, losing as few photons as possible. Our system bases such multiplexing on the use of a diffusing plate, where several LEDs illuminate an almost identical spot from different angles. Ideally, light scatters in all directions, producing an even Lambertian-like distribution on the opposite site of the diffuser. Off-axis illumination influences the acquired picture in several ways. Light from a different direction will change the reflecting conditions for the many surfaces in the sample. Off-axis illumination will also cause features out of focus to be laterally translated in the picture. Therefore, the illumination profile should be as identical as possible for each wavelength to acquire the spectral properties in each pixel. The profile can be improved by using additional diffusers and apertures trading off the throughput. Uneven light distribution over the image can effectively be compensated by fitting low-order polynomial surfaces for each LED.

Fluorescence microscopy is possible but was not demonstrated here. Because fluorescence is a consequence of absorption, it is a powerful method to ensure that contrast arises from molecular absorption and less from refractive index gradients. Spectral information in fluorescence can be provided by different excitation sources or by a spectral analysis of the resulting fluorescence light. Thus, in principle we could provide an excitation-emission matrix in each pixel, multiplexing LEDs, and take advantage of the RGB filters in the color imager. Even LED-based fluorescent lifetime imaging could be performed with a pulsed LED employing the rolling-shutter exposure control of inexpensive CMOS imagers. Imaging in the temporal domain using LEDs has been demonstrated using more expensive equipment.²⁹

In conclusion, we have demonstrated how a low cost setup can be used to illustrate the powerful concepts of multispectral imaging. We chose a microscopy implementation due to cost and educational aspects. The simple equipment suffers from certain deficiencies such as chromatic aberration and stray light and limitations in the available software drivers. Still it was possible to demonstrate advanced multispectral

concepts including multivariate analysis, providing many possibilities for quality inspections and object identification. Such techniques are now being pursued for diagnostics related to malaria.³⁰

The simple case of transmission spectroscopy on grass leaves and flower petals and other specimens is suitable as undergraduate laboratory problems or classroom demonstrations. Multispectral image analysis can also be used for these simple cases or for pollen, spores, and biological cells. The study of human hair strands with all its intricacies regarding various optical phenomena is another example of a possible student project. The bird retina example illustrates that even emerging research in limited-resource environments can be pursued.

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