











# A New Automatic Cancer Colony Forming Units Counting Method

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**Abstract.** Clonogenic assays are an essential tool to evaluate the survival of cancer cells that have been exposed to a certain dose of radiation. Its result can be used in the generation of strategies for the optimization of radiotherapy treatments. The analysis of this type of data requires that the specialist performs the manual counting of colony forming units (CFU), i.e., find every cell that retains the ability to produce a large progeny. This task is time consuming, prone to errors and the results are not reproducible due to specialist subjective assessment. Digital image processing tools can deal with the flaws described above. This article presents a new technique for automatic CFU counting. The proposed technique extracts the regions of interest (ROIs), where a local segmentation algorithm finds and labels the CFUs in order to quantify them. Results show good sensitivity and specificity performance compared to state-of-the-art software used for CFU detection and counting.

**Keywords:** CFU · Colony counting · Cell counting · Cancer · Cell proliferation · Image analysis

## 1 Introduction

Clonogenic cell survival assay is a widely used technique employed to analyze the effects of physical, chemical or environmental conditions on cell survival and proliferation [1]. This method has been used in a large number of studies with diverse kind of

cells and has contributed with information about the effect of ionizing radiation on mammalian cells. The assay measures the cell number that retain the ability to produce a large number of progeny from a single cell, i.e. the cell that achieve at least five or six generations of successive replications to form a colony, composed of, at least, fifty cells, which is called Colony Forming Unit (CFU) [2].

Up to day, the analysis of clonogenic assay data is made primary by an ordinal approach, i.e., comparing two or more culture discs and establishing which has the higher number of colonies [3] or by manual counting of CFUs [4]. Some important technical improvements have been developed to facilitate hematopoietic cells counting, as the STEM Vision™ system, which combines special culture medium, dedicated six well culture plates, and an automated acquisition and image analysis system in order to obtain a fully automated colony counting [5]. Nevertheless, this type of system is expensive, highly consuming in laboratory reagents and materials, and hence, its use is very limited.

Digital image processing is widely used in the automation of processes, allowing to obtain fast, reproducible and unbiased results, and improving the ecosystem of the sample analysis service in cancerology and others research fields. In this way, image processing techniques can be employed to increase the efficiency on the analysis of clonogenic assays, allowing to reduce the time spent in the analysis of the assays, and convert the technique into a high-throughput data analysis tool, increasing in the number of trials and samples processed per experiment. Therefore, in this paper a new methodology for CFU automatic counting is proposed.

## 2 Materials

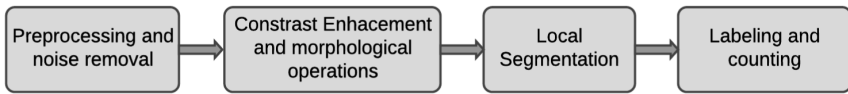
For this project, glioblastoma (U87) and breast cancer (MCF-7) cell lines were used. Cells were cultured in a DMEM medium (Biowest®) supplemented with 10% Bovine Fetal Serum (BFS, Hyclone®), and seeded in Petri culture dishes of 100 mm diameter until reaching a confluence of 70% to 80%. For the clonogenic assay we followed the Plating After Treatment protocol [2]. Briefly, the cells were harvested by incubation with trypsin/EDTA (Gibco®) and counted. 50.000 cell were transferred to 5 mL of medium in 15 mL Falcon tubes. Tubes were irradiated with 6 MV x-ray photons, using a clinical-use linear accelerator, in doses of 0, 1, 2, 4, 6, 8 and 10 Gy. To ensure the electronic balance and a better dose homogeneity in the whole volume, tubes were irradiated in a paraffin physical simulator. After irradiation, 200–2000 cells were seeded per well, in 6-well culture plates, depending on the radiation dose used. Seeding was made by triplicate for every dose. Cells were incubated at 37 °C and 5% CO<sub>2</sub>, to allow colony formation. After 14 days of incubation, the formed colonies were fixed and stained with a mixture of 4% formaldehyde and 1% violet crystal.

