REVIEW

Basic strategies for valid cytometry using image analysis

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Summary

The present review provides a starting point for setting up an image analysis system for quantitative densitometry and absorbance or fluorescence measurements in cell preparations, tissue sections or gels. Guidelines for instrumental settings that are essential for the valid application of image analysis in cytophotometry and cytofluorometry are described. The general principles of the working mechanism of CCD cameras in combination with general methods to improve the behaviour of the cameras are presented. Optimization of illumination of microscopical and macroscopical objects receives special attention because of its importance for valid cytometry. Sources of errors in quantitative measurements are listed and step-by-step charts for tuning the CCD camera, frame grabber and illumination for the optimal use of the systems are described. Suggestions are given for improvement of image arithmetics in difficult imaging situations, such as low fluorescence signals and high absorbance signals.

Introduction

Image analysis has opened wide perspectives as a new tool in cytometry. In principle, it allows fast and accurate measurements and offers a great variety of applications and approaches in cell biology, physiology and pathology. One of the most important advantages that image analysis offers is the possibility of collecting large amounts of quantitative data linked with morphological or structural information on tissues and cells from a single experiment. Digital imaging and image processing always give numerical results. This does not automatically imply that these numbers accurately reflect the situation in the object providing the digital image. Too often, the use of image analysis has seemed deceptively simple, and many errors have been made that could easily have been prevented. An example is the recent discussion on DNA measurements in Feulgen-stained sections of neoplastic tissues using image analysis and the confidence in the corrective power of commercially available insufficiently validated

computer software (Rubin *et al.*, 1994; Cohen & De Rose, 1996; Chieco & Van Noorden, 1996, 1997; Gschwendter & Mairinger, 1997). Therefore, rigid validation of the methods of acquiring data is essential. The criteria for proper cytophotometry, as formulated by Stoward (1980), still apply. In conjunction with these criteria, the settings of the image analysis system that is used have to be checked along the lines set out for absorbance cytometry by Chieco *et al.* (1994) and for fluorescence cytometry by Rost (1991). A list of the main sources of errors in image cytometry and ways and means of avoiding them can be found in Chieco *et al.* (1994). Good general textbooks dealing with image analysis are Inoué (1986) and Shotton (1993).

A proper understanding of the possibilities and limitations of image analysis systems that are presently available is necessary because, in principle, each application requires particular adjustments of the equipment. Proper application of instrumentation alone is no guarantee of valid analysis. The use of software for image processing and image

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analysis also requires considerable understanding. In the present paper, the setting up of image analysis systems for valid absorbance and fluorescence measurements will be reviewed. The purpose of the present consumers' guide is to discuss what exactly is and what is not required for various applications and to provide insight into the main sources of error.

Image analysis systems usually consist of the following components (Fig. 1): (i) the instrument that creates the images, being a microscope or a macroscope with its illumination system; (ii) the camera that captures the images; (iii) the computer, including frame grabber or interface, and software for processing of the images and analysis of data obtained from the images; and (iv and v) the monitor(s) to visualize the images.

The present paper is limited to the application of image analysis to microscopy and macroscopy in life sciences. We assume that the specimens, such as cells or tissue sections, of which images are generated, are available and ready to be analysed, and that the staining applied to the specimens is specific and valid. This aspect of cytometry and cytophotometry is also very important but has been reviewed extensively in the past (Stoward, 1980; Chayen, 1984; Van Noorden, 1989; Chayen & Bitensky, 1991; Lyon, 1991; Stoward & Pearse, 1991; Sutherland *et al.*, 1991; Van Noorden & Frederiks, 1992; Vohradsky & Panek, 1993; Mize, 1994). Morphometric analysis of the shapes and sizes of objects will not be dealt with either because this has been extensively reviewed by others (Marchevsky *et al.*, 1987; Russ, 1994; Nazeran *et al.*, 1995).

First, the various components of image analysis systems are described. Afterwards, we discuss the main errors that are made and how to avoid them.

Optics and illumination

Objects such as electrophoresis gels or large tissue sections of whole organs (macroscopy) or objects such as small tissue sections or individual cells (microscopy) are considered separately for imaging. For the formation of images of macroscopic objects, an objective of a photo camera, e.g. a 50-mm (macro) lens is the best option. These lenses give more homogeneously illuminated images at magnifications up to 2.5 times than microscope objectives. These types of objectives allow imaging of objects as large as gels or adult heart sections at comfortable working distances. For both macroscopical and microscopical purposes, the optics have to be impeccably clean, as described in many microscopy handbooks, such as James and Tanke (1991) and Bradbury (1989). These are well-written handbooks that provide all the information needed for high-quality microscopy. Microscopical errors, such as glare and diffraction (Goldstein, 1975, 1981; Duijndam *et al.*, 1980; Chieco *et al.*, 1994), can play significant roles in quantification but are not specific for image analysis and, therefore, will not be discussed here.

High-quality illumination is one of the major requirements for quantification. Homogeneous illumination of the field of view is even more important for image analysis than it is for photography. The image analysis system is much more sensitive to temporal and spatial variations in illumination than the human eye, because the latter adapts very well to differences in illumination. The better the illumination, the more precise the quantification.

Spatial heterogeneity is encountered particularly when low magnifications are used. When illumination of the field of view is insufficiently homo-

Fig. 1. Hardware requirements for image analysis in cytometry. A microscope or macroscope (I) is used to generate an image of the object on the sensor of the camera (II). An interface unit in the computer (III) translates the image of the camera for the computer and can also be used to control the camera (frame integration, shutter time, etc.). Images are visualized on a monitor (IV). An extra monitor (V) is a convenient means of displaying the data.

geneous, the application of a randomly ordered fibre bundle can be considered (Reitz & Pagliaro, 1994). The fibres in the bundle scramble the light and distribute it evenly over the field of view when placed between the illuminator and the object. External (fluorescent) laser illumination can also be considered, particularly at low magnifications (Wittrup *et al.*, 1994). Remaining unevenness in illumination can be corrected for by flat fielding with the use of a blank image.

