

ColonyCountJ: A User-Friendly Image J Add-on Program for Quantification of Different Colony Parameters in Clonogenic Assay

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Abstract

Objective: There are several commercial automatic colony counters available but they are relatively expensive and associated with several quantifications related problems. Some of the key problems in automated colony counting are clustering of colonies and edge effects. Thus the main objective of this study is to develop a user friendly program for determining colony parameters of clonogenic assay.

Methods: In the present study, a watershed algorithm was employed along with other ImageJ tools to develop ImageJ based add-on program i.e ColonyCountJ to calculate various colony parameters. To test this program, we have used colonies obtained after exposure of MCF-7 cells to different doses of γ -radiation.

Results: The results obtained using this program were compared with the manual counting as well as with automated counting provided by Oxford Optronix GelCount colony counter. It was found that our results corroborated well with the manual as well as with commercial program. As a whole, the performance of ColonyCountJ with regard to processing time and sensitivity was at par with the commercial colony counter.

Conclusion: In conclusion, "ColonyCountJ" add-on program customized for ImageJ with optimized parameters is a reliable method for quantifying the colonies obtained from clonogenic assay. This program will be of great use for researchers working in the area of toxicology, radiation biology, and cancer biology.

Keywords: Clonogenic assay; Image J; Toxicology

Introduction

Clonogenic assay or colony formation assay is extensively used to measure *in vitro* cell survival based on the capacity of a single cell to grow into a colony. When a cell divides and form a cluster of more than 50 cells, known as colony, is regarded as viable and traditionally counted manually [1]. To obtain statistical accuracy such assays require a large number of samples and a large number of colonies per sample. Thus, manual colony counting is extremely tedious and time consuming, particularly when colonies of around 50 cells need be distinguished and/or when experimenters get exhausted after hours of counting. In addition, manual colony counting can be biased, and results can vary significantly depending on the person who is counting. To overcome these key issues associated with the manual counting, automated colony counters have been designed which offer faster and more reproducible results which can be automatically transferred onto a computer. Many groups have developed automatic colony counting methods based on the image analysis principles [2-6], but they have either used commercial software or their uses require in-depth knowledge of computer programming skills. Several colony counting instruments are commercially available, but they are not cost effective. Most of the automatic colony counters that are commercially available use CCD-camera and specialized imaging software. Free colony counting softwares such as Clono-Counter [3-5], ColonyArea [6] are available; however, there are certain drawbacks such as non-availability of quantitative guidelines on how to select key digital counting

parameters such as intensity threshold and colony size. Therefore, still manual counting method is adapted by many researchers for studies involving clonogenic assays as the standard procedure [1,7,8]. Thus the development of user-friendly, semi-automatic or automatic colony counting may aid in reducing the time required for counting and the results are more reliable.

In this paper, we developed a simple and user-friendly colony parameter quantification program which will run on the freely available ImageJ software. The data obtained with this program is validated by comparing with manual counting and commercial colony counter. The ColonyCountJ program gives several colony parameters such as, it determines the number of colonies, % area, average size and intensity weighted colony area percentage (colony intensity percentage) from any digital image of colony formation assay conducted in a single dish or multi-well plate. The program is user-friendly, as it requires selection of a circular region of interest (ROI) that covers wells to be analyzed by the user. "ColonyCountJ" program will definitely be useful for counting colonies in clonogenic assays since it is independent of the image format, does not require any specific equipment for image capturing. It only requires a digital image with the colonies. Furthermore, it uses free software and does not necessitate in-depth knowledge of computer programming skills. This program will be of great use for researchers working in the area of radiation biology, toxicology, cancer biology etc. A free copy of the program can be obtained by sending email at dkmaurya@barc@gmail.com or dkmaurya@barc.gov.in.

Material and Methods

Cell cultures

MCF-7, human breast carcinoma cell line was maintained in DMEM, Dulbecco's modified Eagle's medium (Himedia, India) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (Himedia, India). The cells were incubated in 5% CO₂ humidified at 37°C for growth.

Clonogenic assay

For clonogenic assay, MCF-7 cells (500 cells in 2 ml medium) were seeded in a 6-well plate and were incubated at 37°C overnight for attachment. Next day cells were exposed to different doses (0, 2, 4 and 8 Gy) of gamma radiation and placed in the CO₂ incubator for 12 days. Following incubation, the medium was removed and colonies were fixed and stained with 0.5% methylene blue (dissolved in 50% ethanol). Colonies formed were counted manually, using ColonyCountJ, an add-on program to ImageJ and compared with commercial colony counter (GelCount™). The experiment was carried out twice in triplicates.