Each culture plate was scanned using a HP Scanjet Pro with a resolution of 944 × 636 pixels. To develop the identification process, 216 wells were extracted from

36 RGB images. Wells were manually analyzed and these results were employed as ground truth. 66 images belonged to the U87 and 150 to the MCF-7 cell line.

### 3 Development

A CFU occupy a very small fraction of the total image area (See Fig. 2a). Considering the counted CFUs, the mean area ratio of a colony to the full image size represents only a  $6 * 10^{-5}$  factor. In addition, the background can be very complex due to debris. The proposed method consist of four stages, illustrated in Fig. 1: Preprocessing and noise removal, contrast enhancement and morphological operations, segmentation and, labeling and counting.

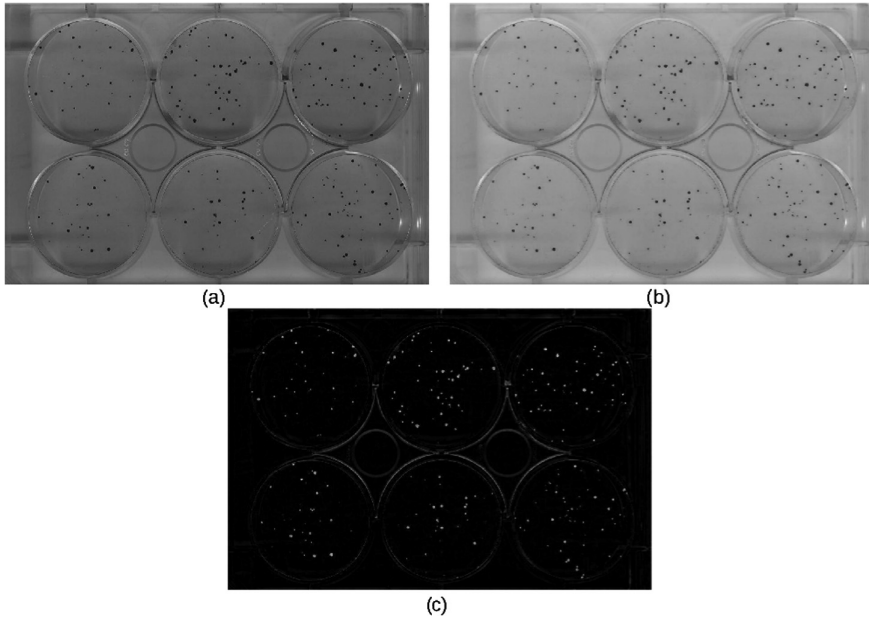


**Fig. 1.** General flowchart of the algorithm.

#### 3.1 Pre-processing

As can be seen in Fig. 2a, image background is not uniform, having a greater intensity in the centre and presenting vignetting at the corners. Therefore, images were pre-processed to correct uneven lighting (Fig. 2b). For this purpose, images were converted to grayscale coding scheme by computing a weighted sum of the linear color components. A gamma correction using  $\gamma = 0.4$  (empirically found), was applied, to increase the separation between CFUs and background. Since some CFUs were not well defined, a morphological opening, using a diamond-shaped structuring element, was employed to obtain the result shown in Fig. 2b. The structural element shape was selected based on the form of colonies and its size (7 pixels) was determined by the smallest colony in the image according to user criteria.

Black top-hat, which is the difference between the original grayscale image and a morphological closing, was applied to images, using a structuring element smaller or equal than the smallest CFU (Fig. 2c). The same diamond-shaped structuring element was used to remove non-significant objects and attenuate background noise. As a result, current image has a bimodal distribution. To enlarge separation between the ROI and the background, another contrast enhancement with  $\gamma = 0.8$  was implemented.



**Fig. 2.** (a) Original grayscale image. (b) Gamma correction and morphological opening. (c) Top-hat filtering and gamma correction.

