A colored view on quantitative pathology
Willemse, Feike

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 2

IMAGE ANALYSIS IN IMMUNOHISTOCHEMISTRY
FACTORS WITH A POSSIBLE INFLUENCE ON THE PERFORMANCE OF VIDAS
VERSION 2.0, A COMMERCIALLY AVAILABLE TRUE COLOR IMAGE ANALYSIS
SYSTEM

F. Willemse, M. Nap, L.B. de Kok, H.F. Eggink
Abstract
This paper describes the evaluation of several factors with possible influence on the performance of a commercially available image analysis system (IAS) capable of true color image analysis. The software used by this system is VIDAS version 2.0. The following factors were evaluated: illumination, power supply, warming up, shading correction, averaging of image intake, hue, luminance and saturation images and relation of illumination to quantification of area percentage (AREA%) of positively staining structures. The first six factors were evaluated by using a macro, with which it is possible to obtain information on variations over the image and over time. The last factor was evaluated by repeated measurement of AREA% of positive staining in a routinely processed tissue section. In our setting, stability of illumination and warmup time of the camera appeared to be the most important factors with influence on the performance of the IAS.
Introduction
In surgical pathology diagnoses are based on the recognition of morphological patterns or combinations of structures in both macroscopy and conventional light microscopy. Apart from making a correct diagnosis, identification of elements that may predict prognosis or therapy response have established a place in tumor pathology. These are, for instance, in patients with breast cancer, hormone receptors and related or other proteins, and markers for cell proliferation in a variety of tumors. Immunohistochemistry offers increasing possibilities to stain for specific cell constituents, making them available for further analysis.

Objective and reproducible quantitative information regarding most of these substances can be obtained by biochemical assays or flow cytometry. A disadvantage of these techniques is loss of relation between morphology and the presence of a substance. Immunohistochemistry in combination with image analysis saves this information and is more objective than visual interpretation 104,115-119.

There is a large variety in price and performance characteristics of Image analysis systems (IASs). The majority of commercially available systems use black-and-white (B&W) cameras. However, true color image analysis may be very useful for the study of histological material 11,15.

The aim of this study was to evaluate to what extent a variety of factors influence the performance of a commercially available true color IAS.

Materials and methods

Image analysis system
The IAS in our laboratory is installed in combination with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany) mounted to a single-chip, color charge coupled device (CCD) video camera (Panasonic WV-CD130, Matsushita Communication Co. Ltd., Yokohama, Japan). Illumination is provided by a halogen light source, connected to a stabilized, adjustable power supply (12V, 100W). Images are digitized with a frame grabber in combination with a 286 AT personal computer, expanded with a 287 mathematic coprocessor. Image processing and analysis is performed with VIDAS version 2.0 (Kontron Elektronik, Eching, Germany), capable of true color image analysis. Digitizing a color image results in three images representing the three color components red, green and blue (RGB). Below we refer, for convenience, to gray levels and gray level images instead of RGB levels and RGB level images.
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Factors considered for evaluation

Image analysis is done on the basis of gray value extraction from original images. Factors that may influence the composition of gray values in the image to be analyzed are: illumination, power supply and warming up. Other subjects for evaluation were: shading correction, averaging of image intake, hue, luminance and saturation (HLS) images and relation of illumination to quantification of area percentage (AREA%).

Illumination

Stability of illumination over the image and in time was evaluated using a macro as the tool (Figure 2.1). With this macro, mean gray values of six horizontal lines in an optical empty image were measured. The lines were evenly distributed over the image. To register possible fluctuations in time the measurement was repeated one hundred times by using a loop. Each time a new image was digitized, measurement was performed on each of the three color components respectively, after (multiplicative) shading correction had taken place. One session took four hours.

Before running the macro, the voltage of the illuminator was set using a look up table (LUT), "tvonline". With this LUT it is possible to visualize whether the dynamic range of the TV interface is exceeded, or in other words: whether there is too much light. The voltage just below this level was chosen. A 16× oil immersion objective with numerical aperture of 0.50 (Zeiss) was used for image intake.

Power supply

Since variation in voltage results in changes in light intensity and hence in variations in measured data, we bypassed the built in power supply of the microscope and connected the halogen illuminator to an external power source (SM 6020, Delta Elektronika, Zierikzee, the Netherlands). The SM 6020 is a constant-voltage source (ripple pulse-pulse, 10 mV; stability: 1.10⁻⁴). The proper voltage was chosen by using the LUT "tvonline", as explained above. After this the macro was run again.