When images are recorded under bright illumination, short exposure times are used to prevent overexposure. When an alternating current (AC) power supply is used, the amount of energy in the illumination may vary from image to image, depending on the onset and duration of exposure (Fig. 2). The quality of images can suffer from this effect when using electronic shutters, which allow exposure times as short as 0.1 ms, which is considerably shorter than the cycle time (20 or 16 ms) of 50 or 60 Hz mains frequency. The best solution for avoiding this type of temporal heterogeneity is the application of direct current (DC) power supply for illumination. These devices, also known as 'stabilized power supplies' also minimize temporal heterogeneity caused by variations in the electricity mains. In environments where heavy-duty loads are switched on and off, as is typical in laboratories and hospitals, transient changes in the intensity of illumination may considerably influence the results of image analysis.

Imaging devices

Images are captured with an image-recording device, for example an electronic camera. At present, the most commonly used type is the charge-coupled

device (CCD) camera. CCD cameras can be cheap and simple video cameras or very expensive when they are sophisticated devices especially developed for high-performance scientific image recording (Hiraoka *et al.*, 1987). The price difference between these cameras can be 10- to 100-fold. The cheap cameras perform amazingly well for routine cytophotometric purposes, but when high spatial resolution, dynamic range or sensitivity are required, high-performance cameras should be used.

Direct capturing of images of an object on an image sensor is the easiest and safest way of digital imaging for quantification. When images are recorded from an intermediate medium, such as radiographic film, photo(micro)graphs or photographic films, extra sources of non-linearity and thus of inaccuracy may be introduced. Results of scanning of these kinds of image carriers are likely to be more dependent on the processes that generated the intermediate images than they are on the properties of the original object. These information sources may be useful for morphometric analysis, but not for cytometry. Therefore, hardware that is capable of recording images of prints, negatives and slides, such as flatbed scanners, X-ray scanners and slide scanners, is outside the scope of the present paper.

Digitization of electronic images

Recorded images have to be digitized in order to be processed and analysed with a computer. A frame grabber is the device that is used to quantify images from analogue video CCD cameras, whereas digital scientific CCD cameras contain a special interface for this purpose. The merits and drawbacks of both systems will be discussed. A set-up procedure for

Fig. 2. Schematic representation of the energy of illumination (area under the curve) of a (microscopical) object when alternating current (AC) power supply is used. Depending on the onset and duration of exposure, the amount of energy may vary (three examples are shown by the hatched areas). Only when entire cycles of the mains frequency are used is a constant amount of illumination energy generated (black areas).

image recording for quantification purposes will also be presented.

Software for image processing and analysis

Selection of a specific type of software depends heavily on priorities. For example, quality of image representation or precision of calculations are features that dictate the choice of software. Options to control peripheral hardware, such as switches for the integrating times of cameras, activation of mechanical shutters for excitation illumination or selection of filter combinations using filter wheels via the software, are other aspects when choosing software. We will also discuss various methods of preventing or circumventing problems that may arise from limited precision arithmetics in software.

Optics and illumination

Densitometry and fluorescence

Two types of measurements of signals from macroscopical or microscopical objects are densitometry and fluorometry. Densitometric measurements are based on the ratio between the amount of incident light and the amount of light passing through the object captured in the image. The difference between the two is caused by either absorbance or scatter of light by the object. Fluorescence measurements are based on capturing light (photons) emitted by fluorescent molecules in the object after being excited by illumination.

Densitometry. In densitometry, the intensity of light passing through an object (I_n) is measured with a detector and related to the amount of light that would have been received by the detector, if no object were in the light path (I_0). I_0 is determined by recording a blank image. Each pixel in this image represents 100% of illumination. This does not mean that each pixel in the image has the same grey value because of uneven illumination, as is discussed above. Densitometric data can be expressed as 'density' or 'absorbance'. Both density and absorbance are calculated as $\log(I_0/I_n)$. This process of density or absorbance calculation implicitly applies flat fielding or shading correction by assuming that $I_0 = 100\%$.

The principal difference between density and absorbance lies in the nature of the object in the light path. An opaque object, such as a silver grain in a photographic emulsion, does not absorb light but blocks it by scatter or reflection, and hence leads to a density value. Density values are measured by densitometers. On the other hand, a coloured precipitate, such as a dye, selectively absorbs (part of) the light of part of the spectrum

(Fig. 3). As a general rule, to measure the amount of dye precipitated accurately, monochromatic light of the wavelength has to be used at which the dye absorbs maximally (Fig. 3C). This is known as absorbance, optical density or extinction. For absorbance measurements, monochrome cameras are more suitable than colour cameras. When a monochrome camera is not available, a three-chip CCD colour camera has to be used. In that case, only one of the three channels (red, green or blue) has to be selected. We will apply 'absorbance' only when a coloured product is measured with the use of monochromatic light. When white light is applied, all light of wavelengths different from that absorbed by the dye passes unhampered through the specimen. This may result in errors in measurements because differences between I_n and I_0 are then relatively small (Fig. 3A and B). Therefore, to determine absorbance as precisely and specifically as possible, monochromatic illumination (Fig. 3C and D) is essential (Goldstein, 1975). Off-peak monochromatic measurements can be made under certain conditions, for example when the dye absorbs too strongly at the peak wavelength (Fand & Spencer, 1964; Goldstein, 1975; Allison *et al.*, 1981). Absorbance measurements can go wrong more easily than density measurements, because white light can be used without any problem for the determination of the densities of opaque objects. Monochromatic filters, such as bandpass filters and interference filters, may be of the incorrect wavelength, they may have other spectral characteristics than assumed or have too large a bandwidth in relation to the absorbance spectrum of the coloured precipitate allowing light of the wrong wavelength to pass unhampered. Bandpass filters and interference filters selectively absorb or reflect most of the visible light, respectively, and only a small part of the light of a particular wavelength passes the filter (Figs 3C and 4). The absorbance value calculated from the transmitted energy is only a correct measure for the amount of precipitate when the correct filters are used. Therefore, we strongly advise always characterizing filters in relation to the absorbance spectrum of the coloured precipitate for applications in cytophotometry with a spectrophotometer, when the suppliers do not provide a transmission curve for the filters. It is advisable to check the filter characteristics on a regular basis (say, once every year). Refer to Goldstein (1975, 1981) for further reading with respect to the use of monochromatic light in absorbance measurements.