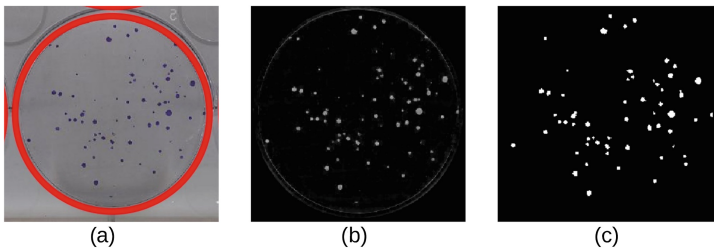
### 3.2 Wells Detection

From the above stage, the images contain a better differentiated ROI from background. However, each image contains one plate of six wells. By taking advantage of the circular shaped form of each well, circular Hough Transform was used for its segmentation (see Fig. 3a). Well radius range is calculated using the relation between wells area and image dimensions. Subsequently, information about centre coordinates and radius  $r$  from each well is used with an Euclidean distance criteria, for keeping pixels located inside the well as shown in Fig. 3b.

One of the main problems in colony counting is the misclassification of noise out of the ROI as colonies, due to intensity similarity of some areas of the recipient, where the wells are placed. This condition can increase the number of false positives and therefore may affect specificity. Hence, isolating the wells from the background eliminates possible false positives detected outside them. At the same time, a one by one well colony counting is required. This is achieved by labeling and counting only the elements that are inside the ROI.

### 3.3 Colonies Segmentation, Labeling and Counting

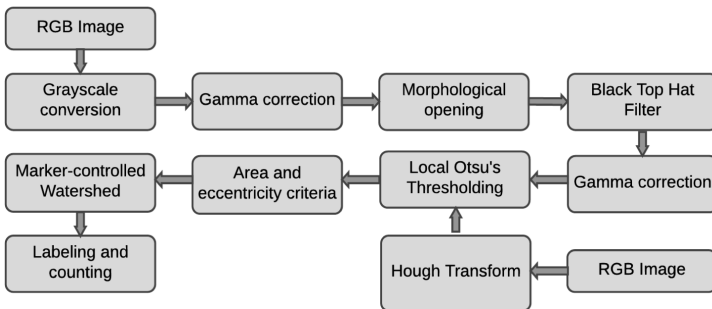
After applying the processing techniques described above, gray level image showed a well-defined bimodal intensity distribution. Therefore, Otsu’s method was used to find optimal threshold for each well separately, in order to achieve its colonies segmentation.



**Fig. 3.** (a) Petri dishes detection by Hough Transform. (b) Euclidean Distance removal. (c) Local thresholding using Otsu’s Method, elimination based on region properties criteria and processing using watershed algorithm.

It was statistically found that a colony, composed of 50 cells, has an average size greater than 7 pixels. Therefore, this value was employed as threshold to reject the regions with a smaller area. In addition, some dish edges were identified as colonies. Given that colonies have a circular shape, objects which eccentricity was greater than 0.9, value found empirically, were eliminated.

In some assays, CFUs overlapped, leading to an underestimated counting. A marker-controlled Watershed algorithm is applied to separate each one of these overlapped CFUs. This algorithm uses as a marker the maxima found by Distance Transform. A general flowchart of preprocessing and noise removal step is shown in Fig. 4.



**Fig. 4.** Detailed flowchart of the algorithm.

## 4 Results and Discussion

It is worth mentioning that global or local thresholding techniques based on intensity histogram are not the best solution for CFUs segmentation from background, given the illumination gradient and the presence of artifacts (i.e., shadow) that can be observed on the inferior part of the dishes. One solution to this particular problem is to improve the acquisition system, which guarantees an even light distribution over the well's area using a chamber with LEDs arranged under [6–8] and/or around them [9], and a digital camera support on the top of it.

### 4.1 Validation

Some general cell counting techniques have been developed, but several parameters must still be adjusted manually. OpenCFU [10] is an open-source software programmed in C++ which uses OpenCV framework functions. OpenCFU has a graphical interface where counting results are shown, but also it allows the user to choose the region of interest, avoiding the detection of false positives outside and at the edges of the discs. It is one of the fastest programs processing an image of  $1.6 \times 1.6$  kpx in approximately 0.69 s. However, in order to get the results, the user has to choose various parameters like the type of threshold (bilateral, inverted or regular) or if a color filter may be used. Image J plugin, CellCounter, allows the user to select a well and adjust a binary threshold for colonies segmentation and counting. In this kind of tools, the user has to follow a process of try and error, and/or modify various parameters to obtain an acceptable result, which can be really difficult if there is no digital image processing background.