Figure 2.1. The macro as it was used in the several tests. When the effect of shading correction was evaluated, the commands for this step were removed. In their place a wait statement was introduced to bring the time needed for one session into line with the time needed for one session of the original macro.
1. DECLARATION OF PARAMETERS

2. CREATION AND OPENING OF DATABASE

3. INPUT REFERENCE IMAGE FOR SHADING CORRECTION

4. START LOOP

5. INPUT RGB-IMAGE TO BE MEASURED

6. SHADING DEFINITION AND CORRECTION RED IMAGE

7. MEASUREMENT OF GRAY LEVELS ON SIX LEVELS IN RED (R) IMAGE

8. SHADING DEFINITION AND CORRECTION GREEN IMAGE

9. MEASUREMENT OF GRAY LEVELS ON SIX LEVELS IN GREEN (G) IMAGE

10. SHADING DEFINITION AND CORRECTION BLUE IMAGE

11. MEASUREMENT OF GRAY LEVELS ON SIX LEVELS IN BLUE (B) IMAGE

12. END LOOP

13. CLOSE DATABASES
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Warming up

Although a certain warmup time of the illuminator prior to measurement is generally accepted, the application is hardly ever mentioned in the literature concerning application of IASs in surgical pathology \(^{15,120}\). One article \(^{15}\) states that noise arising at the sensor in CCD cameras is temperature dependent. No mention is made about necessity of warming up. To investigate the effect of warming up the entire system and the isolated components (i.e. illuminator, personal computer and camera), we used the macro with either all components cold or just one of the components cold while the other two were already warmed up.

As soon as it became clear that the effect of warming up was greatest in the camera, we repeated the measurement with a cold camera. In this repeat experiment the temperature at the camera level was measured simultaneously. Registration was done by the use of a single pen recorder capable of temperature measurement (PM 8251, Philips, Eindhoven, the Netherlands).

Shading correction

The importance of shading correction has been described extensively \(^{48,59}\). However, the conclusions concern systems using a B&W tube camera. It is expected that with CCD cameras shading correction is less necessary than with tube cameras \(^{15,121}\).

Two experiments were done to evaluate the effect of shading correction with our equipment. In the first experiment we used the macro, only this time the shading correction step was omitted. After this modification, one session would only take one hour. To bring the total measurement time into line with the total measurement time of the macro with shading correction, a wait statement was introduced.

In the second experiment the AREA% of an object was measured in nine different positions in the image field. Measurement of the AREA% was done in three ways: (1.) without shading correction, (2.) with multiplicative shading correction, and (3.) with additive shading correction.

Averaging of image intake

Due to noise introduced by the camera, the gray level resolution may deviate from the required 8 bits per pixel of the respective primaries \(^{48}\). This deviation may result in variation in measured gray levels from measurement to measurement. In this case, averaging of images is needed to have 8 bits per pixel available.

The effect of averaging in our setting was evaluated by introducing the function "average input images" into the macro. With this function four images, the maximum number with VIDAS working in the color mode, were used for averaging.
HLS
To evaluate the possibility of using HLS transformation of the color image, we
introduced the function “RGB to HLS” in the macro. Consequently the mean gray
values of the six lines were measured in the HLS images instead of the RGB
images.

Relation of illumination to quantification
To be informed about the actual effect of changes in illumination on the AREA%,
the colored surface in a tissue section stained by immunohistochemistry was
measured. For this purpose we used a formalin-fixed, paraffin-embedded histologi-
cal section of a breast carcinoma with positive staining for pS2 protein \(^{109,122}\). In
brief, the immunohistochemical staining was performed on a 4-\(\mu\)m-thick section
using the anti-pS2 monoclonal antibody (BC4 anti-pS2) (CIS-Bio International, Gif
sur Yvette, France) in combination with a streptavidin-biotin-peroxidase method, as
routinely carried out in our laboratory. The peroxidase label was developed with a 3-
amino-9-ethylcarbazole solution. The presence of pS2 was indicated by a red
staining. To quantify the reaction product as effectively as possible, the section was
not counterstained. The same image field was measured ten times with intervals of
60 minutes in between. The voltage was set as explained above and not adjusted
between measurements. The mean gray value, as a derivative of illumination, of
each RGB image was computed. The AREA% was obtained from a binary image
that resulted from the segmentation step (RGB discrimination). The threshold levels
for segmentation were kept constant in the successive measurements.

Data transformation and graphic displays
Data transformation was performed by using Videoplan Version 2.1 (Kontron), a
program added to the VIDAS software. Lotus 123 Version 2.0 (Lotus Development
Publishing Corporation, Mountain View, California, U.S.A.) were used for graphic
displays.
Results
The results of investigating the stability of illumination with the macro as the tool are shown in Figure 2.2, which demonstrates distributions for the RGB images. The mean gray values are displayed for each of the six lines separately. The blue image shows the highest variation from measurement to measurement, the green the lowest. All three images show a somewhat “wavy” pattern. The fact that the lines are projected more or less on top of each other means that distribution of illumination over the image is relatively even.

The results of the measurements with the external power supply, as well as with application of averaging of image intake, showed a similar distribution as displayed in Figure 2.2.