Density measurements can be performed with monochromatic light of any wavelength or with white light, because they are simply based on blocking incident light by opaque objects (e.g. silver grains). The amount of light blocked is proportional

Fig. 3. Examples of the energy spectra of illumination (hatched areas of A and C) and the energy spectra of light transmitted through an object (hatched areas of B and D) illuminated with white light (A and B) and with monochromatic light (C and D). An imaginary absorbance curve of a particular dye is shown in A (black area). The transmitted energy of illumination as shown in A and C after (partial) absorption by the dye is shown in B and D. The total amount of energy present in these spectra is approximately 95% and 5% of the total illumination energy, when white light or monochromatic light are used respectively. Therefore, measurements with the use of monochromatic light are more precise.

to the area of the opaque objects and proportional to the mass of the silver present in the field of view (Rogers, 1979; Hurter & Driffield, 1890). The opaque objects do not need to be homogeneously distributed over a picture element (pixel). When the opaque object covers 50% of the area of a pixel, the pixel correctly detects a transmission value of 50%, if errors such as glare or light scatter are avoided. When measuring integrated density, glare can be entirely discarded as a source of error, even with heterogeneously distributed silver grains (Chieco *et al.*, 1994). For absorbance measurements, the dye has to be distributed evenly over the pixel to avoid distributional errors (see next section).

Illuminators also emit large amounts of infrared light besides ultraviolet and white light. Spectrophotometers use monochromator prisms or gratings to deflect infrared light, but CCD cameras are used in combination with interference filters or bandpass filters. Gelatin filters are designed to block specific parts of the light in the visible spectrum but do not always block infrared light, which is outside the visible spectrum (Fig. 4). In contrast to the human eye, CCD cameras can detect infrared energy efficiently, and this may lead to erroneous absorbance measurements. Because the energy of infrared

light passing through a gelatin bandpass filter can be relatively large (Fig. 4), infrared incident light has to be blocked by an additional infrared blocking filter. In this respect, the use of glass interference filters is safer than the use of gelatin filters (Fig. 4). Furthermore, gelatin filters cannot deal very well with the energy from the light source. Energy that is absorbed will heat up the filter, and care has to be taken to prevent the filter from melting and becoming unreliable. The same holds for neutral density filters that are used for dimming light without changing the emission spectrum. However, gelatin filters are usually much cheaper than glass filters. When gelatin filters are applied, their spectral characteristics should be checked regularly with a spectrophotometer.

Infrared light can be used to detect opaque particles over a stained tissue, as the coloured product will be fully transparent to the infrared light (P. Chieco, unpublished data).

Distributional errors. Homogeneous distribution of a dye over each single pixel in the object plane is a prerequisite when image analysis is used for absorbance measurements (Chayen, 1984; Van Noorden, 1989). When applying Lambert–Beer's law, the

Fig. 4. Characteristic absorbance curves of a glass interference filter, a gelatin bandpass filter (Wratten 92), a glass infrared (IR) blocking filter and a combination of the gelatin filter and the infrared blocking filter. A considerable amount of energy emitted by common illuminators at wavelengths > 800 nm (infrared light) passes gelatin filters unhampered, because they are designed to block light in the visible range only. When an infrared blocking filter is used as well, the light transmitted through the combination of the filters and available for the illumination of the specimen also approaches monochromatic light 'in the eyes of the camera'.

concentration of a dye in a precipitate can be calculated from its absorbance, when the object thickness (e.g. section thickness) and the molar extinction coefficient of the dye in precipitated form are known. When a dye is heterogeneously distributed, the spatial resolution of the camera should be high enough to realize homogeneous distribution of the dye in each pixel (see, for example Jonker *et al.*, 1995). If this is not the case, the calculation of absorbance will not be correct. This error, which is known as distributional error (Ornstein, 1952), is best explained by an example. Two areas of the same size are completely covered by one pixel and contain the same amount of dye. In the first one, the dye is homogeneously distributed over the entire area, resulting in an average transmission of 10%. In the second case, the dye is distributed over half the area, whereas the other half is empty, resulting in transmissions of 1% and 100% respectively. In the first case, the absorbance is $-\log(10\%) = 1$ and, in the second case, the absorbance is $-\log((100\% + 1\%)/2) = -\log(0.505) = 0.3$ (James, 1983). The size of the pixel on the chip of a CCD camera is usually in the order of micrometres.

Therefore, the size of pixels in the object plane is smaller than the resolving power of the light microscope when objectives with a magnification of 310 or more are used. Thus, the distributional error can be neglected when using CCD cameras and this order of magnification, because the resolving power of light microscopy is the limiting factor. Images of large areas of sections or gels at low magnification but with sufficiently high spatial resolution can be obtained with high-resolution cameras to avoid distributional errors in absorbance measurements (Jonker *et al.*, 1995). A typical example is the CCD camera of Photometrics (Tucson, AZ, USA), which contains a chip of 1317×1035 pixels instead of the usual 768×575 of video cameras. To reduce the amount of storage space for these images, multiple pixels of the image (e.g. 2×2) can be averaged after the calculation of absorbance has been performed (Fig. 5). In this way, distributional errors can be avoided.

Both absorbance and density measurements should always be accompanied by a calibration procedure, in which a set of neutral density filters with known absorbance is recorded. Our advice is

Fig. 5. Enlargement of a detail of an image displayed at two levels of spatial resolution. (A) 70 $\times$ 35 pixel-sized grey value image as recorded at high spatial resolution for the calculation of absorbance. (B) Image of the same area as in A after the calculation of absorbance and gathering an averaged neighbourhood of 2 $\times$ 2 pixels. In this way, a four-fold reduction in memory requirement is accomplished, whereas distributional errors are avoided.

to capture images of the filters routinely at the beginning and the end of a series of measurements, and to store these filter images together with the biological images. This allows calibration of the images in retrospect and avoids variations in measurement caused by the optical system.

In conclusion, densitometric measurements require: (1) a stabilized power supply for illumination; (2) a proper combination of magnification and spatial resolution of the camera that guarantees homogeneity of a dye in a pixel, to avoid distributional errors in absorbance measurements; (3) illumination that is as homogeneous as possible in the field of view; (4) the use of infrared blocking filters and monochromatic filters appropriate for the dye involved for absorbance measurements; and (5) recording of a set of neutral density filters as an internal standard in each series of measurements.

