# Multi-spectral Image Analysis of Plant Cells

G. Fernàndez[1] and J-P. Zrÿd[2]

[1]Signal Processing Laboratory, Swiss Federal Institute of Technology, CH-1015 Lausanne, Switzerland
[2]Plant Cell Genetics Laboratory, University of Lausanne, CH-1015 Lausanne, Switzerland

RÉSUMÉ

*L'analyse polychromatique d'échantillons biologiques peut donner des informations qui restent souvent cachées dans l'analyse monochrome ou couleur. Dans cet article on montre qu'après un choix précis des bandes spectrales, l'information obtenue peut être utilisée efficacement comme outil dans l'analyse d'image (tel que la segmentation) et aussi pour les analyses biochimiques d'absorption. La méthode est basée sur la reconstruction approximative du spectre d'absorption pour chaque point dans l'image. Cette reconstruction nécessite seulement trois observations dans des bandes spectrales non chevauchées. Ces observations sont appliquées à un modèle efficient du spectre d'absorption des échantillons à analyser. Une telle reconstruction établit la base pour faire une segmentation et une analyse des échantillons. Cette technique a été appliquée dans des cellules végétales pigmentées cultivées* in vitro.

ABSTRACT

*The polychromic analysis of biological samples can yield information usually masked in monochrome or even color analysis. This paper shows that after an accurate choice of the spectral bands, the information obtained can be useful for image analysis purposes (such as segmentation) and also for biochemical analysis through absorption or fluorescence measurements. The method is based in the approximate reconstruction of the absorption spectrum at each point in the image. For reconstruction only three observations of non-overlapping bands are used in an efficient model of the absorption spectrum of the samples. Such a reconstruction establishes the basis for the segmentation and also for the analysis of the samples. The application of this technique has been shown to be specially useful for pigmented plant cells cultivated* in vitro.

## 1 Introduction

Image analysis applied to biological samples has been widely studied in computer vision. Most of these studies are concerned with the analysis of specimens obtained by a unique sensor (monochrome images) or sets of RGB sensors (color images). In this paper we present a technique where the samples are analyzed at three non-overlapping wavelengths. This information is used in two ways. Firstly, to produce a segmentation based on this polychrome information, and secondly, to extract biochemical information from samples (pigment concentration per cell).

Cultures of *Beta vulgaris* (red beet) cells are cultivated in vitro in liquid suspension [1]. The main interest of these cells is the presence of pigments useful for food coloring purposes. For a suitable growth control of the cultures, the biologists face the problem of counting and analyzing features of each cell. To have reliable statistical measures they have to count several cultures at different stages of the growth, yielding subjective and imprecise results after hard and tedious work. For these reasons, computer assisted methods appear as the only way able to deal with such measurement.

There is not yet an instrument for the spectral absorption analysis of biological samples in the spatial plane. With the lack of such instrument, biologists still need to extract pigments from culture samples. These analysis do only yield an average value of pigment concentration per cell. Thus we will never be able to assess the proportion of cells accumulating pigment, their morphology, etc... This kind of information is important for the biologists who want to understand the mechanisms of cell differentiation, and adopt the appropriate procedures towards a maximal production of pigment in bioreactors.

The method presented here is mainly based on the approximate reconstruction of the absorption spectrum of the samples using only a set of three basis functions [2] and three unoverlapped spectral measurements, in order to obtain a segmentation and classification of the culture.

## 2 Multi-spectral acquisition

The *Beta vulgaris* cells contain two main classes of pigments, the *betacyanines* that absorb at 536 nm (violet color), and the *betaxanthines* that absorb at 480 nm (yellow color) as shown in Fig. 1.

Combinations of these two pigments can produce other colors as red and orange. An important parameter for the biologists is the pigment concentration per cell which is proportional to the absorption at a given wavelength.