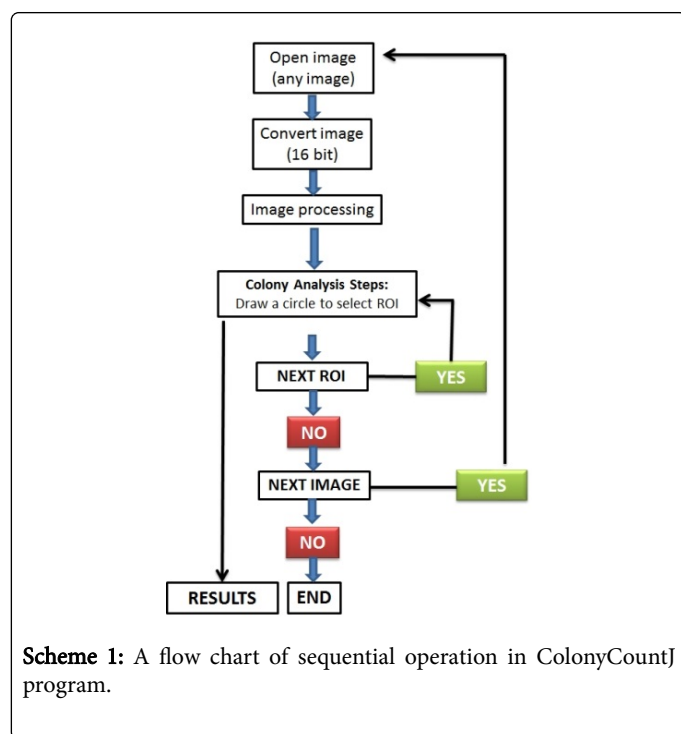
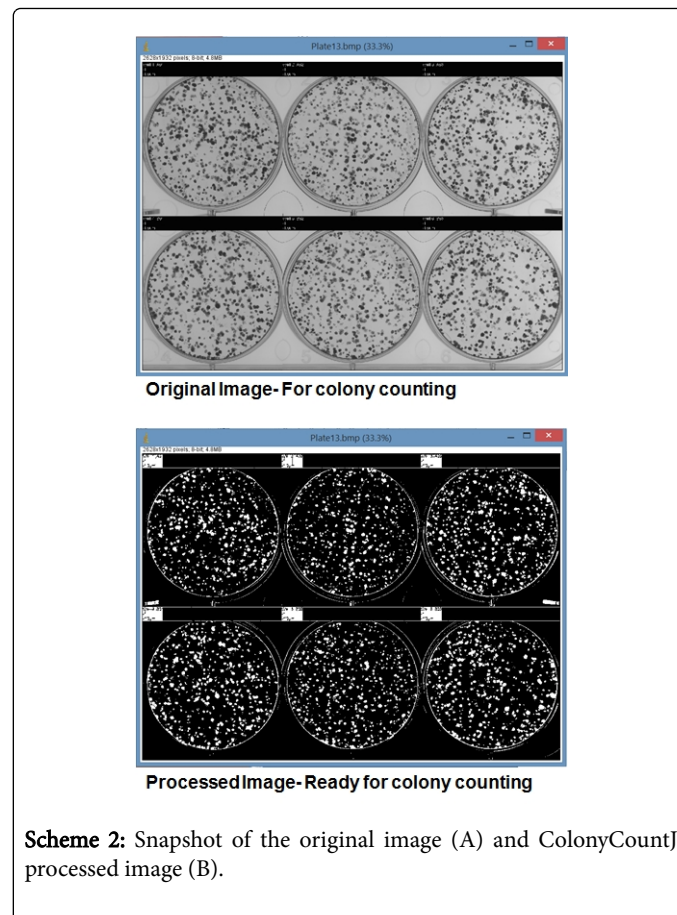


Image acquiring and processing using GelCount: For automated colony counting, images of the plates were scanned using Oxford Optronix GelCount colony counter. The images were transferred, processed and characterized and the data exported using a single integrated hardware/software platform. Colonies were counted using GelCount™ software.

Image processing using ColonyCountJ, an ImageJ add-on program: For assessment of different parameters of colonies, we have developed a "ColonyCountJ" add-on program for public domain image analysis software, ImageJ 1.47v. This program is a semi-automatic program which can provide; i) number of colonies, ii) area covered by the colony, iii) average size of the colony and iv) average intensity of the

colony. To understand the program steps, a flow chart is provided in scheme 1.

The program automatically converts any selected image into 16 bit black and white image followed by several automatic conversions to make individual colony ready for the analysis, by implementing watershed algorithm and other ImageJ tools (scheme 2).



After completing these steps, an action command pop-up window will appear instructing to draw a circle around the region of interest (ROI). Subsequently, next pop-up will appear for selection of another ROI. Continue the above steps until all ROI of the opened field are analyzed. For analyzing new image file: uncheck YES on the pop-up window of NEXT ROI. This will direct the new image. Repeat the above steps until all the ROI in the field are analysed. Continue these steps till all the images are selected and analysed. Results of this analysis will appear on a new result window and will ask for the location to save the results in xls format. The results are returned with the following colony parameters; (i) number of colonies, (ii) area covered by the colony, (iii) average size of the colony and (iv) average intensity of the colony.