Fluorescence. When image analysis is applied to fluorescence, the emission of light from a fluorochrome is measured. A fluorochrome is excited by light of a specific wavelength from an illuminator and emits light of a different (longer) wavelength. A homogeneous field of view can be obtained by imaging a uniformly fluorescent uranyl block or a yellow gelatin filter, which is fluorescent. This can be of help in checking whether or not the illumination by the excitation light is evenly distributed over the entire field of view. It can also be used to obtain an image for flat fielding, which is also called shading correction. An image of the homogeneously luminescent uranyl block reveals variations in the

intensity of the excitation light. When illumination is not homogeneous or when optics are not perfect, the pixel values in the image of the block can be used to correct the images of fluorescent objects. The value of each individual pixel in the image of a fluorescent object is then multiplied by the reciprocal value of the corresponding pixel of the image of the uranyl block. For example, when the periphery of a luminescent uranyl block yields only 80% of the signal that is found in the central area, the signal in these pixels is multiplied by 1.25; when the periphery yields 90% of the central area's intensity, its signal is multiplied by 1.11. In this way, each pixel value is scaled to unity illumination and the calculated pixel value correctly represents the amount of fluorescence. However, improvement of the original image by optimizing the instrumentation always yields better results than mathematical correction of poor images. For an overview of the selection of illumination and detection devices, see Ploem and Tanke (1987), Rost (1991) or Spring (1991).

The image analysis system has to be calibrated for the reliable measurement of fluorescence signals, as is the case for density and absorbance measurements. The response of the camera has to be linear with the concentration of fluorochrome and with exposure time. Microscope and camera can be tuned to optimal performance with the use of fluorescent latex beads. The bright fluorescent beads allow a correct setting of the imaging device to prevent overexposure. The digitized pixels in the bead's image show the maximum value allowed by the system (see flowchart 'camera set-up' in Fig. 6).

Fig. 6. Flow chart for optimized use of the dynamic range of a camera system for densitometric and absorbance image analysis.

Overexposure of the imaging device should be avoided in density measurements, as well as in fluorescence measurements.

We advise capturing a set of images of a range of known concentrations of the fluorochrome with each series of measurements. This allows calculation of the amount of fluorescence in the images and determination of the response of the imaging system. This is a similar procedure to the use of neutral density filters in absorbance measurements.

Distributional errors do not play a role in fluorescence measurements simply because the number of photons that hit a pixel are counted.

Imaging devices

Several types of detecting devices have been developed to prevent distributional errors in absorbance measurements in microscopical images (Deeley, 1955; Goldstein, 1975; Altman, 1975; Smeulders *et al.*, 1987). The traditional scanning and integrating cytophotometers were based on one measuring spot that scanned an area in the microscopical object and subsequent integration of the individual measurements. This integrated signal was the mean absorbance value of that area. These scanning and integrating cytophotometers are based on the same measuring principle as CCD cameras and can be considered as a 'primitive' form of pixel imaging. Each pixel of a CCD camera acts as a single measuring spot. The main difference is that all pixel values are recorded simultaneously as separate values, while an integrating cytophotometer can only determine one average value for the whole area that is measured. Besides scientific digital output CCD cameras, analogue output video CCD cameras are used in image analysis. Both types of cameras have their own merits and drawbacks related to the nature of the images and the procedures used to capture these images. Therefore, these aspects are discussed here in conjunction with the principles of analogue video cameras with their frame grabbers and the principles of digital scientific CCD cameras with their interfaces.

When a single emission wavelength is used in fluorescence imaging or when fluorescence at two or more emission wavelengths is generated and images are captured not simultaneously but sequentially, a monochrome camera is sufficient. When emissions at multiple wavelengths are measured simultaneously, for example in the case of double and triple labelling in fluorescence *in situ* hybridization or immunohistochemistry, a set of filters plus a monochrome camera can be used (see below), or the different emission signals can be measured at once with a colour camera (Tanke *et al.*, 1995). Three-chip cameras are particularly useful for these purposes. Three-chip cameras contain one chip for red, one for green and one for blue light, and each chip completely covers the field of view. The CCD in one-chip colour cameras contains a repetitive square 2 3 2 matrix of pixels sensitive for red, green, blue and green light. As the human eye mainly obtains information on the brightness of an image from green light, the green pixels are repeated more often than the red and blue. Effectively, every set of four neighbouring pixels represents the total intensity

and spectral information of their joint area. Therefore, these cameras readily miss information because a particular fluorochrome in the object plane may not be covered by the corresponding coloured pixel in the chip of the camera. The three-chip colour camera avoids this problem because, after being separated by a prism, each of the colours of the object is detected by a separate chip that covers the entire image. A green fluorochrome hardly produces a signal in red or in blue and vice versa. When three-chip colour cameras are used for the detection of a single fluorochrome, the channel to be sampled should be the one with the highest efficiency for light at the emission wavelength of the fluorochrome to optimize the sensitivity of the detection system.

An important advantage of image analysis is that weak images can be improved by the integration of images in time. Weak specific signals are often encountered, particularly in fluorescence microscopy. This may be either a weak signal in an otherwise empty field or a reasonably strong signal amid a background that is only a little weaker. Analysis of weak signals in a low background demands sensitive low-noise cameras, whereas a signal in a relatively high background demands cameras that have a high dynamic range as well. The weak signal is detected in both cases by time-integrated recording of photons. When the background is low, this is sufficient, because the specific signal increases linearly with time. When the background is high, a camera with a high dynamic range is necessary in order to correct for the high background and to allow integration for a longer period of time. This increases the absolute difference between signal and background.

Two main categories of CCD cameras exist: the CCD camera as in consumer electronics (analogue video cameras) and CCD cameras developed for scientific use (digital scientific cameras). Digital scientific cameras are slow and rather expensive, but are highly sensitive and have a highly dynamic range and spatial resolution. Video cameras combine fast operation (30 frames or images per second) and low cost. This type of camera is connected to the computer by a video frame grabber. Video cameras can be monochrome or colour. Monochromatic images are essential for cytophotometric measurements, so monochrome cameras are the best option. When a filter wheel is applied, the monochrome camera can be used to sample various signals at different narrow bands in the spectrum. When several of these measurements are combined, information on the colour composition of the sample can be obtained and can be expressed as pseudocolours (Chikamori *et al.*, 1991; Tanke *et al.*, 1995). Change of emission filters can lead to errors

caused by 'pixel shift', because each filter has its own optical properties (Tanke *et al.*, 1995).

