RGB CHANNELS (IN)DEPENDENCE IN PHASE CONTRAST MICROPHOTOGRAPHY

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Abstract

Time laps microscopy (microphotography) is one of the important measurements in many biological experiments. To describing life cycle of observed culture, cell division, fusion or comunication, both automaticaly or semiautomaticaly evaulation, is crucial to guarantee optimal camera settings, in case of observing long time experiment run by digital capture device. Monolayer of human cells – often vaguely referred as tissue culture – is the simplest approximation to the model of an organ from which the cells originate. From a small number of cells the culture is allowed to develop into monolayer and this process is observed under the microscope by a series of camera shots, confocal microscope fluorescence readouts etc. In this paper we describe the analysis of camera RGB channels (in)dependence of the most common system, i.e. HeLa cells observed by phase-contrast image enhancement. In a sense, this type of experiment is available to each medical practitioner or biotechnologist. We have chosen this setup as the most obvious example, yet we found out that even partial analysis of the experiment is sufficiently complicated to exploit the capacity limits of current computer technology. The phase contrast microscopy represents the observed object by the way of comparison of the refractivity index of the object (i.e. living cell) interior and refractivity index of free medium. As a result there is obtained image of light interference intensity in the focal point of the microscope. Refractivity index is a sum of non-absorptive, mainly electromagnetic, interactions of light with the matter. The objective assessment of the value of the refractivity index in the living cell interior is practically impossible using the phase contrast but was obtained by different methods. For the purpose of this discussion, it is also important that the refractivity index is wavelength dependent. If the detection system is for example a standard photograph, each of the three channels (Red, Green and Blue) carries different - partly independent - information which may be used for state definition. Therefore, the question of channel independence is important for any further analysis.

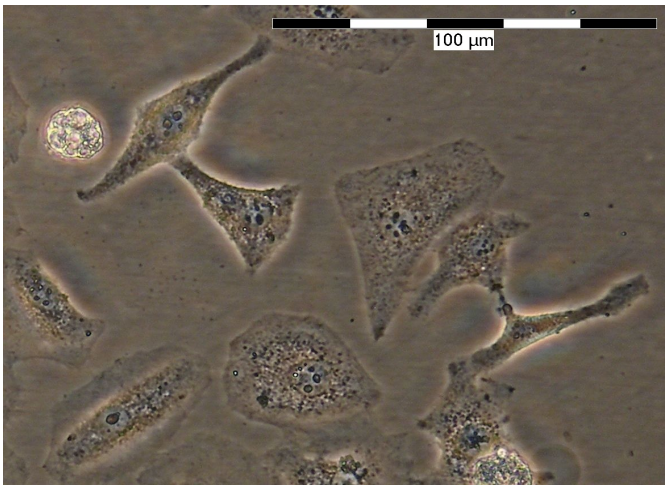

Figure 1: Example of HeLa cells monolayer in phase contrast microscopy.

1. Introduction

Observation of living biological systems (cell culture) in long time as non-invasive technique via microphotography (time lapse) produce huge amount of data (images, frames) exceeding the capacity of subsequent manual analysis. Monitoring live cells and their behavior in time consists of cell to background segmentation, segmentation of individual cells and identification (parameterization) of cell events (division, fusion, death, communication, etc.). These terms are sometimes rather vaguely defined when confronted with information content of the image. To assign

the observable information to cell state events the operator uses both static and dynamical properties of cell interior. The goal of the automation is to recognize corresponding objective observable changes and identify them with biological terms. Those processing demands high computation power especially for proper results in reasonably amount of time. For assign the area of interest it is necessary to locate the time changes as changes between captured images using automated image analysis and identify the events in sense of biological terms. There are plenty of different methods for changes evaluation available and described in the literature. The automated detection tools are capable to detect only the simples structure typess. The complex cell shapes of human tissue cultures represent incomparably more complicated problem. Some of the shapes have names assigned in biological terminology, they may be classifed as phenomenological attributes and variables. The analysis of these complex samples thus relies in most cases on work of human operator. We do not discuss various kinds of genetical or chemical introductions of fluorophores which lead to contrast enhancement but significantly changes the organism itself.