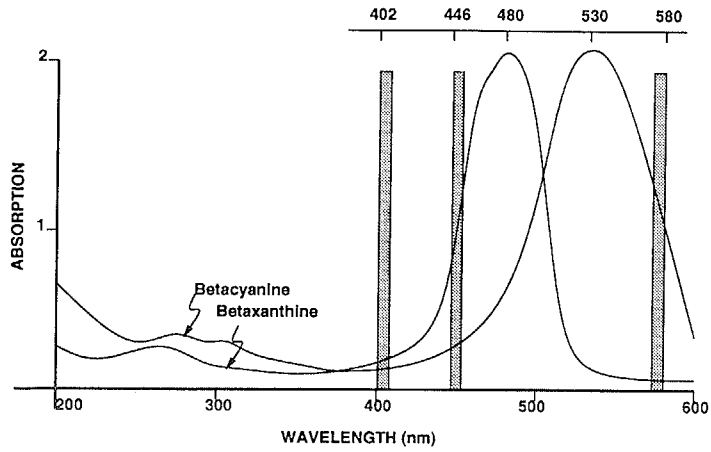


Figure 1: Absorption spectra of yellow and violet pigments, the choice of bands is also shown.

Until now, the measurement of this pigment required its extraction by chemical procedures and a subsequent spectrum analysis (either by spectrophotometry or High Performance Liquid Chromatography). The final result is a global value for the culture. The value of the informations obtained by this procedure is limited in the sense of that there is no knowledge about the pigmented cell distribution in the culture. We do not know how many cells are producing the pigment and how many do not.

The analysis of samples at precise wavelengths is obtained through a set of interference filters held in a filter wheel. These filters allow precise analysis of the spectrum. On the other hand, the total amount of light that can cross the filter is substantially reduced with the consequence that we need a very sensitive camera for the acquisition. The solution adopted for the acquisition is a cooled CCD camera [3] that is able to work in low-light-level conditions. Cooling the CCD, the camera is able to take long time exposures with a reduction of dark current resulting an acceptable SNR at the end.

The combination of interference filters with cooled CCD allows the analysis of the samples at the desired wavelengths. The filters were chosen following the criterion of minimum influence between pigments, giving one filter for *betacyanine* at 583 nm, for *betaxanthins* at 446 nm and finally the filter for calibration at 402 nm as illustrated in Fig. 1. The calibration filter is devoted to detect the absorption due to the cell boundaries. Note that this choice avoids also the maximal absorption peaks that could yield non-linear and camera saturation measurements. Although this is an accurate choice, the spectral overlapping of the pigments produces undesired cross influence. Accordingly, a more complex procedure should be developed to isolate each pigment absorption as explained in section 4.

# 3 Pre-processing

The gray level information obtained through this system will be taken as a measurement of the transmitted light. In order to be able to compare the images they must be calibrated one to each other and pre-processed.

First of all, different camera gains in each wavelength are uniformized. This correction is partly done in the acquisition process by adjusting the exposure time. For a more accurate correction a linear histogram transformation is proposed.

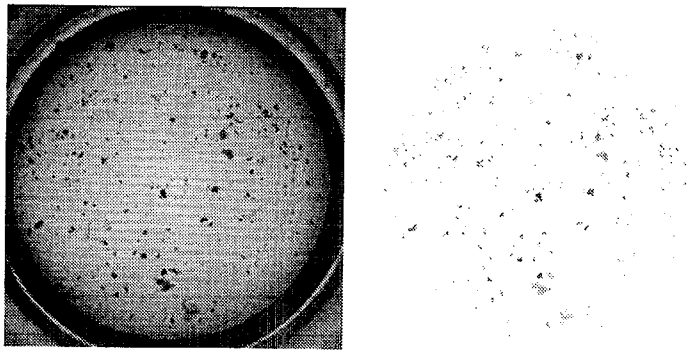

Figure 2: a. Original image of one of the three wavelength under analysis (580nm). b. Enhanced and preprocessed image.