When a monochrome camera is not available, colour cameras can be used for absorbance cytometry, but then one appropriate channel (red, green or blue) has to be selected. Furthermore, application of a monochromatic filter for illumination is a prerequisite, because the bandwidth of each of the three channels is too wide for proper analysis of the absorbance of a dye when using white light. Colour cameras have their main application in the field of fluorescence, for example when multiple fluorescent probes in a specimen can be excited at the same wavelength. As all fluorochromes have their specific emission spectrum, their fluorescence in an image can be separated. Co-localization of two or more fluorochromes each having a specific ratio between the signals in the red, green and blue channel can also be determined without the need to change filters. A multispectral image of all fluorochromes can thus be obtained at once.

CCD cameras capture photons on a chip. The photons excite the silicon and liberate electrons. The electrons are stored in potential wells on the surface of the chip, building up a charge. The charge that is built up in a well can be moved across the silicon surface by selectively coupling adjacent potential wells, hence the name 'charge-coupled' device. Each pixel functions as an individual detector element. For a precise explanation of the physical properties of CCD cameras, see Aikens *et al.* (1989) and Aikens (1990).

Analogue video CCD cameras

The first video cameras were thermionic tube cameras, such as the Vidicon and Plumbicon cameras. These tube cameras were not as accurate as CCD cameras because of the absence of physical pixels. In the recent past, they have been replaced by CCD cameras. The principal characteristics of video cameras are the analogue video signal, in which the image information is packed, and the frequency of images generated, which is in step with the frequency of the mains supply (50 or 60 Hz).

Signals from video cameras can be displayed directly on a video monitor or recorded with a video recorder. Images recorded with video cameras are recorded in analogue signals combined with synchronization pulses, which become available via a video connector. For the analysis of a video image, it has to be converted first into a digital image by a video frame grabber. The video signal is sampled by an analogue-to-digital converter (ADC) on the frame grabber board to decode the image information. Synchronization pulses mixed with the signal by the video camera are used by the frame grabber to keep in step with the video camera. After

each scan line, a horizontal synchronization pulse or hsync is emitted by the camera. This helps the frame grabber to divide each image line into pixels by dividing the signal in time between two hsync pulses. Two images or frames are separated by a vertical synchronization pulse or vsync. With the use of hsync and vsync, the frame grabber can compose a digital image. The digital image is then stored in the computer memory.

Errors of electronic images

Both in digital output cameras and in the frame grabber used for analogue cameras, an image represented by electrons is converted to an image represented by numbers. This conversion can be a source of errors. In encoding of an electronic image on the camera chip in a video signal and in decoding the video signal to produce a digital image, information can be lost and errors can be made. Methods to reduce these errors and to increase the precision of the conversion are discussed below.

Dark current or thermal noise. Liberation of electrons on the silicon surface of the CCD chip by incident photons can also be accomplished by thermal energy. This causes a flow of electrons into the potential wells. This noise is called 'dark current' and is directly related to the temperature of the silicon surface. When the camera chip is cooled, as in scientific CCD cameras, dark current is reduced. This cooling can be passive by convection, by Peltier element (thermoelectric) or by liquid nitrogen (fluid). Video cameras are generally not cooled. Electronic countermeasures to reduce dark current are implemented in some video cameras. Low dark current allows longer periods of integration and, thus, increased sensitivity, because the background signal remains low. This is mainly relevant for the imaging of weak fluorescence signals.

Frame transfer noise. The slower the process of moving a column of electrons in potential wells is executed, the smaller the error of electron redistribution (a particular type of noise). At video rates, a minimum of 30 frames per second needs to be shifted out. When electrons are not given enough time during the shifting process to flow from one potential well to the next, part of the electrons that form the signal are redistributed and thus give rise to inaccuracy. This process takes place when an image is transferred off the CCD chip and hence is called frame (image) transfer noise. Lower transport rates of images, as occur in scientific digital output CCD cameras, enable better separation of electrons in one pixel from those in adjacent pixels.

Amplifier and digitizer noise. Another source of noise present in signals obtained from CCD video cameras is amplifier and digitizer noise. The weak signal formed by the electrons in the potential wells has to be amplified before it can be digitized. The quality of the amplifier determines how much amplifier noise is added to the signal. Subsequently, the amplified analogue signal is converted into a number when it becomes digitized. This number is represented by a shade of grey on the computer monitor. The precision of this conversion depends on the quality of the digitizer. The round-off error that occurs in the digitization process has on average the magnitude of half a grey value. The smaller the voltage range mapped to each grey value, the more precise the digitization. This round-off error made by the digitizing chip is called digitizer noise. Fast one-step converter chips are generally limited to 256 grey values (8 bit) and are generally less precise. Deviation of one grey value is common and means a 0.4% error. Slow converter chips approximate the analogue signal in successive steps and allow higher precision in more grey values, e.g. 4096 (12 bit). Moreover, deviation is usually half a grey value. This means an error of at most 0.012%. The benefits of this high precision are shown in Fig. 7.

Common video digitizer boards may use a special chip set (e.g. the Philips SAA series) intended to digitize colour home videos for multimedia computers. These digitizers use only 176 grey values, while additional grey values are interpolated. For reasonable accuracy in cytometry, this dynamic range is generally not sufficient, because 256 grey values are considered to be the minimum. So, depending on the type of detector, the number of available grey levels is 176 (commonly used in low-cost video frame grabbers found in audio-video inputs of multimedia computers), 256 (in scientific video frame grabbers), 4096 (in medium-grade digital output cameras or scientific frame grabbers) or even 65 536 levels (high-performance digital output cameras cooled by liquid nitrogen).