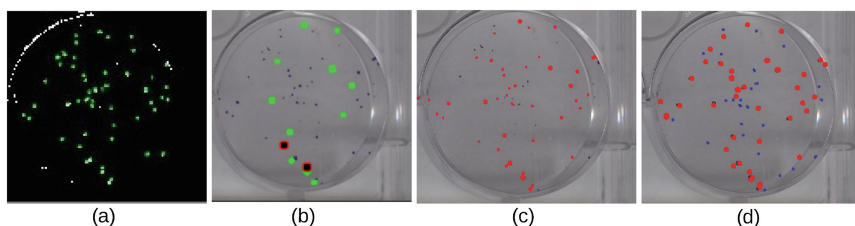
Validation of the system is done by comparing the results obtained by the proposed system and a manual labelling and counting. At the same time, two software programs are used for CFU counting over the same image sets. In OpenCFU, processing was made with a regular threshold and no filter configuration, adjusting the radius in such a way that the best result is obtained for each image. In CellCounter, a circular ROI was selected to be segmented by a threshold adjusted to give the best result. Although other software was employed to carry out this task, they did not give the best results [11].

Sensitivity and specificity are selected as the main validation metrics. Manually labelling consist on placing red marks in image objects that were CFUs, and blue marks in the ones that were not. The resulting image was the ground truth. Then, an automatic comparison between results coming from the proposed system and the ground truth image was performed, in order to find true positives, true negatives, false positives and false negatives. Same process was repeated with the result images obtained by using OpenCFU and CellCounter (Fig. 5).

The program was evaluated in a computer with a CPU Intel Core i5 2.4 GHz, OS Windows 8.1 Pro and 4 GB RAM. It was able to process all the dataset with an average time per image of  $12.94 \pm 1.13$  s. Even though OpenCFU and CellCounter are faster, taking less than 1 s in processing the image, and CellCounter has the better sensitivity and OpenCFU the better specificity of all three software respectively, only our system keeps good average sensitivity ( $>0.78$ ) and specificity ( $>0.78$ ) for the whole image sets analyzed, i.e., our system is better rejecting those objects that do not have at least 50

**Table 1.** Performance of colony counting methods.

Folder	Method	Sensitivity	Specificity
05.04.2016	CellCounter	$0.88 \pm 0.12$	$0.22 \pm 0.16$
	OpenCFU	$0.29 \pm 0.11$	$0.99 \pm 0.02$
	Method proposed	$0.86 \pm 0.11$	$0.78 \pm 0.14$
25.09.2015	CellCounter	$0.61 \pm 0.32$	$0.51 \pm 0.39$
	OpenCFU	$0.47 \pm 0.29$	$0.93 \pm 0.09$
	Method proposed	$0.83 \pm 0.20$	$0.79 \pm 0.27$
26.04.2015	CellCounter	$0.83 \pm 0.22$	$0.11 \pm 0.21$
	OpenCFU	$0.47 \pm 0.27$	$0.94 \pm 0.08$
	Method proposed	$0.78 \pm 0.35$	$0.73 \pm 0.21$

**Fig. 5.** Comparison of results from each algorithm. (a) CellCounter, (b) OpenCFU (c) Proposed system (d) Manual labelling and counting.

cells, in other words, that fulfill the requirements of area and shape simultaneously, preserving the ability to identify those objects that are colonies (true positives) and at the same time, rejecting those that are not (Table 1).

## 5 Conclusions and Future Work

An automatic system able to segment colonies from CFU assay images with multiple wells was successfully developed, keeping a good sensitivity and specificity rather than a good sensitivity with poor sensibility, as in the case of CellCounter, or the opposite, as in the case of OpenCFU, used to validate the results of this work. Future works should aim to validate the algorithm in bigger and different datasets in order to improve it, replacing or adding steps in the processing to make a more robust algorithm.

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