Once the effects of the camera are removed, the next step is the extraction of the useful scene and the removal of the non uniform background absorption. The external circle is obtained by an adaptive form of the *Hough transformation* [4] and the useless part of the scene is removed leaving an image of the cells in liquid suspension. This liquid medium may not have a uniform absorption spectrum along all the wavelengths and the unperfect back illumination yields a non uniform background. All this artifacts can only be removed with a non-linear transformation as the morphological *Top Hat* transformation [5]. Due to the low-light-levels that the camera is acquiring, an additive noise corrupts the images. When the goal is to remove smooth signal variations (background) and dense fluctuations (noise) the so called *Top hat extension* [6] can be used. Results of this preprocessing is shown in Fig.2.

# 4 Recovering the Absorption Spectrum

## 4.1 From gray level to absorption

The intensities measured by the camera represent a function proportional to the final transmitted light by each cell. The transmission factor ($T$) per cell is defined as follows:

$$T = I_t / I_i \tag{1}$$

where $I_t$ is the intensity of the transmitted radiation by the cell and $I_i$ is the intensity of the incident light, i.e. the intensity of the background. This value is absolutely known since in the enhancement part it has been extracted from the cells. Using the Beer-Lambert law [7], the absorption parameter can be easily calculated by:

$$Abs = log(1/T) \tag{2}$$

The reason why we aim at working with absorption instead of transmission lies in two facts: firstly because the pigment concentration is linearly proportional (in most of

the cases) to the absorption, and, secondly, that in a mixture of pigments the final absorption can be decomposed into an addition of the individual absorptions of each pigment.

The measured absorption is the contribution of several phenomena that are present in the cell. These are the cell wall and the presence of the two pigments. Thus, the final absorption can be decomposed in:

$$Abs = Abs_{wall} + Abs_{yellow} + Abs_{violet} \quad (3)$$

These are the hypothesis for the theory explained in the next sections to hold.

### 4.2 Basis function modeling the spectrum

The yellow and violet pigments and the cell wall are the three unknowns that will be recovered from the three measurement.

To this end, we decompose the spectrum in three basis functions, each one representing one of the phenomena described before. For the cell wall a uniform absorption is assumed (constant function) and for the pigments a gaussian approximation has been chosen.

The exact shape of the absorption curves are obtained by spectrophotometry. The violet pigment is easily approximated with a gaussian curve using the MSE criterion. For the yellow pigment, due to its asymmetrical shape a combination of two gaussians has been chosen. The choice of the gaussian approximation lies in the physical basis of the absorption of chemical substances [8].

The spectrum of absorption can be expressed as a linear combination of the mentioned basis functions $(1, vio(\lambda), yel(\lambda)$ below), in the following way:

$$Abs(\lambda) = a + b\ vio(\lambda) + c\ yel(\lambda) \quad (4)$$

The transmittance spectrum of the interference filters have been also analyzed by spectrophotometry and is modeled by a gaussian approximation.

### 4.3 From measurements to spectrum

After modeling the filters and the spectrum, the absorption measured by the camera through the *ith* filter can be expressed as:

$$\begin{aligned} Abs_i &= \log \frac{\int_\lambda I_o\ f_i(\lambda)\ d\lambda}{\int_\lambda I_o\ f_i(\lambda)\ T(\lambda)\ d\lambda} \\ &= \log \frac{\int_\lambda I_o\ f_i(\lambda)\ d\lambda}{\int_\lambda I_o\ f_i(\lambda)\ 10^{-Abs(\lambda)} d\lambda} \end{aligned} \quad (5)$$

where $I_o$ is the light source intensity (constant due to the use of a xenon lamp), $f_i(\lambda)$ is the filter approximation and $T(\lambda)$ and $Abs(\lambda)$ the approximation of the transmission and absorption spectrum respectively.