Reduction of noise errors in weak signals

Some frame grabbers are able to switch particular video cameras to a special integration mode (e.g. the Cohu 4000 camera; Cohu, San Diego, CA, USA) to avoid errors occurring in the registration of weak signals by video cameras. Instead of delivering 30 full frames per second, the control circuit stops this process of continuous video signal output and electrons are accumulated on the chip in the CCD camera. Time integration is expressed in milliseconds or in frames; each frame is usually 1/30th of a second. After a set period of time, the frame grabber switches the camera back to video operation mode

Fig. 7. Differences between an 8-bit (—) and 12-bit (—) camera with similar full well capacity with respect to digitization precision. The camera with the higher digitization precision (12-bit camera) can assign individual grey levels to individual photons, whereas the less precise camera (8-bit) can assign one grey level per 10 photons only. Especially when the background level is close to the signal level, the camera with the higher digitization precision can still discriminate small differences between signal and background, whereas the less precise camera cannot.

and the integrated image is transferred. The contribution of digitizer noise can be reduced by integrations of a number of frames; the signal will increase linearly with the number of integrations, while the contribution of noise, which is random, is linear with the square root of the number of integrations. Signal-to-noise ratio (SNR) can thus be improved by increasing the signal more than the noise levels. This is particularly important when weak signals have to be analysed. An integrated image contains accumulated signals over the number of frames captured, whereas amplifier and digitizer noise are from a single frame. Dark current is not reduced by this integration process. Limiting factors for time integration are the total number of electrons that can be captured (or in other words 'full-well capacity'), digitizing precision and dark current of the camera. However, when the contribution of dark current is known, an image can easily be corrected by subtracting an estimated average dark current value. This estimation can be obtained reliably by averaging dark current images.

A method that resembles integration of frames on chip is the averaging of frames in the computer or frame grabber. A series of images is captured and digitized. The computer calculates the average values of the pixels in these frames and thus reduces the contribution of digitizing and thermal (random) noise in the images.

On-chip integration instead of frame averaging is particularly suitable for reducing digitizer noise in weak signals and for the estimation of dark current and the detection of low signals in general. The quality of a weak-signal image obtained by frame averaging and corrected for frame-averaged background is far less than the quality of an image

obtained by on-chip integration of the image and corrected for dark current estimated by on-chip integration (Fig. 8). With on-chip integration facilities, video cameras benefit from the speed of video and approach the sensitivity of digital output cameras.

Scan line resampling

A CCD image is built up by rows of pixels. A row of pixels in the camera is converted into a video signal, called a scan line. The signals of all individual pixels of that row are present in the continuous video signal and the scan line has to be converted back to individual pixels in the frame grabber. The frame grabber divides the video signal into small time portions and converts each portion to a single pixel. Synchronization pulses generated by the video camera help the frame grabber to decide what the duration of one pixel in the video signal is. When the video digitizer assumes a different pixel number per scan line than is physically present in the CCD chip, pixels are resampled and information from the pixels in the camera will be redistributed over the scan line. Measurements are affected by this resampling; particularly, the sharpness of the images suffers from resampling.

Video standards

Different video systems, such as PAL (European), SECAM (French) and NTSC (American), are not interchangeable, as they use different numbers of scan lines or different numbers of pixels per scan line. Video camera and frame grabber have to be set at the same standard. A programmable frame grabber that can be adjusted to grab the correct number of lines and pixels per scan line is useful in

Fig. 8. Results of averaging or integrating images in time to improve signal-to-noise ratio (SNR). Images recorded with a Cohu 4000 video camera with the facility of on-chip integration of a coffee mug recorded in a darkened room. (A) An average of 64 successively recorded images corrected for the average of 64 dark current images. Each of the 64 signal images and dark current images includes digitization noise. (B) This image has been time integrated on chip for the duration of 64 frames and corrected for a dark current image that was integrated on chip during 64 frames. The higher quality of image B over image A is a consequence of the single digitization step reducing digitization noise by a factor of 64.

environments where more than one type of camera is applied. Different types of cameras can be useful when one system is needed for quantification (e.g. PAL that has a high vertical resolution) and another (e.g. NTSC) for presentation purposes.

Geometric errors

Some video cameras, such as television cameras, have a pixel aspect ratio (ratio of height and width of the pixel) that is not 1. Area and length measurements should be corrected for this aspect ratio. In the case of an aspect ratio that is not unity, the length of a line in an image when measured as a number of pixels is not the same when it is oriented vertically or horizontally in the image. In most software packages, a correction factor for the aspect ratio is available. When an area is measured in pixel units, the appropriate conversion to metrical units is applied automatically (see Chieco *et al.*, 1994).

Video camera and frame grabber set-up

Video cameras and video digitizers have to be tuned for optimal performance. The setting up of analogue video equipment is usually more difficult than the setting up of digital output cameras. The latter cameras are specifically designed for optimal quantitative performance in a laboratory and need only minor adaptations. When using CCD video equipment, it can happen that pixel values do not seem to be out of range, while in fact the CCD camera is overexposed and a low amplification setting actually hides the problem. In this light, it should be realized that automatic gain control (AGC) and gamma

correction are switched off (see also Chieco *et al.*, 1994).

The process of subdividing the video signal into temporal samples, each of which is the equivalent of one image pixel, has already been mentioned. The voltage level in these samples represents the intensity of light detected by the camera pixel and consists of several components (Fig. 9). A constant voltage of, say, 0.6 V, called a pedestal, is always present as the base level and is defined by industrial standards. Synchronization pulses are negative when compared with this pedestal. Dark current of the video camera is a small signal of a few grey levels above pedestal. The specific signal resulting from illumination of the chip varies between pedestal level and white level. White level is the voltage that represents maximal illumination. When the intensity of the light exceeds the allowed maximum, a voltage increase will not be observed because the video signal is already at its maximum level (signal clipping) (Fig. 9).

A convenient tool offered by almost every image processing software package for observing the grey levels in an image is the histogram (Fig. 10). A histogram shows grey levels on the horizontal axis and the number of pixels containing a particular grey value on the vertical axis. Most pixels in a dark current image have very low grey values (Fig. 10A). A correctly tuned illumination image has a symmetrical peak at high grey levels. The highest grey levels should not be used (Fig. 10B). When too much light is used for illumination, the chip is overexposed and many pixels contain the highest grey value (Fig. 10C). A histogram of an image containing an object contains a wide range of

Fig. 9. Graphical representation of a video signal in time. The different levels indicated are the start of a scan line (synchronization pulse or sync), one scan line, a clipped signal (at the white level) and dark current. Dark current is the difference between dark current level and pedestal.

grey levels when the illumination is correctly tuned (Fig. 10D).