Manual colony counting

The manual counting of the colonies was performed using a light box and pen method. Two independent operators counted the colonies and the average is plotted.

Statistical analysis

The graphs were plotted as Mean \pm SEM (standard error of the mean). We evaluated correlations between different colony parameters of manual count, GelCount™ and ColonyCountJ using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla California USA). A p value less than 0.05 was considered significant.

Results and Discussion

Due to wide applicability of clonogenic assay in the area of toxicology, cancer biology and radiation biology, a user friendly and automated and semi-automated colony counting program is required for fast data analysis over manual counting.

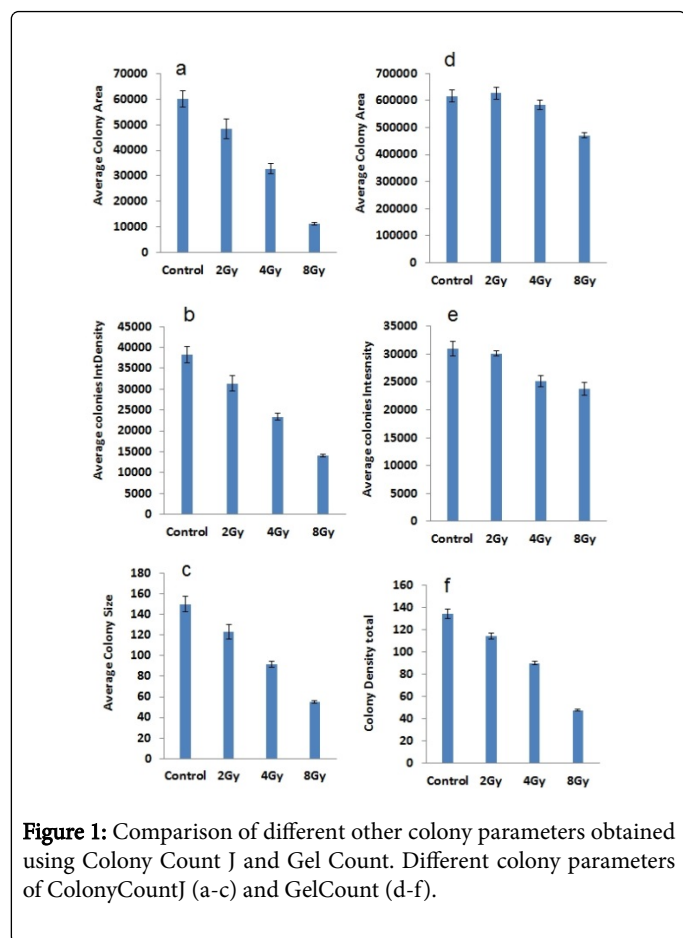


Figure 1: Comparison of different other colony parameters obtained using Colony Count J and Gel Count. Different colony parameters of ColonyCountJ (a-c) and GelCount (d-f).

To overcome the manual counting problems, several semi-automated and automated colony counters have been designed [2-6] and each has certain limitations. Here we have developed a user-friendly and semi-automatic program for gathering colony parameters in clonogenic assay. Scheme 1 depicts the flow chart of newly developed ColonyCountJ program. The program does several automatic conversions to make individual colony ready for the analysis, by implementing watershed algorithm and other ImageJ tools (scheme 2) followed by a manual drawing of a circle around the region of interest (ROI). This will complete the analysis of a single ROI. We have evaluated this ImageJ ad-on program using clonogenic assay images of MCF-7 cell line exposed to different doses (2, 4 and 8 Gy) of gamma-radiation. Different colony parameters were measured by the

GelCount™ and ColonyCountJ along with manual counting for colony numbers (Figures 1 and 2).