Due to the narrowness of the interference filters, the variations of $T(\lambda)$ can be considered to be constant within the interval of integration. Then,

$$Abs_i \simeq \log \frac{I_o\ \int_\lambda f_i(\lambda)\ d\lambda}{I_o\ 10^{-Abs(\lambda_i)} \int_\lambda f_i(\lambda)\ d\lambda} = Abs(\lambda_i) \quad (6)$$

being $\lambda_i$ the central wavelength of the $i$th filter. The error of this approximation has been shown to be of 14% in the worst case and typically of 5% (note that it depends on the parameters of the model). We can then define the matrix $K$ with,

$$k_{ij} = b_j(\lambda_i) \quad (7)$$

where $b_j(\lambda)$ is the $j$th basis function $(b_j = \{1, vio(\lambda), yel(\lambda)\})$. Let $S$ be the matrix of measurements and $A$ that of the parameters, the simultaneous linear equations can be expressed by:

$$S = KA \quad (8)$$

and the solution is,

$$A = K^{-1}S \quad (9)$$

of course, knowing the value of $A$, the full spectrum reconstruction per pixel is possible substituting the values to Eq.(4). The three input images are transformed to the *pigment space* by Eq.(9).

## 5 Pigment Based Segmentation

The images obtained from the camera are transformed to the *pigment space* having each of the absorptions expressed in Eq.(4) separated. Taking as reference the violet and yellow pigment, cells are segmented. Since the pigment may not be present in all parts of the cells, the boundaries contribution is added to each pigment. After that, the cells are segmented by a majority filter between pigments as shown in Fig.3.

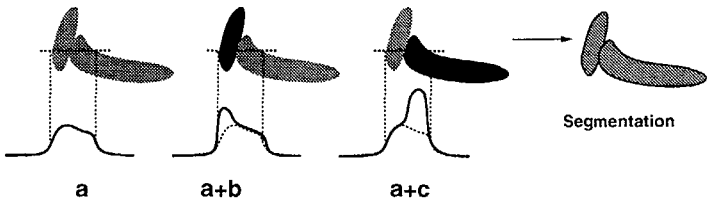


Figure 3: Pigment based segmentation

After this rough segmentation between pigments, each class is segmented again following morphological criteria [9]. Once the cells are segmented, morphological features and also color features are extracted for an efficient classification.

## 6 Pigment Classification

The classification of the culture is done into three main classes: the violet, yellow and unpigmented cells. In a culture of *Beta vulgaris* a typical problem is the loss of pigment through time. The proportion of unpigmented cells is then an important parameter in order to know the state of the culture.

From the pigment information, cells presenting small quantity of pigment are selected by a fixed threshold.The content of each pigment is compared to each other, by the fuzzy $\pi$ membership function [10].

The result of this function is the *fuzzy unpigmented feature*. Cells will be classified as unpigmented if this function is larger than 0.5.

Further classifications are done depending on the shape and size (big aggregates, small aggregates, long isolated and

round isolated) by the morphological analysis. All these classifications give at last a characterization of the culture in 12 classes.

## 7 Results

In order to check the robustness of the presented method, a mixture of violet and yellow cells was prepared. The samples were acquired with a Peltier-cooled ($-25^0$C) CCD camera at a resolution of $1035 * 1035 pixels$ and $12 bit/pixel$. The three spectral images were pre-processed and the absorption of each pigment was discriminated, this information was used to perform the pigment based segmentation. The goodness of the segmentation was checked by color prints of the same sample. Further morphological segmentation was obtained. All the morphological and spectral parameters were collected for the final classification of the culture. Results were correlated with manual counting, High Performance Liquid Chromatography pigment measurements and other biochemical measurements finding more than 90% of correlation.