Unfortunately, both automatic or semi-automatic methods ewre often devoloped for binary (black and white) images, and then extended also for grayscale images. Usualy, there was done none conditional analysis on colour representations. In many techniques, is the processing of colour images based on independent processing of each single colour channel (Red, Green, Blue). In this paper, we want to show how is the condition of channel independence fulfilled in phase contrast microphotography of living HeLa cells.

2. Materials and methods

2.1. HeLa cells

On 4th of October 1951 in Johns Hopkins Hospital in Baltimore died Henrietta Lacks, a black woman in age 31. The reason of her death was cervival cancer. Without her knowledge, the sample of tumor cells were taken by the researches. This cell line survived to the nowadays and is still used in laboratories as a model for human cells in thousands of biological experiments, contributing to the understanding of disease processes. HeLa cells were propagated into an immortal human cell line by George Otto Gey, scientist from Tissue Culture Laboratory at Johns Hopkins Hospital. The word "HeLa" was devised by him using the first two letters of Mrs. Lacks' first and last names to keep her real name in a secret. Lacks' cancer cells have evolved into a self-replicating, single-cell life-form and to HeLa cells were given the new species name: *Helacyton gartleri*. The cells are the genetic chimera of human papillomavirus HPV18 and human cervical cells.

Figure 2.: Henrietta Lacks, source: http://wikipedia.org/.

2.2. Phase contrast microscopy

The method of phase contrast allow to observe soft, colourless, transparent objects, especially living cells. The phase differences of light beams are passing through the cell and converted into differences in amplitude. That made them visible for human eye. The principle of phase contrast for microscopy was first time proposed by Dutch scientist Frederik Zernike in 1932. His idea was awarded with the Nobel price in Physics in 1953. Between specimen and observer is situated the phase-plate consist of thin annulus, changing the phase by angle $\pi/2$ or $-\pi/2$ for negative or positive phase contrast. For positive phase contrast ($-\pi/2$ phase-plate) appear the thicker parts darker.

Unfortunately, there are also some important disadvantages. When the specimen was strongly refracted, a halo effect occurs. That means, very shiny boundary overlapped the real object boundaries. The next limitation is disappearing of absorbing saturated objects for the observer.

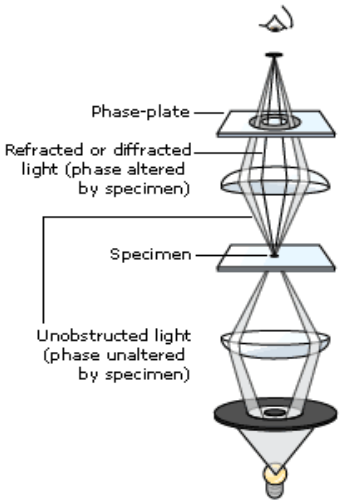

Figure 3.: Scheme of phase contrast microscope, source: http://nobelprice.org/.

The HeLa cells were growing for experiments focused on cytotoxity in vitro in Laboratory of tissue cultures, Academic and University Center of Nové Hrady. The RGB images were taken by Olympus camera C7070 on inverse phase contrast microscope Olympus IX51. The resolution was 6Mpx and the background on all images is the bottom of the NUNC, plastic cultivation ware.. All images were processed in Matlab R2008b.

Figure 4.: Microscope OLYMPUS IX51, source: http://olympuseurope.com/.

2.3. Colour and grayscale representations

Most common colour representation using by machine for vision or display is RGB (Red, Green, Blue) colour space, where each pixel of image is represented by triplet (r,g,b). Value of colour channel (triplet element) is equal to intensity of its colour. The value for each channel is usually situated in interval <0,255> or <0,1>.