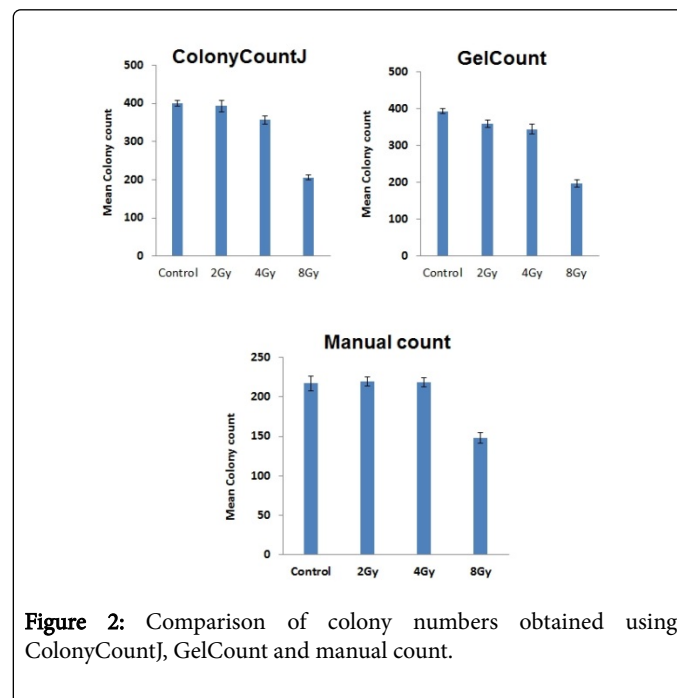


Figure 2: Comparison of colony numbers obtained using ColonyCountJ, GelCount and manual count.

Both these programs showed a dose dependent decrease in the colony parameters upon exposure to radiation. These include colony number, average colony size, average colony intensity. As shown in the Figure 1, both GelCount™ and ColonyCountJ yielded similar results in terms of number of colonies but at the same time ColonyCountJ showed better dose dependent reduction in the colony parameters as compared to GelCount™ (Figure 1). Further, these results were in agreement with the manual counting method indicating that ColonyCountJ developed by us provided reliable results (Figure 2). As shown in the Figure 2, the number of colonies counted by manual counting is lower than colonies counted by the software. This may be due to error involved in the manual counting. Different colony parameters obtained by GelCount™ and ColonyCountJ showed good correlation ($R^2 > 0.9$) with each other (Figures 3 and 4). We have also used this program for analysing the colony parameters using several other cell lines such as A549, INT 407 and L132. We have found good correlation between manual counting and counting performed by ColonyCountJ. It is well-known that semi-automated and automated programs have advantage over manual counting because automated programs provide several colony parameters such as colony intensity, density, morphology etc. apart from colony number. All these additional parameters are useful over just colony numbers obtained by manual counting. Several colony counting instruments are commercially available, but they are expensive. Most of the automatic colony counters that are commercially available use CCD-camera and specialized imaging software.

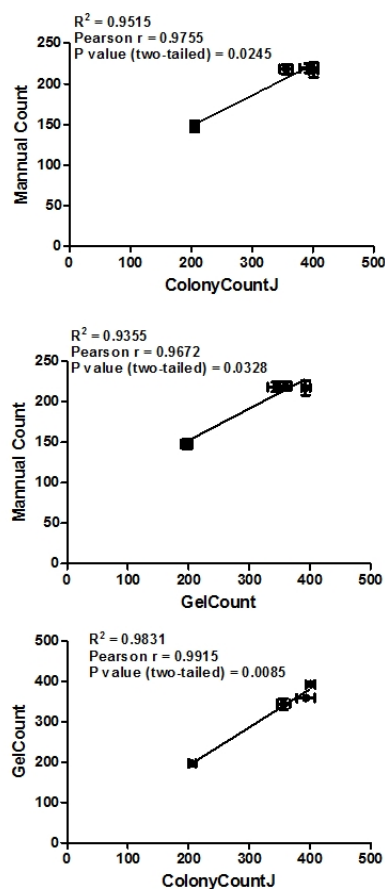


Figure 3: Correlation between colony number obtained by manual count, ColonyCountJ and GelCount.

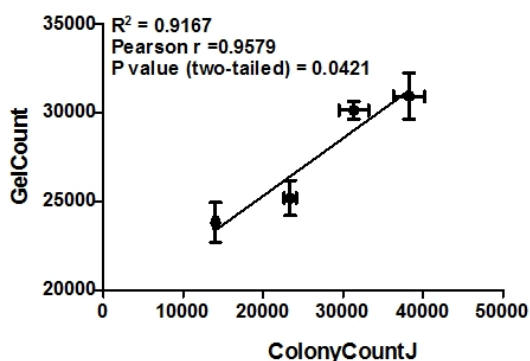


Figure 4: Correlation between colony intensity obtained using ColonyCountJ and GelCount program.

There are also freely available colony counting software's such as Clono-Counter (3), ColonyArea (6), however, they have certain drawbacks such as no quantitative guidelines to select critical digital counting parameters like intensity threshold and colony size. Therefore, manual counting method is still popular among research community working in the area of cancer biology and radiation biology. The existing methods have their own associated merits and de-merits such as they require specific hardware and training on the software. This newly developed program has taken care of the problems associated with the existing colony counting programs.

In conclusion, the semi-automatic colony counting program, ColonyCountJ developed here is user friendly and does not require any specific hardware and extensive training on the software. It requires a digital image of the colonies in any image format. ColonyCountJ gave better information about colony parameters (sensitivity and time required) as compared to manual and other commercial software.

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