Conversion of voltage to numbers (grey values) by the frame grabber depends on two parameters. The lower limit of a frame grabber, also called 'offset', should be set at pedestal voltage and the upper limit, or 'gain', should be set at the white level. When the lower limit is set just below pedestal, dark current can be measured. When the upper limit is set just below white level, overexposure and signal clipping can be detected. The voltage difference between the upper and lower limits in conjunction with the number of available

digitizing levels (grey values) determines the digitizing range. When a CCD video camera and frame grabber are tuned by the procedure we describe here (Fig. 11), the widest dynamic range of the system is obtained. This is demonstrated here with an example. Let us assume that a completely white signal is represented by 1.6 V, the pedestal by 0.6 V and the dark current voltage level in our example is 0.61 V. Frame grabbers divide the range between white and pedestal or 1.0 V into 256 steps or grey levels of $1/256$ V each. When the software is capable of indicating underexposed and overexposed pixels, these pixels can be highlighted in

Fig. 10. Histograms of grey scale images after rescaling of the pixels with the highest grey values. (A) Image of obscured camera contains grey levels with pedestal (offset) and dark current only. (B) Correct illumination results in a narrow, symmetrical peak near the maximum grey level. (C) When the illumination is too bright (overexposure), the peak of the histogram is shifted to the right-hand side of the histogram, leaning to that side. (D) A histogram of the grey levels of an image of an object with proper illumination.

Fig. 11. Flow chart for optimized performance of density or absorbance measurements with an image analysis system. The procedure applies for microscopical as well as macroscopical measurements.

bright colour. When the lens of the camera is covered, i.e. no light reaches the chip, the only signal that remains is the pedestal plus dark current. The lower limit of the frame grabber has to be adjusted in such a way that the digitized image (when the camera lens is closed) never contains grey values of 0 (zero). When zero values are encountered in the image, it is likely that the lower limit of the frame grabber is set too high.

Dark current cannot be measured in that case, because it is out of the measuring range. A substantial amount of light is then needed to generate an image. In this way, weak signals are lost and stronger signals are underestimated. On the other hand, when low values are not present in the image, the lower limit is probably set too low and the measuring range is unnecessarily restricted. Large differences in grey values in the image of an obscured camera indicate that the dark current of the camera varies considerably.

When the lower limit is set properly, all light incident on the chip is detected as signal. In our example, the lower limit should be set at 0.6 V and the upper limit at 1.6 V. The dark current of 0.61 V is measured as 0.01 V above pedestal, corresponding with an average grey value of 3 (256 steps = 1 V and thus 0.01 V is 2.56 or approximately three steps). An impression of the dark current can be obtained by grabbing a dark field image and calculating the mean and the standard deviation. When the mean value is altered and the standard deviation remains the same, this means that the dark current is in the measuring range. However, when the standard deviation changes with the mean, the dark current is not measured properly.

Next, we determine the correct setting of the upper limit of the frame grabber. This allows the detection of overexposure. To accomplish this, the lens cap is removed and the camera is directed to a bright light source. The upper limit is reduced or the gain is increased, respectively, in such a way that the software indicates (incorrectly) that the camera is not overexposed. Then, the upper limit is slightly reduced, while still aiming the camera at the bright light source, so that the software can correctly indicate overexposure. Once obtained, the settings should be saved and used from then on in that combination of camera and frame grabber.

Digital output CCD cameras

Digital output CCD cameras deliver digital numbers rather than analogue signals. The conversion of electrons into numbers takes place inside the camera or near the camera chip. This is a relatively slow but accurate process. This process is directed by a special controller. Electrons representing the signal of (a group of) wells are directed to a precision amplifier by the controller. The signal is amplified by a constant factor and handed over to a high precision ADC. This ADC directly converts the signal (which is linear to the number of electrons present in a potential well) to a numerical value that is transported to a computer. Therefore, the noise that is introduced by conversion to and from video signals is absent in digital output cameras. Interference signals that can be picked up from the environment by video

cabling are absent in digital output cameras, since the signals travel as numerical entities between camera and computer. Also, cross-talk that may influence signals travelling in two adjacent analogue cables is not present in signals that travel as digital numbers. The controller can adjust a programmable amplifier in the digitizer or selectively sample certain areas of the chip to pool photons that are detected by several pixels. Pooling of electrons generated by very weak signals over a number of pixels allows amplification of weak signals at the cost of spatial resolution. This process is called binning. The binning factor or the number of pixels taken together can be specified individually for the horizontal and vertical direction. If binning factors for horizontal and vertical are set as h and v , respectively, the electrons of each $h \times v$ pixel area are pooled. Both signal and dark current are amplified by $h \times v$. The resolution is reduced to $width/h \times height/v$, where *width* and *height* are the size of the CCD chip in pixels. A favourable side-effect of binning is the reduction of integration time and faster transport of the reduced digital information by a factor of $h \times v$. The image size is also reduced by a factor of $h \times v$. Quantification takes place only once instead of $h \times v$ times, so the quantification error is reduced by a factor of $h \times v$. On the other hand, the signal is amplified $h \times v$ times, while the dynamic range of the camera remains the same, so over-exposure becomes a serious risk.

Digital output camera set-up

Adjustment of digital output cameras is rather simple, as they generally provide a programmable gain and are equipped with fast mechanical shutters enabling integration times in the order of milliseconds. As soon as maximum output values are approached, various measures can be taken. Binning factors, shutter time or gain can be reduced to keep the output from overflowing. A point that deserves special attention is the shutter time. Although any shutter time may be selected, the relation between shutter time and signal has to be assessed. When images are recorded with constant illumination and decreasing shutter times, a linear decrease in the signal must be found. Below a certain shutter time, the signal does not decrease linearly anymore with the specified shutter time, but levels off and eventually becomes constant (Fig. 12) when the physical limitation of the shutter is reached. Too short shutter times should be avoided, as this will result in an overestimation of the signal.

Short exposure times

In contrast to the integration mode of video cameras, which allows exposures longer than 1/30th of a second, some (video) cameras are equipped with either electromechanical shutters (a metal curtain

Fig. 12. Determination of the physical limit of a mechanical shutter of a digital camera. Decreasing shutter times leads to decreasing average signals as detected by the camera. Below 25 ms, the shutter cannot be opened for shorter periods. Quantitative data in relationship to shutter times can only be obtained when these are > 25 ms.

operated by an electromagnet), electro-optical shutters (liquid crystal panels that are electrically activated; Verwoerd *et al.*, 1994) or electronic shutters (which activate or block the chip) that allow short exposure times. To facilitate the capture of fast-moving objects, the shutter time may be varied electronically from 1/30th of a second to 1/10 000th of a second or even shorter. However, the output of these images is at video rate. When images are overexposed at single frame grabbing (1/30th of a second) with correct frame grabber settings, the best solution is to use the programmable electronic shutter. It should be checked that the camera signal is linearly related to the shutter times (Fig. 12). The digitized signal obtained at 1/10 000th of a second should be corrected by multiplying by 10 000/30 to enable comparison with images captured at regular video operation. We recommend that not only a series of calibration images are captured with the images of the experiments, but also that the settings of offset, gain and shutter times are recorded.