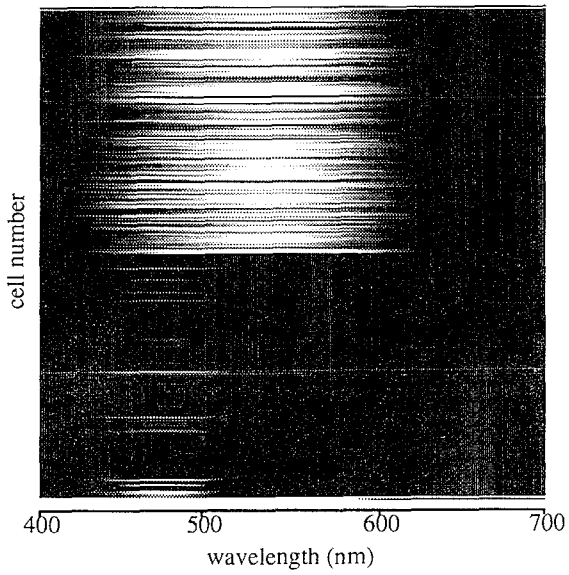


Figure 4: Approximate spectrum reconstruction per cell

Since the pigment absorption per cell was known, it was possible to reconstruct the approximate spectrum absorption of all the cells. In Fig. 4 we show the spectrum of each cell after segmentation. Two classes are present in the culture, a main peak in the violet absorption and a smaller absorption for yellow cells are clearly visible. The culture is about 50% of each class, which is coherent since the original artificial mixture was done with exact proportions of each culture. This representation of the data suggests us new ways of classification of the cells in more refined classes depending on its pigment.

## 8 Conclusions

In this paper we presented segmentation and analysis of biological samples through a multi-spectral acquisition system at three different unoverlapped bands. The method is based on the approximative spectrum reconstruction of the samples. This spectrum reconstruction has been obtained after an efficient modeling by choosing the appropriate set of basis functions. It has been shown to be effective to the pigment discrimination problem. Results on segmentation and classification of the cells depending on their pigment show the spectral information as a good criterion, specially combined with a morphological segmentation. The interpretation of the final data suggest new ways of classification that must be explored in the future.

## References

[1] R. Leathers, C. Davin, and J-P. Zryd. Betalain producing cell cultures of beta vulgaris l. var. bikores monogerm (red beet). *In Vitro Cell. Dev. Biol.*, 28:39–45, 1992.

[2] G. Healey and T.O. Binford. A color metric for computer vision. In *Proceedings IEEE Conf. on Computer Vision and Pattern Recognition*, pages 10–17, Ann Arbor, MI, June 5-6 1988.

[3] Photometrics Ltd. *Charge-Coupled Devices for quantitative electronic imaging.* Tucson, Arizona, 1992.

[4] R. Duda and P. Hart. Use of the hough transformation to detect lines and curves in pictures. *Communications of the ACM*, 15:11–15, 1972.

[5] F. Meyer. *Cytologie quantitative et morphologie mathématique.* PhD thesis, Ecole Nationale Supérieure des Mines de Paris, Fontainebleau, 1979.

[6] P. Salembier. *Multiscale image analysis and modeling using rank order based filter - application to defect detection.* PhD thesis, Ecole Polytechnique Fédérale de Lausanne, Lausanne, June 1991.

[7] C.N.R. Rao. *Ultra-violet and visible spectroscopy, chemical applications.* Butterworths, London, 1967.

[8] A.F. Drake. Optical spectroscopy, principles and instrumentation. In *Microscopy, optical spectroscopy and macroscopic techniques*, pages 151–171, Totowa, NJ, 1994. Humana Press. coll. Methods in Molecular Biology, vol.22.

[9] P. Brigger and M. Kunt. Morphological plant cell analysis allowing a better control of the bio-process. In *Compte-rendus 14 me colloque GRETSI sur le traitement des signaux et images*, pages 1255–1258, Juan-les-Pins, France, September 1993.

[10] S.K. Pal and D.K. Dutta Majumder. *Fuzzy mathematical approach to pattern recognition.* John Wiley & Sons, 1985.