Warming up the camera was especially important in affecting the measurements. A major decrease in gray values over the first 40 measurements (100 minutes) could be seen. After this a plateau was reached. The results of the measurements starting with the other components cold did not show such a pattern. There the distributions were similar to those seen in the measurements with a warmed up system (similar to Figure 2.2). The results of measurement with a cold camera and simultaneous temperature registration are shown in Figure 2.3, where the solid line represents the temperature and the symbols represent the values of line A measured in the green image. The decrease in gray values over the first 40 measurements indeed appears to be an effect of warming up. As soon as the plateau in temperature is reached, the gray values become more or less constant.

In the first experiment concerning shading correction there was an increase in variation of the gray values from measurement to measurement. This was especially visible in the blue image partition. However, the gray values remained between 216 and 229, 220 and 230 and between 217 and 238 for the red, green and blue images, respectively. This experiment showed that there is to a certain extent correction of uneven illumination over the image by the shading correction procedure.

The results of the second experiment are displayed in table 2.I. No significant difference was found between the three methods of shading correction used in this experiment (Student’s t-test).

Figure 2.2. Results of one measurement session of the macro. Due to even distribution in the image, the data of the several lines are more or less projected on top of each other. The 100 measurements took four hours.
Figure 2.3. Results of the measurement session with simultaneous temperature measurement on the camera level. The decrease in gray values over the first 40 measurements is shown to be an effect of warming up.

Figure 2.4. Effect of illumination on quantification of AREA%. The symbols represent the actual measured data; these points were connected for better visualisation of the effect. On the X axis are the consecutive measurements.
Table 2.1. Comparison of methods of shading correction

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</table>

Results of measurement of AREA% taken in by an object. This object was positioned in nine different positions in the image, starting at the upper left and ending at the lower right. At each position the AREA% was measured without shading correction (NO), with additive shading correction (ADDIT) and with multiplicative shading correction (MULTI).

Transformation of RGB images to HLS images resulted in gray value distributions comparable to those of the RGB images for the L image only. For reasons we do not know, variations over the image per measurement and in time in the H and S images were much larger than in the luminance extraction. This makes them less suitable for actual measurement. Consequently, the L image would be the only image remaining available for further processing.

The results of investigation of the effect of variation in illumination are displayed in Figure 2.4, in which × represents the AREA% and + the mean gray value in the green image. The mean gray values of the red and blue images, display a similar pattern as those of the green image. Since the voltage was not changed during this test, we concluded that the variation in AREA% most probably was a result of the variations seen in Figure 2.2.
Discussion

The results of the experiments described above show that the most important factors are stability of illumination and warming up the camera. A warmup time of at least 100 minutes is needed. In a setting where measurement takes place regularly, one can consider to leave the camera on permanently to avoid waiting nearly two hours before measurements can be started. The camera we used has a built in power supply. When a camera with an external power supply is applied, the effect of warming up will be less pronounced. Note that in this study the effect of warming up was tested only on a halogen illuminator. The conclusions do not concern other kinds of illuminators (e.g., a mercury illuminator).

The necessity to keep the illumination as stable as possible became most clear in the last experiment. We concluded that the differences in AREA% were the result of variations in illumination, as seen in Figure 2.2. These variations appeared not to be an effect of changes in voltage in the built in power supply of the microscope. The exact source of the variations remains unclear.

Since VIDAS is not capable of real-time display of strength of illumination we ultimately adopted the following procedure to achieve control of strength of illumination. Before the first measurement, the light is set using the LUT "tvonline". Then a reference microscopic field in a control slide with known AREA% of positive staining is used. Control between measurements is performed by computing the AREA% and the mean gray values again. Adjustments are made by repeating this procedure.

The run of the macro without shading correction showed that uneven illumination over the image is, to a certain extent, corrected successfully. However, the experiment in which AREA% of an object is measured in nine different positions showed that there is no significant difference between measurement with or without shading correction. This indicates that in a procedure where AREA% of positive staining is quantified, shading correction may not be absolutely necessary in our setting. In a situation where densitometric measurements are done, shading correction, using averaging, might be preferable.121

Because the RGB model does not directly relate to the human intuitive color notions of hue, saturation and brightness, models using the HLS model were developed.10 We find the use of the transformation to HLS images less suitable for the following reasons. First, the transformation of RGB to HLS images is time consuming. Second, due to the high variations in the H and S images only the L image remains present for processing and measurement. Despite the fact that only one image suffices for further processing HLS images, we would prefer the possibility to choose from three instead of one. Third, if the HLS images would be used for further processing and measurement, one has to take into account that...
segmentation and other procedures take place on basis of B&W images containing
gray values corresponding to notions of color. In this way our preference for
detection of targets on basis of color would be lost.

In conclusion, this paper gives a concise methodology to document the
characteristics of an IAS in a specific laboratory environment. This information
cannot be obtained from the documentation accompanying the separate
components. In our opinion, before actual measurements are carried out on patient
material, one should make this type of information available. With this knowledge it
is possible to fine tune the measurement system and improve the reliability of the
data.