0	0	0	
255	0	0	
0	255	0	
0	0	255	
0	255	255	
255	0	255	
255	255	0	
255	255	255	

Table 1.: RGB colours .

Grayscale representation is an image in 256 shades of gray <0,255>. There are three ways how to create grayscale image from RGB image. First one is accorded to the relative sensitivity of human eye for primary colours:

$$Y1 = 0.3 * r + 0.59 * g + 0.11 * b \tag{1}$$

In the second one, just combination of intensity of all three channels is done with the same weight coefficient:

$$Y2 = \frac{1}{3} * r + \frac{1}{3} * g + \frac{1}{3} * b \tag{2}$$

The last one is weight channels relative to each other:

$$Y3 = \frac{r^2 + g^2 + b^2}{r + g + b} \tag{3}$$

RGB *Y1*

Y2 *Y3*

Figure 5.: RGB and grayscales, source: J.Urban.

During the grayscale transformation are the information about colour lost and can not be restored back from grayscale image.

2.4. 1D and 2D histograms

The Histogram function H (p) is an intensity function, shows count of pixel f(i,j) with the intensity equal p independently on the position (i,j).

$$\begin{aligned} H(p) &= \sum_{i,j} h(i,j,p) \\ h(i,j,p) &= 1 \; if \; f(i,j) = p \\ &= 0 \; if \; f(i,j) \neq p \end{aligned} \tag{4}$$

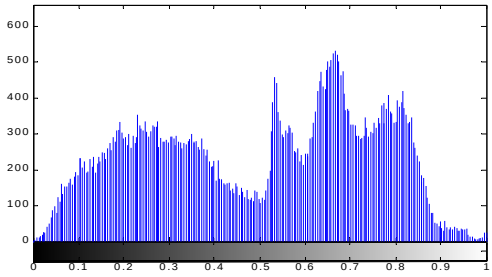

Figure 6: Histogram function for Y1.

In case of colour images are histogram functions normally computed independently for each colour channel, without considerations about relations between each other.

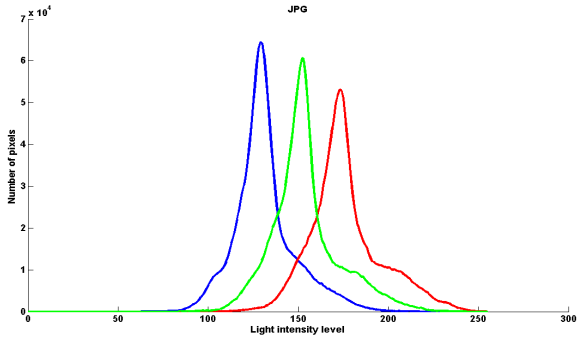

Figure 6: Example of RGB histogram function.

The (in)dependence of colour channels should be easily examined via 2D histograms:

Step1:. Basic 'grayscale' histogram *h* is computed for given colour channel from the image.

Step2: For *k-th* constituent of histogram h domain of definition (n intensity values) are pinpointed the pixels in original colour image, where intensity value of the pixel in given channel is equal to the *k*-th constituent.

Step3: Basic 'grayscale' histogram *h2* of one of the remaining colour channels is computed only from pinpointed pixels.

Step4: Construct 2D histogram of dependence of histogram *h* on histogram *h2* as image of resolution *n* x *n*, where on *k-th* row are occurrence values of histogram *h2*.

Step5: Repeat step 2, 3 and 4 for whole domain of definition of the first given channel histogram *h*.

Step6: Repeat step2, 3, 4 and step5 for rest of the colour channels for dependence of histogram *h*.

Step7: Repat all steps for all colour channels.

Therefore are obtained six 2D histograms *n* x *n* for three channel image:

- Dependence of Red on Green,
- Dependence of Red on Blue,
- Dependence of Green on Red,
- Dependence of Green on Blue,
- Dependence of Blue on Red,
- Dependence of Blue on Green.