Software techniques for image processing and analysis

Image mathematics

Once valid images are obtained, calculations with these images can be performed. Calculations have to be performed with precision high enough to avoid loss of information. Some image-processing programs take advantage of the fact that cameras have 256 grey values (8 bit). These grey values can be stored in just one byte, which is historically the

common unit of computer memory. The display of 256 grey levels is about the maximum the eye can distinguish and, in that respect, 256 levels are sufficient. When more than 256 values are to be displayed, display scaling, false colour scaling or other tricks of the trade must be used to present the image in a useful way. Another advantage of having only 256 grey values is that manipulation of bytes is fast and 8-bit images need little storage space. A drawback of having only 8 bits available, allowing 256 different values, is particularly noticed when calculations are performed with these images.

An application of image mathematics is the correction for lens aberrations in fluorescence imaging, as shown in Table 1. Here, five pixel values are shown in an image of a homogeneous fluorescent object. The grey values should all read 254 when ideal optics were used. However, in practice, lower values are obtained. A correction factor can be derived from the imperfect values to approximate the ideal image. These factors can then be used to correct the measured values, as shown in Table 1. Both imperfect and corrected pixel values can be in the range of 0–255 and, therefore, in theory result in $\pm 65\,536$ (256^2) different values. In practice, only a small number of values are encountered, which should be distributed as economically as possible over the 256 values available. The result of a multiplication of numbers between 100 and 150 will be in the range of 10 000–22 500. The minimum grey value (0) is then used to represent 10 000 and the maximum grey value (255) to represent 22 500. Each intermediate grey value represents a 'real world value' of 48.8 (calculated as $(22\,500 - 10\,000)/256$).

In case a larger result 'image' is made, each pixel is represented by a 16- or 32-bit 'word' or a floating point value. Programs working with higher precision, such as 16-bit integers or floating point numbers, are generally slower and require more memory and processing power. Typical applications that require high-precision arithmetics are high-

density absorbance measurements and weak signal imaging in fluorescence.

High-density absorbance images

According to Lambert–Beer's law, the amount of dye present in an object is linearly related to the absorbance, $A = \epsilon \cdot c \cdot d$. The amount of dye is thus logarithmically related to transmission, since $A = -\log(I_0/I_n)$. A small change in the amount of dye in an environment, in which only a small amount of dye is present, may change the transmission value from 99% to 98% ($-\log 0.99 = 0.004$, $-\log 0.98 = 0.009$), giving a change in absorbance of 0.005. At high absorbances, however, the amount of absorbance needed to change the output value from 2% transmission ($-\log 0.02 = 1.699$) to 1% transmission ($-\log 0.01 = 2$) is considerably higher, while the step is still 1%. The change in absorbance in this example, 0.301 ($2 - 1.699$), is much larger.

The log function used in the calculations of absorbance partially overcomes this peculiarity in the intermediate transmission calculation steps. Nevertheless, the round-off error in measurements of low transmission values can be expected to be high. Measurement of transmission by the camera is in the linear, and not in the logarithmic, domain. When transmission values are low, the SNR of these weak signals decreases. When high precision is needed, longer exposure times have to be used.

Interval step as an additional method for improving calculation precision at lower transmission values

When the maximum transmission of an object is 50% at most and the detector exhibits a linear exposure time to signal response, integration time can be doubled or two frames can be combined to obtain more precise results. The integration of two frames then results in a signal that is within the limits of the camera. Say the two highest transmission values found in an image are 49% and 50%. After the integration of two frames, transmission in these two pixels is 98% and 100% in the integrated image

Table 1. An example of image mathematics to correct the signal in an image obtained with imperfect optics in five individual pixels

	<i>Measured value of 100% fluorescence</i>				
	240	246	252	242	238
Correction factor	1.06	1.03	1.01	1.05	1.07
Normalized value of 100% fluorescence	254	254	254	254	254
Measured signal	220	205	145	35	25
Correction factor	1.06	1.04	1.01	1.05	1.07
Approximated true signal value	234	213	147	37	27

respectively. When the transmission value is calculated by dividing the integrated image by 2, the result is 49% and 50%. There will also be pixels in the integrated image that have a value of 99%, which is 49.5% when corrected for the integration of the two frames. In this way, precision is increased. For transmissions of at most 25%, four frames can be integrated, and so on. In systems with non-monotonous signals, i.e. when signal levels go up or down in time, care has to be taken that the integrated signal is well within digitization limits. In this way, a well-controlled arithmetic gain can be implemented.

This should not be mistaken for the analogue automatic gain control available on some CCD video cameras. This automatic gain is a highly undesirable feature, as the signal obtained from a camera with automatic gain control switched on is totally unreliable for quantification (Chieco *et al.*, 1994). Depending on transmission, the camera changes its gain setting in such a way that the output video signal has the highest dynamic range possible. Therefore, this setting and thus the output signal changes constantly.

A special point of attention in fluorescence imaging is the fading and bleaching of fluorochromes. When the software of an image analysis system enables hardware control, this can be used to control excitation time by operating a shutter in the illumination system. This allows the limitation and standardization of exposure time to minimize and to obtain uniform fading and bleaching.

Conclusions

The most common caveats and pitfalls when setting up an image analysis system have been discussed above. It should be relatively easy now to start using an image analysis system for quantitative purposes. The guidelines given do not solely apply to the use of a microscope in densitometry, absorbance and fluorescence cytometry, but can also be used for the quantitative imaging of larger objects, such as large areas in tissue sections and gels, e.g. after electrophoresis, as is commonly used in biomedical research.

We have indicated where high-performance cameras are necessary and where they are to be preferred, but we have also shown that the cheaper video cameras can be used equally well in many cases, albeit with caution and with the understanding of possible improvements to their standard operation methods.

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