The assumption that dependence *h1* on *h* is just the inversion of dependence *h* on *h1* is negative or zero and will be also tested. There should be no evident pattern, if the relation between

two channel is strictly independent (random). The artificially created 2D histogram of that random independence is shown on Fig.7.

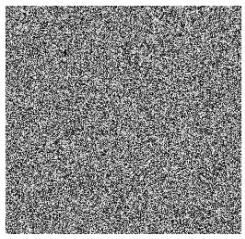

Figure 7: 2D histogram of independent channels.

On the other side, imagination of totally dependent 2D histogram is simple. If channel *b* is dependent on channel *a* totally, then for each *k* of the histogram *h* of channel *a* is in histogram *h2* (of channel *b* for pixels with intensity *k* in channel *a*) allowed only short interval of intensities in channel *b*. Therefore, 2D histogram of totally dependent channels will be similar to the one on Fig.8. and differs only in the slope.

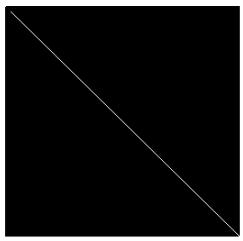

Figure 8: 2D histogram of totally dependent channels, *h2 = k, for all k in h*.

3. Results and discussions

The algorithm, how to obtain 2D histogram was described in section 2.4. According to the phase contrast properties described in section 2.2. is expected partial channel dependence in phase contrast microphotography of HeLa cells. The results similar to the Fig. 7. of section 2.4. stand for independence. The results similar to the Fig. 8. of section 2.4. stand for strong dependence.

Algorithms were tested on images of real non-microscopic scene, to examine dependence level of common snapshots. 2D histogram on Fig. 9. shows interesting results. Presence of the dependence in daily images is more general then special case, although usual dependence is not as strong as on 2D histogram on Fig. 8. of section 2.4.

Figure 8: 2D histogram of partial dependence in RGB image from Fig. 5.

Tested HeLa cells images were images of growth of cell monolayer from Fig. 1. For better visibility of HeLa 2D histograms were the histograms enhanced by false colours (Matlab Jet) to show the occurrence values of *k* histograms *h2* (rows). Consequently, Fig. 9. shows clearly that dependence between two channels is very significant in in first half of histogram *h* – low intensities *k* (upper part

of 2D histogram) and arise into more spread but still strongly dependent behavior in the higher intensities. The difference between Fig. 9. A and Fig. 9. B also disproves the inversion dependences *h* on *h2* and *h2* on *h*.

A B

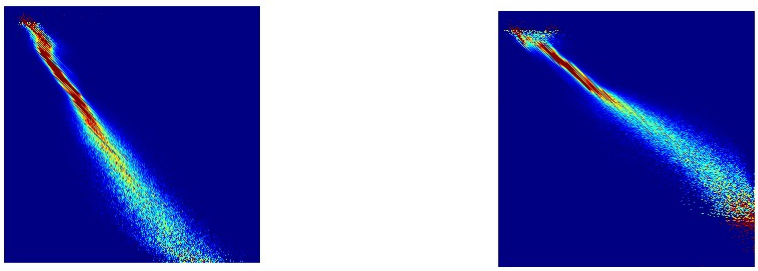

Figure 9: Dependence of Red on Blue in panel A, Dependence of Red on Blue in panel B.

Thus, phase contrast microphotography captured in colours produce images with strong dependence between the colour channels. This property has to be considered in any further analysis using histogram functions like thresholding, entropy contribution or segmentation.

Methods of 2D histograms easily and illustratively shows the level of channel dependence and allow to computational solution of conditional probabilities. However, let us note that 2D histograms as well as 1D histograms are still simplification of whole conditional space. Full evaluation of all conditional probabilities require multidimensional space including all dimensions of the problem (all colour channels), which is partially out of scope of this article.

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